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AN INTEGRATED MOLECULAR, IMMUNOLOGICAL AND AI-BASED APPROACH TO PRECISION ONCOLOGY

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Abstract

Background: Precision oncology increasingly relies on the characterization of tumor-specific molecular alterations and patient-specific immune profiles to support individualized therapeutic decision-making. Next-generation sequencing (NGS) provides valuable information regarding somatic mutations and their potential clinical relevance, while immune profiling offers complementary information on the systemic immune status of the patient. However, the interpretation of these heterogeneous datasets is frequently performed independently, potentially limiting the comprehensive assessment of individual patients.

Objective: This study aimed to evaluate the feasibility and potential added value of an AI-based Expert System Software (ESS) for integrating molecular, immunological, biomarker and clinical information into a comprehensive patient-specific report supporting personalized therapeutic orientation.

Methods: Five patients with solid tumors, including two endometrial cancers and three ovarian cancers, were investigated. Tumor samples were analyzed by targeted next-generation sequencing using an AmpliSeq HotSpot panel, while peripheral blood samples were characterized by spectral flow cytometry. The resulting molecular and immunological data were integrated with available biomarker and clinical information within the AI-based Expert System Software. Identified genetic alterations were classified according to their clinical relevance, and potential therapeutic implications were assessed using evidence-based clinical guidelines. The system also provided PubMed identifiers (PMIDs) for scientific publications relevant to the identified mutations, immune parameters and biomarkers.

Results: NGS and immune profiling independently provided complementary information regarding tumor molecular characteristics and patient immune status. Integration of these datasets within the Expert System generated a comprehensive patient-specific profile by establishing correlations between genomic alterations, immune parameters, biomarkers and clinical characteristics. Compared with the interpretation of individual datasets, the integrated analysis provided additional context for the assessment of clinically relevant molecular alterations and generated broader possibilities for patient-specific therapeutic orientation. Linking the integrated findings with relevant scientific literature further supported evidence-based interpretation.

Conclusions: This proof-of-concept study demonstrates the potential value of integrating molecular, immunological, biomarker and clinical information within a single AI-based decision-support platform. Such an approach may facilitate a more comprehensive, patient-centered interpretation of complex oncological data and support personalized therapeutic orientation. Further studies involving larger patient cohorts and clinical outcome assessment are warranted to validate the clinical utility of this integrated precision oncology approach.

Key words: NGS, immune profile, Expert System Software, precision oncology

Introduction

Cancer is characterized by substantial molecular and biological heterogeneity, even among tumors arising from the same anatomical site. This heterogeneity contributes to differences in disease progression, treatment response and resistance, emphasizing the need for approaches that move beyond conventional population-based treatment strategies toward patient-specific therapeutic decision-making. Precision oncology aims to address this challenge by integrating biological and clinical characteristics of individual patients to identify diagnostic, prognostic and therapeutic opportunities that are relevant to their particular disease profile [1,2].

Next-generation sequencing (NGS) has become an important component of precision oncology by enabling the simultaneous assessment of multiple cancer-associated genes and the identification of somatic genomic alterations with potential diagnostic, prognostic or therapeutic relevance. The clinical utility of NGS, however, extends beyond the detection of mutations. The interpretation, prioritization and assessment of the clinical actionability of identified alterations are essential for translating genomic information into clinically meaningful conclusions [2,3]. Current European Society for Medical Oncology (ESMO)

recommendations emphasize that clinical genomic reports should integrate genomic alterations with their functional annotation, clinical actionability and potential therapeutic implications, while presenting the main findings in a format that facilitates clinical interpretation [3]. Multigene NGS is already recommended in selected solid tumors, including ovarian cancer, where genomic characterization can contribute to the identification of therapeutic opportunities [2].

Tumor genomic characteristics represent only one dimension of cancer biology. The interaction between malignant cells and the host immune system plays a fundamental role in tumor progression and therapeutic response, making characterization of the patient's immune status an important complementary component of precision oncology. Multiparametric flow cytometry allows the simultaneous identification and quantification of multiple immune cell populations and phenotypic characteristics within heterogeneous biological samples and is widely used for the characterization of immune responses in cancer [4]. In particular, peripheral blood immune parameters have attracted considerable interest as minimally invasive biomarkers that may provide information about systemic immune status and potentially complement tumor-derived biomarkers [5,6]. Nevertheless, peripheral immune biomarkers remain an evolving field, and their clinical utility requires appropriate analytical and prospective clinical validation [5,6].

The increasing availability of molecular, immunological and clinical data creates an important challenge: how to transform multiple independent analytical results into an integrated representation of the individual patient. NGS may identify clinically relevant tumor mutations, while immune profiling may describe the patient's systemic immune status; biomarkers and clinical characteristics provide additional layers of information. When these datasets are considered separately, each provides valuable but inherently partial information. A more comprehensive approach requires the correlation of these complementary domains within a common analytical framework.

The integration of heterogeneous biomedical datasets has consequently become an important direction in precision oncology. Multimodal data integration can combine molecular diagnostics with clinical and other biological information, potentially generating a more comprehensive representation of individual patients than single-modality analyses [7]. Recent developments in artificial intelligence (AI) and machine learning have further expanded the possibilities for integrating high-dimensional and heterogeneous datasets, including genomic, transcriptomic, proteomic and clinical information [8,9]. Such approaches may facilitate patient stratification, biomarker interpretation and treatment-related decision support. However, the translation of AI-based approaches into clinical practice requires careful attention to data quality, validation, interpretability, generalizability and appropriate clinical oversight [8,9].

Recent work has demonstrated the potential of AI-enabled systems to integrate patient-specific molecular and clinical information with external biomedical knowledge to support precision oncology decision-making. For example, the Knowledge Connector decision-support system was developed to integrate individual molecular and clinical data with broader biomedical knowledge and to support the interpretation of complex molecular findings within multidisciplinary precision oncology workflows [10]. These developments support the concept that the value of complex molecular profiling may be enhanced when individual findings are not interpreted in isolation but are connected to other patient-specific parameters and relevant scientific evidence. In this context, we developed and applied an AI-based Expert System Software (ESS) designed to integrate molecular, immunological, biomarker and clinical information into a comprehensive patient-specific report. The system incorporates tumor genomic data generated by NGS and immune profiling obtained by spectral flow cytometry, together with available biomarker and clinical information. Through integrated algorithms, the ESS correlates these parameters and evaluates the clinical relevance of identified genomic alterations and their potential therapeutic implications. An additional feature of the system is the incorporation of relevant scientific literature through PubMed identifiers (PMIDs) associated with identified gene mutations, immune characteristics and biomarkers, thereby facilitating access to the scientific evidence supporting the interpretation of patient-specific findings.

The present study describes a proof-of-concept application of this integrated approach in five patients with solid tumors, including two patients with endometrial cancer and three with ovarian cancer. The objective was not to validate a therapeutic intervention or demonstrate clinical outcome benefit, but to explore how the integration of complementary molecular and immunological datasets within a single AI-based platform can provide a broader patient-specific perspective than the independent interpretation of each dataset. We hypothesized that the integrated analysis could facilitate the interpretation of complex oncological information, connect patient-specific findings with relevant scientific evidence and support a more comprehensive, patient-centered approach to therapeutic orientation.

Materials and Methods

Flow Cytometric Evaluation

Peripheral venous blood (4 mL) was collected from oncologic patients after obtaining written informed consent using the Informed Consent Form developed within the CrossCare project. Blood samples collected in Serbia were transported to Romania for further processing. Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation using Ficoll-Paque. The isolated PBMCs were cryopreserved in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen until analysis.

For immunophenotypic evaluation, thawed PBMCs were analyzed using a Cytex Northern Lights spectral flow cytometer. Cells

were stained with a panel of fluorochrome-conjugated monoclonal antibodies directed against CD45-FITC, CD3-APC-H7, CD4-PerCP-Cy5.5, CD8-PE-Cy5, CD19-PE, and CD56-APC (all antibodies purchased from BD Biosciences). Following antibody staining, the cells were washed and resuspended for acquisition. Spectral flow cytometry was performed by collecting the complete fluorescence emission spectra generated by the fluorochromes rather than measuring fluorescence within discrete conventional filter channels. The acquired spectral signatures were unmixed using the instrument software, allowing the individual fluorochrome signals to be separated and enabling multiparameter identification of the major lymphocyte populations. The immune profile was subsequently characterized based on the expression of CD3, CD4, CD8, CD19, and CD56, with CD45 used for leukocyte identification.

Next-Generation Sequencing and Bioinformatic Analysis

Fresh tumor tissue samples obtained from five oncologic patients, comprising two endometrial and three ovarian cancer cases, were subjected to targeted next-generation sequencing (NGS) for the identification of somatic genetic alterations. Genomic DNA was purified from fresh tumor tissue using the PureLink™ Genomic DNA Mini Kit (Invitrogen, Thermo Fisher Scientific), according to the manufacturer's instructions. DNA concentration was determined using a Qubit™ 4 Fluorometer and the Qubit™ dsDNA High Sensitivity (HS) Assay Kit (Thermo Fisher Scientific).

Targeted sequencing libraries were prepared using the Ion AmpliSeq™ Cancer Hotspot Panel v2 (Thermo Fisher Scientific), targeting recurrently mutated regions in cancer-associated genes. Library preparation and templating were performed using the Ion AmpliSeq™ Library Preparation and Templating Kit on the Ion Chef™ System (Thermo Fisher Scientific), according to the manufacturer's protocol. The prepared libraries were subsequently loaded onto Ion 530™ Chips and sequenced using the Ion GeneStudio™ S5 System (Thermo Fisher Scientific), employing Ion semiconductor sequencing technology and the corresponding sequencing reagents.

Following sequencing, the generated data were stored on the Ion Torrent™ server and processed using Torrent Suite™ Software for primary data processing and quality assessment. Variant identification and initial interpretation were performed using Ion Reporter™ Software (Thermo Fisher Scientific). The identified genetic variants were subsequently evaluated according to their molecular characteristics and potential clinical relevance and were incorporated into the integrated analysis performed by the Expert System Software.

Expert System Software v2 Data Integration and Analysis

The molecular, immunological, biomarker, and clinical information obtained for each patient was integrated and analyzed using the Expert System Software version 2 (ESS v2), developed within the CrossCare project (RO-SRB Cross-border cooperation to foster the resilience of clinical management in cancer patients by establishing best practices in personalized molecular-based diagnostics, treatment, and long-term care, RORS00040/2024; Ethics Committee Agreement 553/15.07.2025). For each patient, the NGS results generated by Ion Reporter™ Software were exported as individual Excel (.xlsx) files and imported into ESS v2. The software processed the identified genetic variants and associated molecular information to generate a patient-specific molecular profile.

The immune profile was introduced into ESS v2 based on the percentage of the major peripheral blood lymphocyte populations identified by flow cytometry, including total T cells (CD3+), helper T cells (CD4+), cytotoxic T cells (CD8+), B cells (CD19+), and natural killer (NK) cells (CD56+). Each immune parameter was classified as low, normal, or high according to predefined reference intervals incorporated into the ESS v2 analysis. The interpretation was based on established flow-cytometric immunophenotyping principles and published reference ranges for peripheral blood lymphocyte subsets. Published studies have demonstrated that reference intervals for CD3+, CD4+, CD8+, CD19+, and NK-cell populations may vary according to population characteristics, age, sex, and analytical methodology; therefore, the classification was performed using the reference values implemented in the software rather than applying a universal threshold [11-13].

The biomarker module of ESS v2 incorporates a predefined list of cancer biomarkers derived from the National Cancer Institute (NCI) "Tumor Marker Tests in Common Use" database. The NCI list includes molecular, genetic, protein, and other tumor-associated markers used for cancer diagnosis, prognosis, treatment selection, and monitoring, and is explicitly described by the NCI as a non-comprehensive list that is updated as new markers become available. Biomarkers relevant to the individual tumor type and molecular profile can be selected from this predefined database. In addition, biomarkers not included in the predefined list can be manually introduced into ESS v2 when supported by the patient's clinical, pathological, or molecular information [14].

NGS variant interpretation in ESS v2 is performed using an automated oncogenic variant analysis pipeline integrating multiple molecular and clinical knowledge sources, including the National Comprehensive Cancer Network (NCCN) and European Society for Medical Oncology (ESMO) Clinical Practice Guidelines, Clinical Interpretation of Variants in Cancer (CIVIC), ClinVar, the Genome Aggregation Database (gnomAD), Combined Annotation Dependent Depletion (CADD), recurrent cancer mutations from the Memorial Sloan Kettering Cancer Center (MSK) Cancer Hotspots database, and the COSMIC Cancer Gene Census (CGC). Variant classification follows the 2017 Standards and Guidelines for the Interpretation and Reporting of Sequence Variants in Cancer established by the Association for Molecular Pathology (AMP), American Society of Clinical Oncology (ASCO), and College of American Pathologists (CAP).

Based on this integrated evidence framework, identified variants are classified into four tiers (Tier I–IV) according to their clinical significance and therapeutic relevance. ESS v2 subsequently identifies potential mutation-associated therapeutic options and provides regulatory and guideline indicators. These include U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) marketing authorizations for oncology indications, as well as recommendations from NCCN and ESMO Clinical Practice Guidelines. For NCCN recommendations, the system incorporates the corresponding evidence categories (Category 1, 2A, 2B, or 3), while ESMO recommendations are characterized according to levels of evidence (I–V) and grades of recommendation (A–E). These indicators provide contextual information regarding the regulatory status, strength of evidence, and level of clinical recommendation associated with each therapeutic option.

The mutation-based therapeutic information generated by ESS v2 is subsequently correlated with the patient's immune profile and selected biomarkers. This integrated analysis combines molecular, immunological, biomarker, and clinical information within a patient-specific framework, allowing therapeutic options to be considered in the context of the overall biological profile rather than on the basis of individual molecular alterations alone. Based on these correlations, ESS v2 provides additional therapeutic indications and potential contraindications or considerations associated with different treatment approaches. The system also provides PubMed identifiers (PMIDs) corresponding to relevant scientific publications and clinical trial reports, facilitating retrieval of the scientific evidence supporting the interpretation of identified molecular alterations, biomarkers, immune characteristics, and potential therapeutic approaches.

Results

Disturbed immune profile in oncologic patients

Flow-cytometric analysis revealed heterogeneous peripheral immune profiles among the five ovarian cancer patients. MM_44 showed normal B-cell, CD3+ and CD4+ T-cell proportions, accompanied by reduced CD8+ T cells and NK cells. KS_108 presented normal B-cell, CD3+ and CD8+ T-cell proportions, with reduced CD4+ T cells and NK cells. PR_96 displayed reduced B-cell and NK-cell populations, while the CD3+, CD4+, and CD8+ T-cell populations remained within the normal range. In contrast, SV_111 showed a normal distribution of all analyzed lymphocyte subsets. SD_91 exhibited a more pronounced alteration of the immune profile, characterized by reduced B cells, CD3+ and CD8+ T cells, while CD4+ T cells and NK cells remained within the normal range. Overall, the observed differences in T-cell, B-cell, and NK-cell populations demonstrated substantial inter-patient variability in peripheral immune status, providing complementary information to the molecular profiles obtained by NGS.

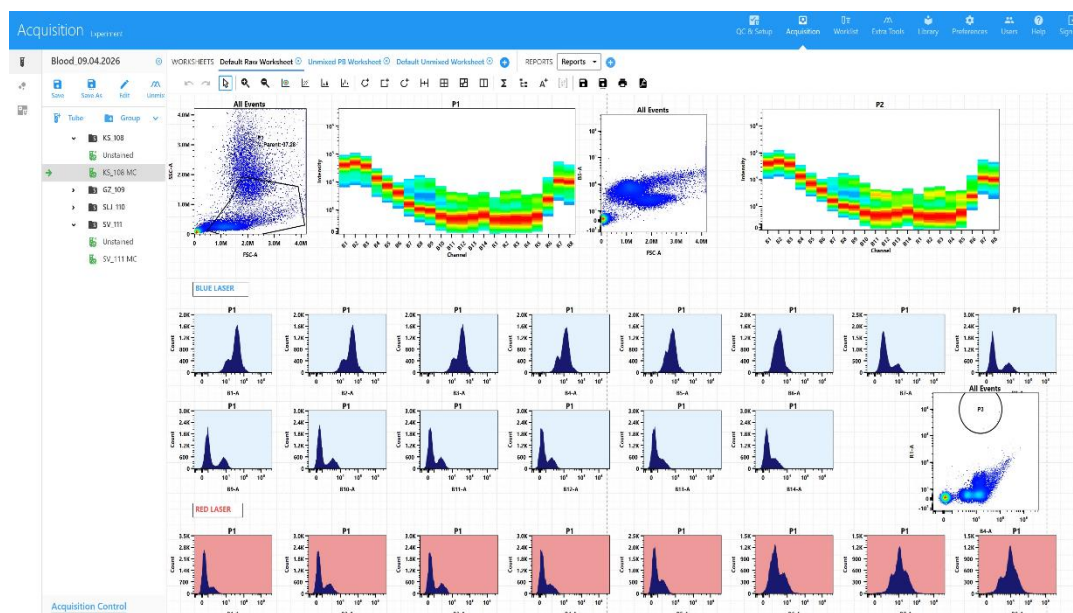


Fig. 1. Aspects of the raw data obtained by spectral flowcytometry. Analysis of peripheral blood.

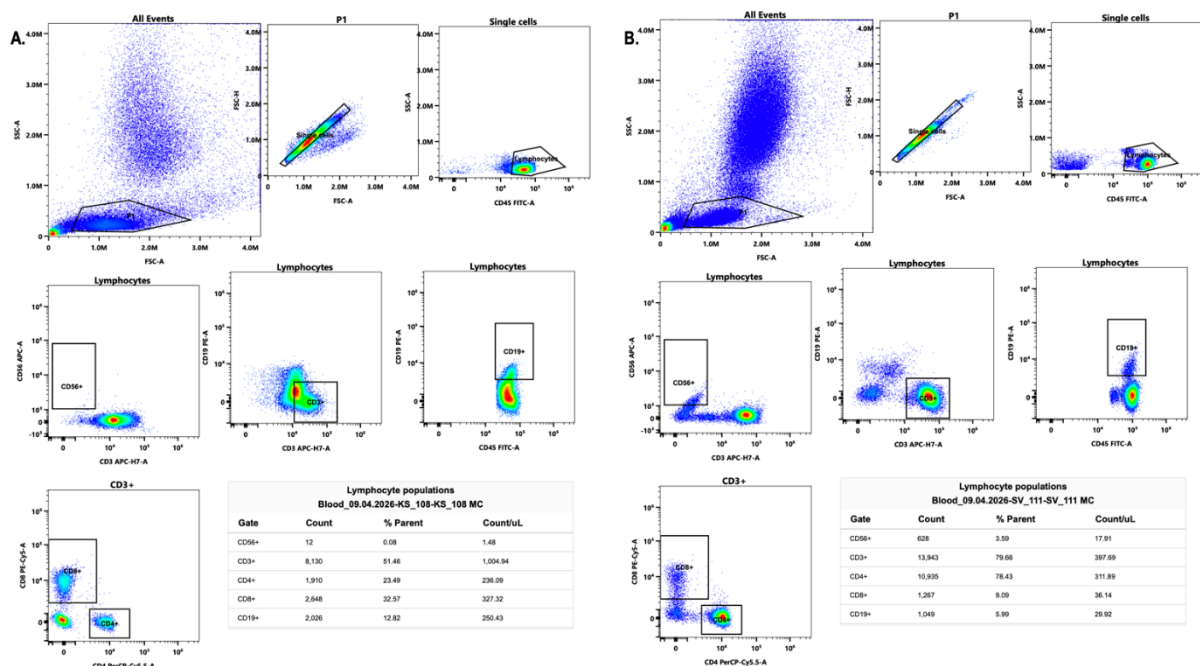


Fig. 2. KS_108 (A) and SV_111 (B) flowcytometric immune profile in endometrial cancer patients

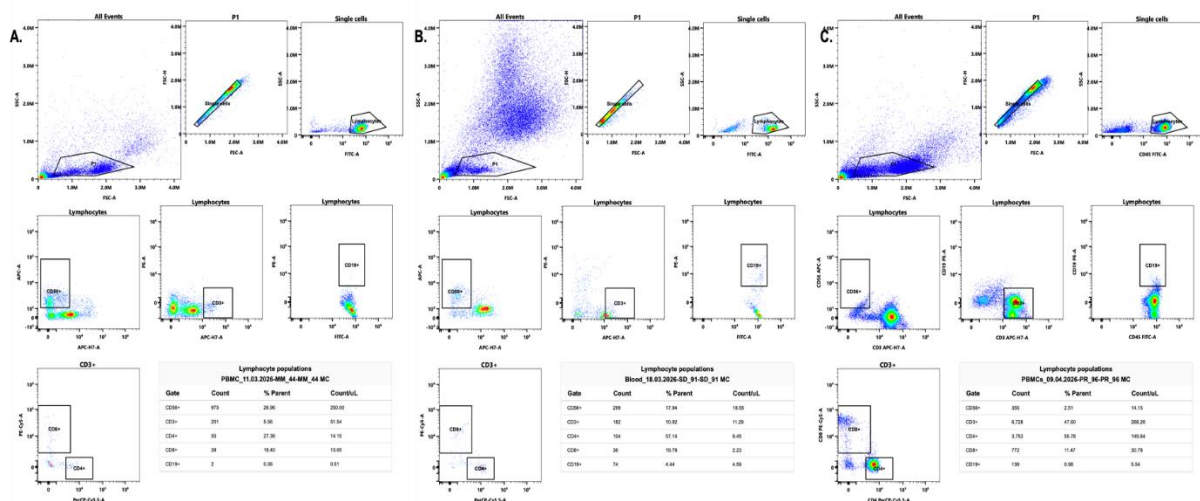


Fig. 3. MM_44 (A), SD_91 (B), PR_96 (C) ovarian cancer patients immune profile

Integrated analysis of molecular and immune profiles

The following results were obtained by integrated all the parameters into the Expert System Software v2:

Patient Summary. KS_108

This patient has ovarian cancer with an IDH2 p.Arg159Cys activating hotspot mutation at low VAF (5.3%) and an STK11 p.Gly276Asp likely loss-of-function alteration at higher VAF (48.2%). The supplied evidence does not identify any guideline-supported or clinically validated mutation-directed targeted therapy for either alteration in ovarian cancer; the only treatment-linked signal is preclinical evidence suggesting possible reduced carboplatin sensitivity in STK11-altered ovarian models. Immune profiling shows low CD4 and low NK cells, which may be relevant for infection risk and treatment tolerance monitoring.

Patient Summary. SV_111

The provided profile contains normal lymphocyte subset measurements and no listed somatic variants, cancer diagnosis, stage, histology, receptor status, or prior treatment history. Because no actionable tumour molecular alterations or disease context are available, a precision oncology treatment recommendation cannot be made from the supplied data.

Patient Summary. PR_96

The provided profile contains immune cell subset data showing low B cells and low NK cells with otherwise normal T-cell markers, but no somatic variants, cancer diagnosis, stage, histology, receptor status, prior therapies, or actionable molecular alterations are provided. Without a defined malignancy context or gene-level evidence digests, no evidence-based precision oncology treatment recommendation can be made.

Patient Summary. SD_91

The patient has a somatic MET p.Thr1010Ile missense variant at a VAF of 47.8% in the setting of ovarian neoplasm. Available evidence indicates no on-label actionable support for MET-targeted therapy in this disease context, and the off-label literature for MET T1010I is not sufficient to establish it as a validated therapeutic driver. Immune profiling shows low B cells, low CD3 lymphocytes, and low CD8 T cells, which may be relevant when considering immunotherapy tolerance and expected immune responsiveness. Met-Targeted Therapy - Not recommended for this patient because the available evidence does not establish MET T1010I as a validated actionable driver in ovarian neoplasms, and there is no on-label or guideline-supported basis for MET inhibitor use in this setting.

Patient Summary. MM_44

The patient has ovarian cancer with a somatic TP53 p.C275F missense mutation (VAF 35.4%), a pathogenic DNA-binding domain alteration consistent with loss of normal p53 function. In ovarian cancer, TP53 mutation is primarily a prognostic and treatment-response biomarker, with literature suggesting reduced or heterogeneous sensitivity to platinum agents rather than a validated directly targetable alteration; immune profiling shows low CD8 and low NK cells, which may be relevant for immunotherapy trial interpretation but does not create a standard treatment indication.

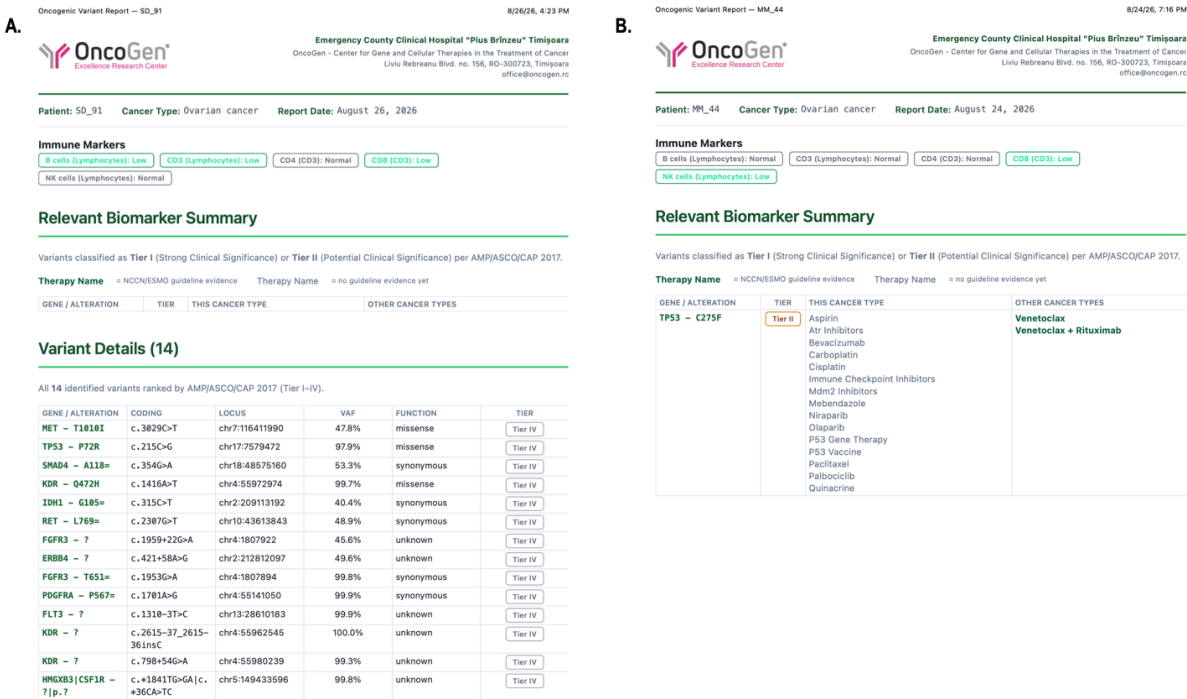


Fig. 4. ESS v2 report on 2 patients. A. SD_91 ovarian cancer; B. MM_44 ovarian cancer

The integrated analysis performed using ESS v2 demonstrated substantial heterogeneity among the five ovarian cancer patient profiles. Molecular analysis identified distinct genetic alterations, including IDH2 p.Arg159Cys, STK11 p.Gly276Asp, MET p.Thr1010Ile, and TP53 p.C275F, whereas no somatic variants were identified in the remaining patients for whom molecular data were available. Importantly, none of the identified alterations resulted in a validated, guideline-supported mutation-directed therapy for ovarian cancer, illustrating the importance of distinguishing the detection of a molecular alteration from its established clinical actionability.

Integration of the molecular findings with peripheral immune profiling provided additional patient-specific information. Several patients exhibited reduced CD4+ T-cell, CD8+ T-cell, CD3+, B-cell, and/or NK-cell populations, while one patient presented an overall normal lymphocyte profile. These immune alterations did not independently generate standard treatment indications but provided additional biological context for the interpretation of potential therapeutic strategies and for consideration of immunotherapy-related tolerance and clinical trial settings.

Overall, ESS v2 demonstrated the feasibility of integrating tumor genomic alterations with immune-profile characteristics and biomarker information to generate a more comprehensive patient-specific therapeutic assessment. In cases where no actionable molecular alteration was identified, the system was able to indicate the absence of sufficient evidence for targeted treatment rather than assigning an unsupported therapeutic option. Thus, the integrated approach provides information beyond the individual NGS or immune-profile results and may support a more cautious and evidence-oriented therapeutic orientation in precision oncology.

The ESS v2 analysis demonstrated that the presence of a somatic mutation does not necessarily translate into a clinically actionable therapeutic target and that integration with disease context and immune profiling is essential for a more appropriately contextualized therapeutic orientation.

Comparative molecular analysis of ovarian cancer profiles

To further contextualize the patient-derived NGS findings, the ovarian cancer cell line SKOV3 was also characterized by targeted NGS. Four somatic missense alterations were identified: PIK3CA p.His1047Arg, KDR p.Gln472His, FBXW7 p.Arg505Leu, and FLT3 p.Ala450Val. The SKOV3 molecular profile therefore involved alterations affecting several oncogenic signaling pathways, including PI3K/AKT and receptor tyrosine kinase signaling, providing a molecular background distinct from that observed in the analyzed patient tumors.

Comparison with the patient profiles demonstrated both molecular heterogeneity and convergence at the level of cancer-associated signaling pathways. The MET p.Thr1010Ile alteration identified in patient SD_91 and the KDR and FLT3 alterations detected in SKOV3 represent changes involving receptor tyrosine kinase signaling, although the specific genes and variants differed. Importantly, ESS v2 indicated that the MET alteration in SD_91 did not represent a validated actionable driver in ovarian cancer, illustrating that the identification of an alteration within an oncogenic signaling pathway does not necessarily translate into a clinically supported targeted therapy. In contrast, the TP53 p.C275F alteration identified in patient MM_44 represented a distinct mechanism associated with loss of normal p53 function, while patient PR_96 did not present a reportable somatic mutation but showed alterations in immune-cell subsets.

Overall, the comparison highlighted the molecular heterogeneity of ovarian cancer and demonstrated that distinct genetic alterations may converge on related oncogenic processes while having different levels of clinical actionability. The inclusion of SKOV3 further illustrated the potential of the integrated NGS–ESS v2 workflow to characterize molecular profiles in both patient-derived tumors and experimental ovarian cancer models, while emphasizing the need to distinguish molecular pathway involvement from clinically validated therapeutic targets.

Discussion

Precision oncology has progressively shifted from the identification of individual molecular alterations toward the integration of heterogeneous biological and clinical information to support patient-specific therapeutic decisions. Next-generation sequencing (NGS) has become an important component of this approach, particularly in tumor types in which actionable genomic alterations may inform treatment selection. In ovarian cancer, the European Society for Medical Oncology (ESMO) recommends the use of tumor multigene NGS, reflecting the increasing relevance of molecular characterization in clinical management [15]. However, the detection of a genomic alteration does not, by itself, establish clinical actionability. Interpretation requires consideration of the specific variant, tumor type, level of evidence, available therapies, and clinical context [16,17]. The present study illustrates this distinction and proposes an integrated workflow in which NGS-derived molecular information is evaluated together with peripheral immune profiling, biomarkers and clinical information using the Expert System Software version 2 (ESS v2).

The NGS analysis of the five ovarian cancer cases demonstrated considerable molecular heterogeneity. Alterations involving IDH2, STK11, MET and TP53 were identified in individual patients, whereas no reportable somatic variants were identified in the other analyzed cases. Importantly, the presence of a molecular alteration did not automatically translate into a mutation-directed therapeutic option. For example, although KS_108 harbored IDH2 p.Arg159Cys and STK11 p.Gly276Asp, neither alteration provided a validated ovarian cancer-specific targeted treatment indication within the evidence framework used by ESS v2. Similarly, the MET p.Thr1010Ile alteration identified in SD_91 was not interpreted as a validated actionable MET driver in ovarian cancer, despite its relatively high variant allele frequency. TP53 p.C275F identified in MM_44 represented a pathogenic alteration affecting the DNA-binding domain of p53, but its presence does not currently constitute, by itself, an indication for a specific TP53-directed therapy in ovarian cancer. These findings emphasize an important principle of precision oncology: molecular detection and clinical actionability are distinct analytical levels.

This distinction is consistent with established recommendations for somatic variant interpretation. The AMP/ASCO/CAP framework emphasizes classification of cancer-associated variants according to their clinical significance and recommends that molecular findings be interpreted in the context of histological and clinical information rather than considered in isolation [16]. Similarly, recent ESMO recommendations for genomic reporting emphasize that clinical genomic reports should include functional annotation, assessment of clinical actionability and matching of alterations to potential therapy indications [17]. In this respect, the ESS v2 workflow extends the interpretation of the primary NGS result by organizing molecular information

according to evidence level and by contextualizing potential therapeutic associations using clinical guidelines, curated knowledge sources and scientific literature.

An important observation from the present study was therefore not the identification of a specific targetable alteration, but the ability of the integrated system to explicitly identify situations in which the available molecular evidence was insufficient to support a targeted therapeutic recommendation. This is particularly relevant in precision oncology, where large sequencing panels can generate numerous alterations of uncertain or limited clinical relevance. An effective decision-support framework should therefore not only identify potential therapeutic matches, but also prevent overinterpretation of molecular findings. In the present series, ESS v2 generated a more conservative interpretation for alterations for which evidence did not establish a validated therapeutic relationship. Such negative or non-actionable findings are clinically relevant because they can prevent the interpretation of a molecular alteration as equivalent to a treatment indication.

The peripheral immune profiling provided a complementary dimension to the molecular characterization. Substantial inter-patient variability was observed in the distribution of CD3+, CD4+, CD8+, CD19+ and CD56+ lymphocyte populations. MM_44 showed reduced CD8+ T-cell and NK-cell populations, KS_108 had reduced CD4+ T-cell and NK-cell populations, PR_96 showed reduced B-cell and NK-cell populations, and SD_91 demonstrated reduced B-cell, total T-cell and CD8+ T-cell populations. In contrast, SV_111 exhibited a normal distribution of all analyzed lymphocyte subsets. These findings illustrate that patients with the same broad tumor diagnosis may display substantially different systemic immune profiles.

The relevance of immune profiling in oncology derives from the recognition that tumor behavior and therapeutic response are influenced not only by tumor-intrinsic molecular characteristics but also by the immune context in which the tumor develops [18]. Increasing evidence has demonstrated that immune-cell composition can provide information relevant to prognosis and treatment response, although the biological and clinical meaning of individual peripheral immune-cell populations is highly context dependent. High-dimensional immune profiling studies have further demonstrated that immune parameters can contain predictive information regarding response to immunotherapy, although such associations require disease-specific validation and cannot be inferred from isolated lymphocyte measurements alone [19]. Accordingly, the reduced CD4+, CD8+, CD3+, B-cell or NK-cell populations observed in the present study should be regarded as patient-specific biological context rather than as independent indications for a particular therapy.

The integration of immune information with molecular data represents an important conceptual advantage of the proposed workflow. NGS provides information primarily related to tumor genomic alterations, whereas peripheral flow cytometry provides information regarding the systemic immune landscape. These datasets answer different biological questions and therefore cannot be considered interchangeable. Their integration may nevertheless provide a broader representation of the individual patient. For example, KS_108 combined two molecular alterations without an established ovarian cancer-specific targeted treatment indication with reduced CD4+ and NK-cell populations. Similarly, SD_91 combined a non-validated MET alteration with reductions in B cells, total T cells and CD8+ T cells. The integrated interpretation does not transform these findings into a therapeutic indication; rather, it provides a more comprehensive description of the patient's molecular and immunological context.

This distinction is particularly important when considering immunotherapy. Although immune profiling is increasingly incorporated into cancer research, the presence or absence of a particular circulating lymphocyte population should not be interpreted as a validated predictor of treatment response in the absence of appropriate clinical evidence. Therefore, the role of immune profiling within ESS v2 is better understood as a contextual layer that may contribute to therapeutic assessment, identification of potentially relevant considerations and selection of hypotheses for further clinical evaluation, rather than as a standalone treatment-selection algorithm.

The analysis of the SKOV3 ovarian cancer cell line further illustrated the molecular heterogeneity that can occur within ovarian cancer. The identification of PIK3CA p.His1047Arg, KDR p.Gln472His, FBXW7 p.Arg505Leu and FLT3 p.Ala450Val demonstrated alterations involving PI3K/AKT and receptor tyrosine kinase-related signaling pathways. Comparison with the patient profiles showed that molecular alterations may converge at the level of biological pathways while remaining distinct at the level of specific genes and variants. The MET alteration identified in SD_91 and KDR/FLT3 alterations in SKOV3 provide an example of this pathway-level relationship. Importantly, however, pathway involvement should not be equated with clinical actionability. The SKOV3 findings therefore serve primarily as a molecular reference for an experimental ovarian cancer model and further illustrate why interpretation of individual variants must remain disease- and variant-specific.

The broader significance of the present approach is related to the increasing need for multimodal data integration in precision oncology. Cancer is characterized by biological heterogeneity across multiple levels, and isolated analytical modalities provide only partial representations of this complexity. Multimodal integration has consequently emerged as an important direction for precision oncology, with the objective of combining genomic, molecular, immune and clinical information into more comprehensive patient representations [20]. Recent developments in artificial intelligence further emphasize the potential of computational approaches to integrate high-dimensional molecular and clinical datasets and facilitate their interpretation [21]. Similarly, recent decision-support systems such as the Knowledge Connector demonstrate the feasibility of integrating patient-specific molecular information with external biomedical knowledge to support and document precision oncology decision-making [22,23].

Within this context, the principal contribution of the present work is not the introduction of NGS, flow cytometry or artificial

intelligence as individual technologies. Rather, it is the implementation of a workflow in which these heterogeneous data types are brought together within a patient-specific analytical framework. The ESS v2 system integrates NGS-derived variants, immune-cell subset information, biomarkers and clinical information and connects the resulting profile with relevant clinical guidelines and scientific literature. This creates a conceptual progression from data acquisition to integration, interpretation and therapeutic orientation. Such an approach may be particularly valuable because the output is not limited to a list of molecular alterations but instead attempts to place each finding within the broader clinical and biological context of the individual patient.

Another relevant feature of the present approach is the use of evidence-oriented interpretation. ESS v2 incorporates multiple knowledge sources, including clinical guidelines and curated molecular databases, and applies the AMP/ASCO/CAP framework for variant classification. This is important because the rapidly changing nature of cancer genomics makes interpretation dependent on continuously evolving evidence. The need for standardized and clinically contextualized reporting has also been emphasized by recent ESMO recommendations, which highlight the importance of presenting genomic alterations together with their functional annotation, clinical actionability and potential therapeutic implications [17]. The incorporation of links to relevant scientific publications within ESS v2 may additionally facilitate evidence retrieval and provide traceability for the biological and clinical interpretation of individual findings. Importantly, these literature links should be regarded as a mechanism for evidence retrieval and contextualization rather than as a substitute for multidisciplinary clinical judgment.

The present study has several limitations. First, the number of analyzed patients was small, with only five ovarian cancer cases, and therefore the results cannot be used to establish clinical validity, diagnostic performance, treatment efficacy or patient-outcome benefit. The heterogeneous molecular and immune profiles observed in this small cohort should be considered illustrative rather than representative of the ovarian cancer population. Second, the immune analysis was based on a limited panel of circulating lymphocyte populations and does not provide a comprehensive characterization of the tumor immune microenvironment. Third, the absence of complete clinical information for some cases limits the extent to which molecular findings can be translated into individualized clinical recommendations. Fourth, the present analysis does not compare ESS v2-generated therapeutic orientations prospectively with decisions made by multidisciplinary tumor boards or with patient outcomes. Such comparisons will be necessary to determine whether the integrated approach improves interpretation, reduces uncertainty or contributes to clinically meaningful decision-making.

Despite these limitations, the study provides a proof-of-concept for the feasibility of integrating heterogeneous patient-level information into a unified precision oncology workflow. An important strength is that the system does not equate the presence of a mutation with the existence of a targeted treatment. Instead, the examples presented demonstrate the value of identifying both actionable and non-actionable findings and of placing genomic results in the context of immune and clinical information. This conservative interpretation is particularly important in clinical environments in which increasingly comprehensive sequencing generates a growing number of molecular findings whose therapeutic relevance may remain uncertain.

Future studies should evaluate the platform in larger and clinically annotated cohorts, incorporate longitudinal clinical and treatment-response data, and compare ESS v2 outputs with multidisciplinary expert interpretation. Expansion of immune profiling, incorporation of additional molecular and pathological biomarkers, and systematic updating of the underlying evidence sources may further increase the clinical utility of the platform. Prospective evaluation will ultimately be required to determine whether integrated molecular-immunological decision support can improve the consistency, efficiency and clinical relevance of precision oncology workflows.

In conclusion, this proof-of-concept study demonstrates the feasibility of integrating tumor NGS, peripheral immune profiling, biomarkers and clinical information within an AI-supported analytical framework. The findings highlight that the principal value of such integration is not simply the accumulation of additional data, but their contextual interpretation at the individual-patient level. By distinguishing molecular alterations from clinically actionable findings and by incorporating immune characteristics and external evidence, ESS v2 provides a broader patient-centered framework for therapeutic orientation. Although clinical benefit cannot be established from the present small cohort, the approach provides a basis for further prospective validation of integrated molecular, immunological and AI-assisted precision oncology.

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O ABORDARE INTEGRATĂ MOLECULARĂ, IMUNOLOGICĂ ȘI BAZATĂ PE INTELIGENȚĂ ARTIFICIALĂ ÎN ONCOLOGIA DE PRECIZIE

Rezumat

Context: Oncologia de precizie se bazează din ce în ce mai mult pe caracterizarea modificărilor moleculare specifice tumorii și a profilului imun specific pacientului pentru a sprijini deciziile terapeutice individualizate. Secvențierea de nouă generație (NGS) furnizează informații valoroase privind mutațiile somatice și potențiala lor relevanță clinică, în timp ce profilarea imunologică oferă informații complementare privind statusul imun sistemic al pacientului. Cu toate acestea, interpretarea acestor seturi de date heterogene este realizată frecvent în mod independent, ceea ce poate limita evaluarea comprehensivă a fiecărui pacient.

Obiectiv: Studiul a avut ca obiectiv evaluarea fezabilității și a potențialei valori adăugate a unui Software Expert System (ESS) bazat pe inteligență artificială (IA), destinat integrării informațiilor moleculare, imunologice, privind biomarkerii și clinice într-un raport comprehensiv, specific pacientului, care să sprijine orientarea terapeutică personalizată.

Metode: Au fost investigați cinci pacienți cu tumori solide, dintre care doi cu cancer endometrial și trei cu cancer ovarian. Probele tumorale au fost analizate prin secvențiere de nouă generație (NGS) țintită, utilizând un panel AmpliSeq HotSpot, iar probele de sânge periferic au fost caracterizate prin citometrie în flux spectrală. Datele moleculare și imunologice obținute au fost integrate cu informațiile disponibile privind biomarkerii și datele clinice în cadrul Software-ului Expert System bazat pe IA. Modificările genetice identificate au fost clasificate în funcție de relevanța lor clinică, iar potențialele implicații terapeutice au fost evaluate utilizând ghiduri clinice bazate pe dovezi. Sistemul a furnizat, de asemenea, identificatori PubMed (PMID) pentru publicațiile științifice relevante pentru mutațiile, parametrii imunologici și biomarkerii identificați.

Rezultate: Analizele NGS și profilarea imunologică au furnizat în mod independent informații complementare privind caracteristicile moleculare ale tumorii și statusul imun al pacientului. Integrarea acestor date în cadrul Expert System a generat un profil comprehensiv, specific fiecărui pacient, prin stabilirea unor corelații între modificările genomice, parametrii imunologici, biomarkerii și caracteristicile clinice. Comparativ cu interpretarea individuală a fiecărui set de date, analiza integrată a oferit un context suplimentar pentru evaluarea modificărilor moleculare relevante din punct de vedere clinic și a generat posibilități mai ample de orientare terapeutică specifică pacientului. Corelarea rezultatelor integrate cu literatura științifică relevantă a susținut, de asemenea, interpretarea bazată pe dovezi.

Concluzii: Acest studiu de tip "proof-of-concept" demonstrează potențiala valoare a integrării informațiilor moleculare, imunologice, privind biomarkerii și clinice într-o singură platformă de suport decizional bazată pe IA. O astfel de abordare poate facilita o interpretare mai comprehensivă și centrată pe pacient a datelor oncologice complexe și poate sprijini orientarea terapeutică personalizată. Sunt necesare studii suplimentare, care să includă cohorte mai mari de pacienți și evaluarea rezultatelor clinice, pentru validarea utilității clinice a acestei abordări integrate de oncologie de precizie.

Cuvinte cheie: NGS, profil imunologic, Software Expert System, oncologie de precizie

METHODOLOGICAL EVALUATION OF MEDIA COMPOSITION AND SERUM DEPRIVATION KINETICS ON THE PROLIFERATION OF HUMAN PRE-B LYMPHOBLASTS

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ABSTRACT

Background: Human tumor cell lines serve as vital preclinical models for deciphering cancer biology and evaluating therapeutic interventions. While environmental parameters heavily influence *in vitro* phenotypes, a precise mathematical understanding of how basal media architecture and serum availability govern proliferation dynamics remains critical. This study provides a rigorous methodological evaluation of media composition and serum deprivation kinetics using the NALM-6 human pre-B lymphoblast model.

Methods: NALM-6 suspension cultures were monitored longitudinally over an 8-day timeline. In the first cohort, proliferation was evaluated in RPMI 1640 versus DMEM/F12, both supplemented with 10% fetal bovine serum (FBS), alongside a serum-free baseline. In the second cohort, serum deprivation kinetics were mapped by titrating FBS concentrations (10%, 5%, and 1%). Proliferation dynamics were explicitly quantified through daily calculations of population doubling numbers (N_d) and doubling times (T_d), coupled with plasma membrane integrity tracking via trypan blue vital staining.

Results: In nutrient-replete conditions (10% FBS), both RPMI 1640 and DMEM/F12 sustained robust exponential growth kinetics with cell viability remaining above 90% through Day 8. Conversely, total serum deprivation triggered a progressive breakdown in cellular homeostasis, resulting in a linear decline in survival down to 50%. Serum titration dynamics revealed a distinct kinetic divergence: while a critical threshold of 1% FBS failed to support log-phase growth, a 5% FBS concentration successfully preserved near-optimal population doubling metrics and high viability (>85%), demonstrating a non-linear cellular response to serum withdrawal.

Conclusions: These findings clarify the kinetic boundaries of pre-B lymphoblast proliferation *in vitro*. The mathematical characterization of NALM-6 doubling dynamics demonstrates that serum deprivation kinetics follow a threshold-dependent pattern rather than a strict linear decline, offering key methodological insights for standardizing suspension cell models in translational research.

Keywords: NALM-6 cell line; Pre-B lymphoblasts; Proliferation kinetics; Serum deprivation; Basal media composition; Population doubling time.

INTRODUCTION

Although recent advances in proteomics and genomics have opened new avenues for translational research and enabled the direct evaluation of clinical samples, reliable preclinical models remain indispensable for testing therapeutic strategies. Human tumour cell lines represent foundational models currently utilized in laboratories to investigate cancer biology and evaluate the therapeutic efficacy of anticancer agents. The cultivation of tumour cell lines constitutes one of the most widely employed techniques for a variety of *in vitro* pathogenic models and has formed the cornerstone of our current understanding of cancer biology.

Cell culture: a brief historical overview

Animal cell culture possesses a history spanning over a century. Ross Harrison, Wilhelm Roux, and Leo Loeb established the earliest tissue cultures during the 19th and early 20th centuries. In these pioneering animal cell culture experiments, tissue fragments were cultivated in a mixture of blood and warm saline solution. It was observed that certain cells initiated growth and aggregated at the culture surface. Subsequently, specific cells were isolated and continued to divide for a limited number of generations. However, these primary cells could not be maintained in long-term culture [1]. Montrose Burrows and Alexis Carrel were the first researchers to cultivate small tumours *in vitro*, utilizing scaffolds (such as fragments of silk or cotton) for tissue isolation and cultivation [2]. Also in 1911, Warren Lewis investigated and characterized the influence of factors responsible for cell survival and growth within the culture medium. In 1916, Francis Peyton Rous and F.S. Jones introduced trypsin solution for the first time to prepare cell suspensions [3].

During the 1940s, Wilton Earle at the National Cancer Institute designed glass tube culture vessels with conical tips ("T-flasks"), developed a chemically defined medium for cell culture, and established the first continuous cell line, namely mouse fibroblasts (L cells) [4]. In 1948, the first mouse cell strain, L929, was cloned and remains widely used in laboratories worldwide. These

cells were grown in a balanced salt solution supplemented with serum, ascitic fluid, and chick embryo extract [5]. Another milestone in cell culture history was the development of a cell culture medium by Harry Eagle in 1955 [6], which was subsequently modified by Renato Dulbecco (known as Dulbecco's Modified Eagle Medium - DMEM). Instead of containing over 50% serum or tissue fluids, the cell culture medium became more than 80% chemically defined basal medium.

Undoubtedly, a major breakthrough in mammalian cell culture development was the isolation of HeLa cells in 1951 by George Gey. Utilizing HeLa cells, researchers successfully achieved the *in vitro* propagation of the poliovirus, leading to the development of the polio vaccine [7]. HeLa cells have been employed across numerous research domains, including oncology and the evaluation of toxic substances or radiation effects on living systems. Following the establishment of HeLa cells, multiple human cell lines—both normal and malignant—were isolated from various tissues. Some cell lines retained the phenotypic characteristics of their tissue of origin. For instance, HepG2 cells, derived from hepatocellular carcinoma, express numerous hepatic markers and secrete albumin [8].

In 1961, Leonard Hayflick and Paul Moorhead discovered that primary human cells in *in vitro* cultures can replicate only a finite number of times. The researchers investigated the *in vitro* replicative capacity of primary human fibroblasts and concluded that the aging process occurs at the cellular level, contradicting the prevailing paradigm of that era, which asserted that *in vitro* cells could replicate indefinitely [9]. Since the inception of cell culture, autoclaving has been utilized to sterilize liquids, consumables, and equipment. However, saline solutions and other components, including nutrient mixtures containing tissue fluids, could not be easily sterilized via heat due to their thermal lability. The commercialization of nitrocellulose and the manufacture of ultrafiltration membranes enabled the sterilization of complex nutrient mixtures, significantly advancing cell research.

RPMI 1640, commonly referred to as RPMI medium, is a standard growth medium utilized in cell culture. This medium was developed by George E. Moore, Robert E. Gerner, and H. Addison Franklin in 1966 at the Roswell Park Memorial Institute, from which its name is derived. It represents a modification of McCoy's 5A medium (or RPMI 1630). It was originally formulated to support lymphoblastoid cells in suspension cultures, though it can also support a wide variety of adherent cells. It has traditionally been used for the propagation of human lymphocytes. This medium contains an elevated concentration of phosphate and is formulated for use in a 5% carbon dioxide (CO₂) atmosphere. It was historically employed for expanding human lymphoid cells in the absence of fetal serum. RPMI 1640 utilizes a bicarbonate buffering system and differs from most culture media by maintaining a pH of approximately 8. When appropriately supplemented with serum, RPMI 1640 facilitates the cultivation of numerous cell types, particularly human T or B lymphocytes, other hematogenous bone marrow cells, and cell hybridomas [10].

Fetal bovine serum (FBS), also referred to as fetal calf serum (FCS), is harvested from bovine fetuses in abattoirs using a closed-circuit collection system. Fetal serum is the most widely utilized supplement for the *in vitro* culture of eukaryotic cells. This preference is due to its low antibody levels and high concentration of growth factors, which facilitate diverse applications in cell culture systems. Bovine serum albumin (BSA) constitutes a major component of fetal bovine serum. The rich variety of proteins in fetal serum maintains cultivated cells in an environment that supports survival, growth, and proliferation. However, FBS is not a fully defined component, resulting in significant batch-to-batch compositional variability. Nonetheless, it remains widely used because the efficacy of serum-free media is often limited [11].

Based on these considerations, the present study aimed to conduct a rigorous methodological evaluation of the *in vitro* proliferation and kinetic behaviour of a human leukemic cell line. Specifically, the dynamics of a standardized suspension model, the NALM-6 line, were mapped across culture environments of varying composition and serum availability. NALM-6 cells serve as excellent models for B-cell differentiation, central nervous system (CNS) infiltration, metastasis, and pre-B leukemia research. Phenotypically, these cells express both CD24 and CD19 markers, making NALM-6 cells ideal candidates for evaluating the *in vivo* efficacy of chimeric antigen receptor (CAR) T cells and other immunotherapies. Particular attention was directed toward characterizing the baseline kinetic thresholds and proliferation limits under conditions of progressive serum deprivation.

MATERIALS AND METHODS

The NALM-6 cell line, a human pre-B lymphoblast line originally isolated from the peripheral blood of a 24-year-old male patient diagnosed with acute lymphoblastic leukemia, was utilized. The cell line was purchased from the DSMZ (German Collection of Microorganisms and Cell Cultures GmbH).

Experimental design overview

To systematically evaluate the proliferation boundaries and adaptive mechanics of the NALM-6 cell line, a two-phase experimental matrix was established.

- **Phase I (basal media kinetics):** Focused on evaluating the impact of basal media composition on lymphoid expansion. Cell suspensions were monitored concurrently in RPMI 1640 versus DMEM/F12, both supplemented with standard 10% Fetal Bovine Serum (FBS), alongside a serum-free baseline control (RPMI 1640 without FBS) to map cellular homeostasis breakdown.

- **Phase II (serum deprivation kinetics):** Aimed to isolate the non-linear kinetic threshold driven by serum withdrawal. Based on Phase I profiles, lymphoblasts were subjected to a progressive serum titration gradient (10%, 5%, and 1% FBS) utilizing the optimal basal medium.

Both experimental phases were carried out over a standardized 8-day longitudinal timeline under identical environmental parameters (37°C, 5% CO₂). Cellular kinetics were strictly quantified via automated mathematical metrics (doubling parameters) alongside plasma membrane stability tracking.

Cell thawing, passaging, and seeding

All experiments were conducted under strict operating conditions, adhering to safety and aseptic protocols. Work surfaces, instruments, reagent bottles, culture media, and culture plates were decontaminated prior to each experimental step. All open handling procedures were performed exclusively within a laminar flow hood.

In the initial phase, NALM-6 cells (stored in cryovials at passage 7) were thawed using the manufacturer-recommended medium (RPMI 1640 + 10% FBS + 1% P/S). Following thawing, the culture vessel (T25 flask) was placed in an incubator at 37°C in a humidified atmosphere of 95% O₂ and 5% CO₂. Cell viability and the number of thawed cells were assessed after 24 hours. Cell counting and viability assessments were performed using vital dye staining (trypan blue), with live and dead cells quantified via a hemocytometer.

To obtain a sufficient cell number for the planned experimental design, two successive passages were executed. Finally, cells were seeded at a standard initial density of 2×10^5 cells/mL in multi-well culture plates, with 3 mL of cell suspension allocated per well.

Evaluation of basal media cohorts

During this stage, three distinct culture media environments were compared as follows:

- **Medium 1:** RPMI 1640 + 10% FBS + 1% penicillin/streptomycin (P/S)
- **Medium 2:** RPMI 1640 + 1% penicillin/streptomycin (P/S) [Serum-free control]
- **Medium 3:** DMEM/F12 + 10% FBS + 1% penicillin/streptomycin (P/S)

Cells were seeded in 24-well culture plates at a density of 2×10^5 cells/mL (3 mL of cell suspension per well). Cell proliferation and morphological changes were assessed daily over an 8-day period through macroscopic examination, inverted light microscopy, and cell counting.

Serum titration cohorts

Following the baseline evaluation, the longitudinal procedure was repeated to map the kinetics of progressive serum deprivation by titrating the FBS concentration of Medium 1 as follows:

- **Medium 1A:** RPMI 1640 + 10% FBS + 1% P/S
- **Medium 1B:** RPMI 1640 + 5% FBS + 1% P/S
- **Medium 1C:** RPMI 1640 + 1% FBS + 1% P/S

The seeding protocol and kinetic monitoring intervals were completely identical to those employed in the baseline phase.

RESULTS

1. Proliferation kinetics in different basal media

Microscopic examination using an inverted light microscope focused on evaluating the morphological appearance of the suspension cells and identifying potential signs of cellular distress. Under physiological conditions, NALM-6 lymphoblasts exhibit a stable spherical morphology and grow strictly in suspension without adhering to the surface of the culture vessel. Throughout the 8-day experimental period, no deviations from normal cell morphology were detected in serum-replete cohorts. Cells were counted daily, and the primary proliferation curves are reflected in Figures 1, 2, and 3.

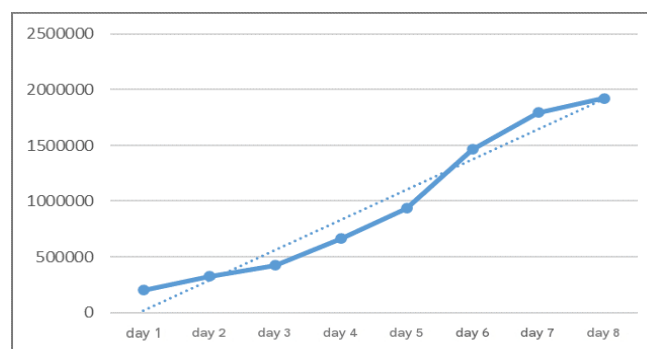


Fig. 1: Daily proliferation kinetics of NALM-6 cell cultivated in Medium 1

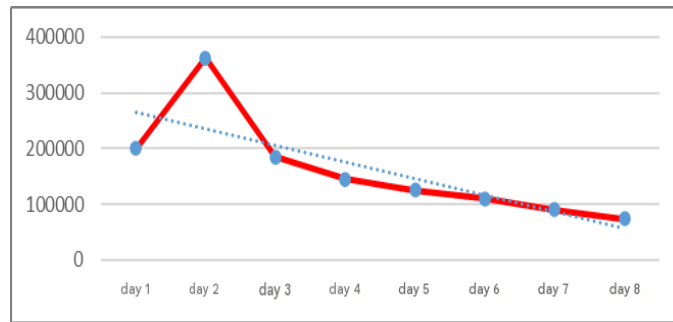


Fig. 2: Daily proliferation kinetics of NALM-6 cell cultivated in Medium 2

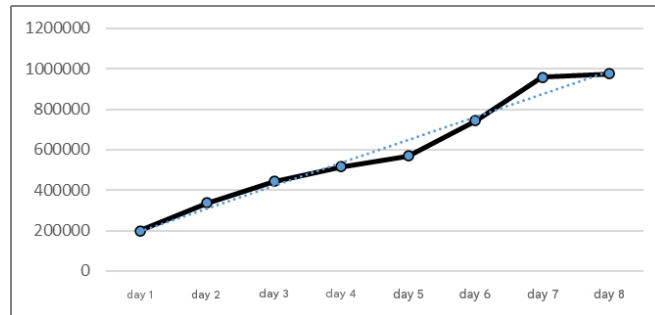


Fig. 3: Daily proliferation kinetics of NALM-6 cell cultivated in Medium 3

To achieve a precise mathematical analysis of proliferation dynamics, the population doubling number (N_d) and population doubling time (T_d) were calculated daily relative to the initial cell density. Population doubling was determined using the standard equation:

$$N_d = \frac{\ln\left(\frac{X}{X_0}\right)}{\ln 2}$$

where N_d represents the number of population doublings within a given time frame, X_0 represents the number of viable cells at $t = 0$, and X is the number of viable cells at time t .

The population doubling time was subsequently resolved as follows:

$$T_d = \frac{\Delta t}{N_d}$$

These descriptive kinetic parameters are compiled in Tables I and II, and illustrated in Figures 4 and 5.

Table I. Daily cell population doubling values (N_d) relative to Day 1 across different basal media

Medium	Day 2 vs 1	Day 3 vs 1	Day 4 vs 1	Day 5 vs 1	Day 6 vs 1	Day 7 vs 1	Day 8 vs 1
Medium 1	0.689	1.087	1.733	2.229	2.870	3.166	3.267
Medium 2	0.858	-0.112	-0.464	-0.672	-0.862	-1.152	-1.434
Medium 3	0.755	1.146	1.365	1.511	1.892	2.263	2.285

Table II. Daily cell population doubling times (T_d , days) relative to Day 1 across different basal media

Medium	Day 2 vs 1	Day 3 vs 1	Day 4 vs 1	Day 5 vs 1	Day 6 vs 1	Day 7 vs 1	Day 8 vs 1
Medium 1	1.450	1.839	1.730	1.794	1.741	1.895	2.142
Medium 2	1.165	-7.778	-6.466	-5.949	-5.797	-5.208	-4.880
Medium 3	1.324	1.745	2.198	2.647	2.642	2.651	3.062

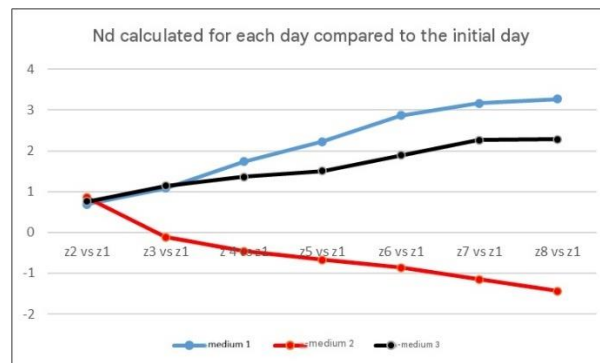


Fig. 4: Daily evolution of the cell population doubling number (Nd)

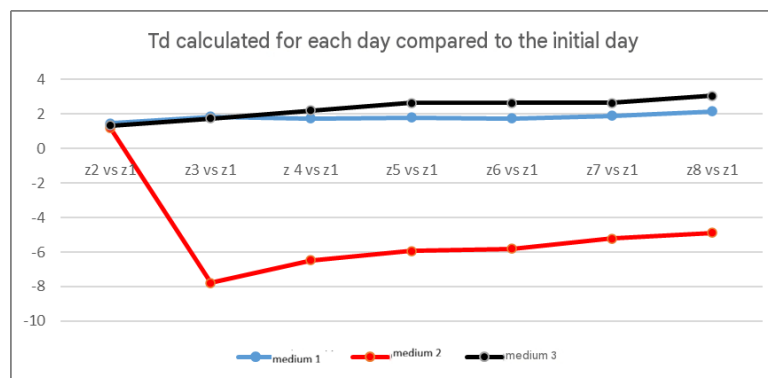


Fig. 5: Daily evolution of the cell population doubling time (Td).

Hemocytometer cell counting was performed concurrently with plasma membrane integrity assessments. Non-viable cells with compromised membranes permit the entry of the vital dye, appearing deep blue under the microscope, whereas viable cells remain unstained and bright. Microscopic evaluation of the counting chamber revealed that high cell viability was maintained in Medium 1 and 3 throughout the 8-day experiment, whereas Medium 2 exhibited a progressive decline in cell survival. Cell viability was calculated as the percentage of live cells relative to the total number of analyzed cells. The findings are detailed in Table III and visually represented in Figure 6.

Table III. Mean viability percentages of NALM-6 cells during the 8-day experimental timeline

Medium	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Medium 1	92%	95%	94%	95%	93%	90%	90%
Medium 2	90%	85%	70%	65%	65%	60%	50%
Medium 3	90%	92%	95%	95%	95%	94%	92%

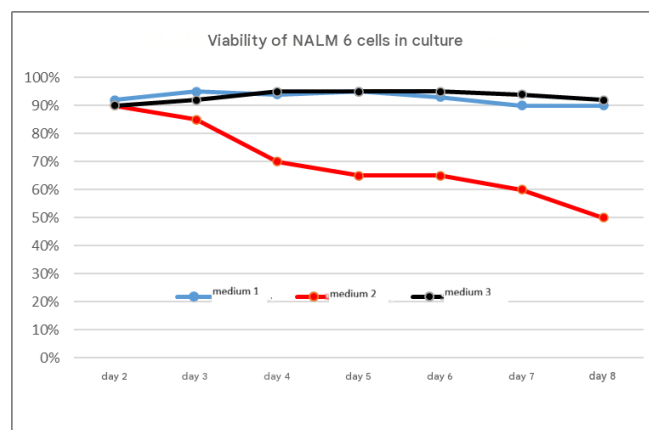


Fig. 6: Dynamics of cell viability as a function of the culture medium

2. Serum deprivation kinetics and titration

Preliminary data analysis from the first experimental set indicated that serum components are indispensable for maintaining positive log-phase kinetics in NALM-6 cells. To explore the mathematical boundaries of this requirement, a serum titration assay was established. Using RPMI 1640 as the constant basal platform, the FBS concentration was systematically adjusted to 10% (Medium 1A), 5% (Medium 1B), and 1% (Medium 1C), with kinetics tracked longitudinally over an additional 8-day window.

The absolute cell count trends under progressive serum withdrawal are displayed in Figure 7.

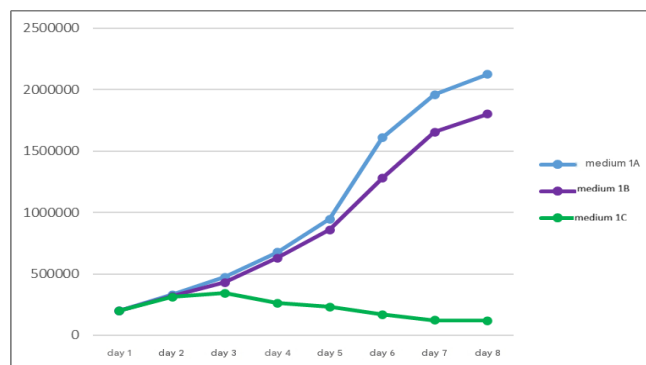


Fig. 7: Daily proliferation kinetics of NALM-6 cells subjected to varying FBS deprivation gradients

Concurrently, cellular viability parameters were evaluated for all three serum titration groups. The quantitative results are shown in Table IV and Figure 8.

Table IV. NALM-6 cell viability under progressive serum titration gradients

Medium	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Medium 1A (10% FBS)	94%	96%	95%	95%	94%	93%	93%
Medium 1B (5% FBS)	95%	95%	95%	90%	90%	85%	85%
Medium 1C (1% FBS)	90%	85%	85%	80%	75%	65%	55%

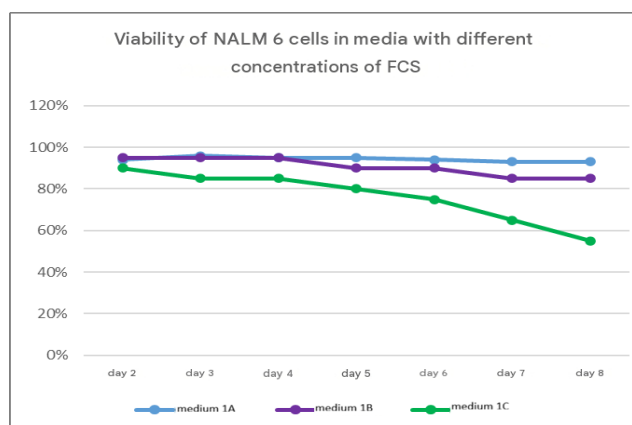


Fig. 8: Cellular viability tracking under progressive FBS concentration gradients

DISCUSSION

An immortalized cell line was selected for these experimental procedures because stable, fully characterized cultures eliminate confounding variables that could negatively impact reproducibility. A suspension cell culture was chosen because, unlike adherent cells, suspension-growing lines tend to display higher sensitivity to subtle alterations in parameters driven by culture medium composition and experimental handling.

Daily macroscopic and inverted light microscopic examinations revealed no signs of contamination, confirming the correct execution of work protocols. Regarding the microscopic appearance of NALM-6 cells, they consistently grew in suspension without adhering to the vessel walls, despite being maintained in a standard incubator rather than a shaking incubator. Even

in culture media whose composition did not favour cellular replication, the majority of cells exhibited normal morphology, characterized by clear cytoplasm and well-defined membranes.

Signs of cellular deterioration typically include peri-nuclear granularity, cell detachment (for adherent lines), and cytoplasmic vacuolation. Such deterioration can originate from various causes, including culture contamination, cell line senescence, the presence of toxic substances, or nutrient depletion caused by an expanding cell population [12]. In the case of nutrient depletion, a shift in pH is frequently observed; this was macroscopically detectable only in Medium 3, which includes a colorimetric pH indicator (the medium shifting from deep pink to yellowish).

Analysis of the data from the first experimental set regarding NALM-6 proliferation demonstrates that in media supplemented with 10% fetal serum - whether RPMI (Medium 1) or DMEM/F12 (Medium 3), the cells exhibited robust proliferation rates. Their numbers increased exponentially, aligning with standard growth curves characteristic of immortalized cell lines. Conversely, in the serum-free condition (Medium 2), following an apparent initial increase on Day 2, the total cell count trended negatively, exhibiting a slow but progressive decline. Kinetic calculations of population doublings and doubling times further corroborate these findings.

Monitoring *in vitro* growth and cell status in mammalian cell cultures is critical for achieving reproducible and reliable results, as well as for the timely detection of contamination. Microscopy remains the classical monitoring method, preceding the establishment of the first *in vitro* mammalian cell cultures. An inverted phase-contrast microscope is a staple instrument in cell culture laboratories. The primary distinction between an inverted microscope and conventional configurations is that the objectives are positioned beneath the stage rather than above it. This design facilitates the observation of suspension cells and adherent cultures within standard vessels. Through routine microscopy, cells can be monitored prior to manipulation, allowing the biological dynamics of the culture to be observed over time [13].

Microscopy enables researchers to precisely determine optimal timing for cell passaging. Following a lag phase, cells enter an exponential growth phase. Before reaching confluence or encountering density-dependent inhibition, cells are subcultured, diluted according to growth rates, and re-seeded into new vessels at the desired density. Visual confluence estimation via microscopy is particularly relevant for adherent cells.

Quantification of NALM-6 viability across the three media types revealed marked impairment in the absence of fetal serum. The application of a vital dye allows for the precise quantification of viable versus compromised or dead cells, providing an objective, quantifiable distinction between these subpopulations compared to simple visual inspection under an inverted microscope.

While various cell counting and viability assessment techniques exist [14], most rely on vital dyes (typically trypan blue) that are excluded by the intact membranes of viable cells. Cells are quantified using either automated cell counters or a manual hemocytometer. Another approach involves dual-fluorescence staining to differentiate live cells (e.g., using Calcein-AM or fluorescein diacetate) from dead cells (e.g., via propidium iodide). The primary advantage of this method is its speed (10–15 min), though it is inherently semi-quantitative. Indirect viability assays depend on measurements of cellular metabolism. In these assays, a substrate solution is incubated with cells, during which it is reduced by intracellular enzymes, and the product concentration is subsequently measured. Reaction substrates and products can be quantified via colorimetric assays (e.g., the MTT assay) or fluorometric assays (e.g., Alamar Blue).

A comparison of proliferation and viability data between the two media supplemented with 10% fetal serum (Medium 1 and Medium 3) indicates that both formulations support robust cell growth and maintain high viability, with percentages remaining above 90% through Day 8. However, examination of the growth curves reveals that while RPMI medium favours a slightly higher proliferation rate, DMEM/F12 satisfies the metabolic and functional requirements of the cells more effectively during the second half of the experimental window (Days 4–8) (Fig. 9). This trend is likely attributable to a more balanced chemical composition tailored to cellular demands; nevertheless, the divergence between the two media types was not statistically significant.

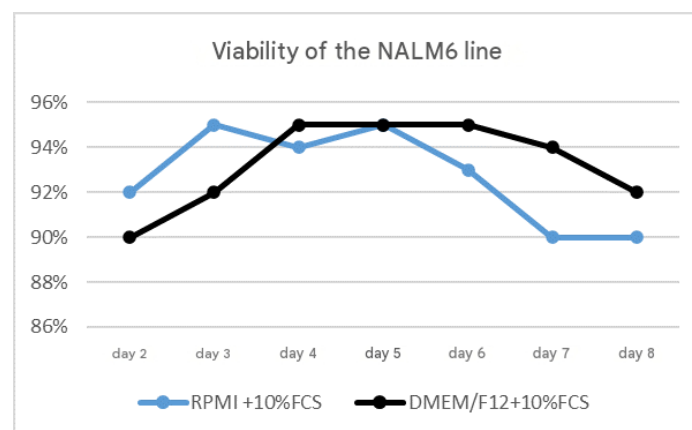


Fig. 9: Comparative overview of cell viability between serum-replete cohorts (10% FBS)

The second experimental model evaluated the impact of progressive FBS reduction. While the absolute absence of FBS does not support cell growth or viability maintenance, reducing the serum percentage could offer a viable strategy to lower experimental costs while preserving cell line integrity. RPMI 1640 was selected as the basal medium for this phase because it demonstrated a superior baseline proliferation rate compared to DMEM/F12 in the initial experiments.

Data interpretation revealed divergent growth curves (Fig. 10): sustained exponential curves were observed in media containing 10% and 5% FBS, whereas curves flattened initially and then declined in conditions either totally deprived of serum (Medium 2) or partially deprived (1% FBS, Medium 1C). Although the 10% FBS supplement yielded the optimal growth curve, experimental data and viability analysis indicate that the *in vitro* survival and proliferation of NALM-6 cells can be successfully maintained even when the FBS supplement is halved to 5%.

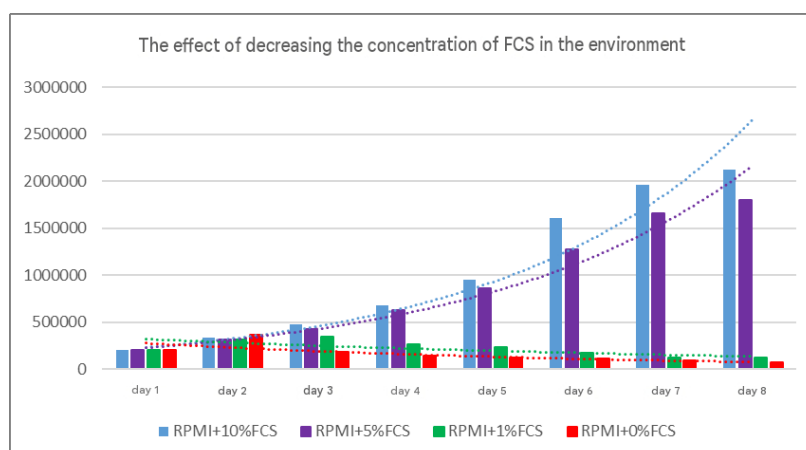


Fig. 10: Kinetic divergence of cell proliferation curves under progressive FBS(FCS) withdrawal

The capacity to maintain and expand animal and human cells has been a prerequisite for numerous breakthroughs in cellular and molecular biology, including the identification of chromosomal aberrations in cancer and the production of monoclonal antibodies. Cell culture media serve as the primary element ensuring the survival, continuous propagation, and differentiation of freshly isolated or transformed cells. These media supply energy sources, deliver oxygen, maintain osmotic pressure, and provide a physiological pH beneficial to the cultures while facilitating the clearance of metabolic waste. Furthermore, media formulations deliver signalling molecules essential for survival and growth, effectively replicating the natural fluid environment of the cells.

Prior to the development of chemically defined formulations, natural components such as plasma, serum, lymph, and tissue extracts derived from adult tissues, embryos, or amniotic fluid were utilized. A major limitation of these natural sources remains batch-to-batch and organism-to-organism variability. Additional constraints of serum as an additive include its undefined nature and the potential presence of microparticles or exosomes that are difficult to remove.

Importantly, animal-derived serum is unsuitable for cultivating cells or tissues intended for human transplantation due to the risk of eliciting an immune response against xenogeneic proteins. This adverse effect has been documented in patients following the transplantation of cells expanded in the presence of fetal calf serum [15]. Consequently, poorly defined natural sources have continued to provide critical stimuli in serum-supplemented cultures. Additional functions of natural ingredients include detoxification through the binding and inactivation of waste products, maintenance of colloid osmotic pressure, increased viscosity to protect cells from mechanical damage, and buffering against non-physiological pH fluctuations.

In classical cell culture media, sufficient quantities of plasma proteins are typically supplied via serum supplementation. Compared to adult bovine or equine serum, fetal serum is a richer source of growth factors and is highly suited for sustained cell growth. Conversely, when conducting cell differentiation experiments, serum harvested from adult animals is recommended because it does not stimulate proliferation to the same extent. For primary cell cultures, such as epidermal equivalents from skin explants, fetal serum may not be mandatory, as targeted substitution of specific required factors can be adequate [16].

In clinical scenarios where xenogeneic components are prohibited, such as the *ex vivo* expansion of human cells for cellular therapies, media can be supplemented with human-derived additives, including human plasma or human platelet lysate, thereby preventing allosensitization against animal proteins. The development of completely serum-free media advanced significantly when three primary components became available in recombinant or purified forms: albumin, transferrin, and cholesterol. The most common proteins and peptides in cell culture media are albumin, transferrin, and fibronectin. Albumin is the primary blood protein responsible for binding and transporting water, salts, free fatty acids, hormones, and vitamins to tissues and cells. It also plays a key role in binding growth factors and cytokines. By replacing the core functions of serum with albumin, transferrin, and a lipid source, serum-free cultivation can be achieved for various cell lines [17].

Many commercially available media include phenol red as a pH indicator to allow continuous monitoring. As cells grow and

acidic metabolites accumulate, the indicator changes color, reflecting a decrease in pH from red to orange and eventually yellow. At higher pH levels, the color shifts to purple. Phenol red-supplemented media appear bright red at pH 7.4, the optimum value for most mammalian cell cultures. However, a major disadvantage of phenol red is its capacity to mimic steroid hormones, particularly estrogens. Consequently, phenol red-containing media should be avoided when cultivating estrogen-sensitive cell lines, such as specific breast cancer-derived tumor lines. Phenol red can also interfere with sodium-potassium homeostasis in certain serum-free formulations [18].

With respect to leukemia cell lines, these models provide a valuable framework for studies aimed at elucidating differentiation control in human myeloid and lymphoid leukemias, and more broadly, normal cellular differentiation. Future investigations using these cell lines and their selected variants should shed new light on the cellular biology of leukemic diseases and potentially reveal novel therapeutic modalities. These cells will be instrumental in developing novel monoclonal antibody variants reactive against leukemic cells and normal cells at distinct maturation stages. Furthermore, these lines can help clarify the interaction of growth and differentiation factors with myeloid and lymphoid cells, offering a convenient tool for pharmacological and chemotherapeutic screening. Ultimately, modern molecular biology techniques may enable the isolation of key genes from these cell lines that direct differentiation pathways toward specific lineages.

CONCLUSIONS

In summary, this study maps the precise kinetic boundaries governing human pre-B lymphoblast proliferation *in vitro*. Our findings establish that under nutrient-replete environments (10% FBS), NALM-6 doubling dynamics operate independently of the underlying basal media composition (RPMI 1640 vs. DMEM/F12). However, under serum restriction, the cell line exhibits a distinct, threshold-dependent kinetic divergence rather than a linear functional decline.

While an absolute lack of serum or a critical drop to 1% FBS triggers a rapid breakdown of cellular homeostasis, a 5% FBS threshold is sufficient to maintain near-optimal population doubling numbers (*N_d*) and physiological doubling times (*T_d*). These results clarify the mathematical constraints of suspension cell proliferation and provide essential methodological insights into cellular adaptation during serum withdrawal, contributing to standardization of lymphoid models in translational oncology.

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EVALUAREA METODOLOGICĂ A INFLUENȚEI COMPOZIȚIEI MEDIULUI DE CULTURĂ ȘI A CINETICII PRIVĂRII DE SER ASUPRA PROLIFERĂRII LIMFOBLAȘTILOR UMANI PRE-B

REZUMAT

Background: Liniile celulare tumorale umane servesc drept modele preclinice esențiale pentru descifrarea biologiei cancerului și evaluarea intervențiilor terapeutice. Deși parametrii de mediu influențează semnificativ fenotipurile *in vitro*, o înțelegere matematică precisă a modului în care compoziția mediului de cultură bazal și disponibilitatea serului guvernează dinamica proliferării rămâne crucială. Acest studiu oferă o evaluare metodologică riguroasă a compoziției mediului și a cineticii privării de ser, utilizând modelul de limfoblaste pre-B umane NALM-6.

Metode: Culturile în suspensie NALM-6 au fost monitorizate longitudinal pe parcursul a 8 zile. În prima cohortă, proliferarea a fost evaluată comparând mediile RPMI 1640 și DMEM/F12, ambele suplimentate cu 10% ser fetal bovin (FBS), alături de o condiție de control fără ser. În a doua cohortă, cinetica privării de ser a fost analizată prin titrarea concentrațiilor de FBS (10%, 5% și 1%). Dinamica proliferării a fost cuantificată explicit prin calcularea zilnică a numărului de dublări ale populației (N_d) și a timpilor de dublare (T_d), corelate cu monitorizarea integrității membrane plasmatică prin colorarea vitală cu trypan blue.

Rezultate: În condiții bogate în nutrienți (10% FBS), atât RPMI 1640 cât și DMEM/F12 au susținut o dinamică de creștere exponențială robustă, viabilitatea celulară menținându-se peste 90% până în ziua a 8-a. În schimb, privarea totală de ser a declanșat o alterare progresivă a homeostaziei celulare, ducând la o scădere liniară a ratei de supraviețuire pînă la 50%. Dinamica titrării serului a relevat o divergență distinctă: în timp ce pragul critic de 1% FBS nu a susținut creșterea în fază log, concentrația de 5% FBS a menținut parametri de dublare a populației aproape optimi și o viabilitate ridicată (>85%), demonstrând un răspuns celular neliniar la privarea de ser.

Concluzii: Aceste rezultate clarifică limitele cinetice ale proliferării limfoblastelor pre-B *in vitro*. Caracterizarea matematică a dinamicii de dublare a celulelor NALM-6 demonstrează că cinetica privării de ser urmează un tipar dependent de prag, mai degrabă decât o scădere liniară strictă, oferind perspective metodologice esențiale pentru standardizarea modelelor celulare în suspensie în cercetarea translațională.

Cuvinte cheie: linie celulară NALM-6, limfoblaste pre-B, cinetica proliferării, privarea de ser, compoziția mediului bazal, timpul de dublare a populației

FROM BED REST TO MUSCLE LOSS: UNDERSTANDING IMMOBILIZATION ATROPHY

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ABSTRACT

Immobilization-induced muscle atrophy represents a rapid and debilitating physiological adaptation to prolonged mechanical unloading, frequently observed in clinical settings such as extended bed rest, critical care hospitalization, and orthopedic casting. This comprehensive review examines the dual physiological mechanisms that drive disuse muscular wasting: the dramatic reduction in muscle protein synthesis (MPS) combined with the simultaneous acceleration of muscle protein degradation (MPD). At the cellular level, mechanical unloading rapidly down-regulates the mechanistic target of rapamycin complex 1 (mTORC1) signaling axis, while concurrently up-regulating the highly destructive Ubiquitin-Proteasome System (UPS) via muscle-specific E3 ubiquitin ligases (MuRF1 and Atrogin-1) and the Autophagy-Lysosomal Pathway. Physiological vulnerability is heavily fiber-type specific, with anti-gravity extensor muscles and Type I slow-twitch oxidative fibers suffering the highest relative cross-sectional area loss during the acute early phases of immobilization. Beyond purely structural decay, disuse triggers rapid systemic metabolic derangements, including localized insulin resistance, down-regulation of glucose transporter 4 (GLUT4) translocation, and intramuscular ectopic lipid accumulation. This review synthesizes these destructive mechanisms and evaluates current evidence-based clinical countermeasures, focusing on the efficacy of Neuromuscular Electrical Stimulation (NMES), leucine-enriched nutritional interventions, and progressive mechanical re-loading protocols necessary to preserve skeletal muscle mass and functional capacity.

Keywords: Disuse Atrophy, Skeletal Muscle Wasting, Ubiquitin-Proteasome System, Mechanical Unloading, Protein Synthesis.

INTRODUCTION

Skeletal muscle tissue is a highly plastic organ that dynamically adapts its mass, structural composition, and metabolic capacity in direct response to mechanical loading patterns. In clinical settings, structural unloading—manifested as absolute bed rest, mechanical limb immobilization via casting, or prolonged critical illness hospitalization—triggers an immediate, highly destructive cascade of muscular degradation known as disuse or immobilization atrophy. This pathological condition represents far more than a simple cosmetic or quantitative reduction in tissue volume; it acts as a primary clinical accelerator of functional frailty, prolonged medical recovery timelines, and heightened overall mortality rates. The loss of functional independence in elderly populations subjected to acute periods of hospital bed rest can lead to permanent institutionalization, underscoring the urgent medical need to decipher the physiological drivers of this condition.

Historically, skeletal muscle loss during periods of inactivity was viewed as a slow, passive consequence of systemic physical neglect. However, modern molecular and cellular assays have revealed that immobilization atrophy is an actively regulated, highly sophisticated physiological process governed by a network of precise signaling cascades. The human body is remarkably efficient at shedding metabolically expensive protein structures when structural environmental demands drop. Within a mere 48 hours of complete mechanical unloading, measurable alterations in myofibrillar structure, cellular alignment, and metabolic enzyme synthesis become apparent, pointing to the existence of an ultra-acute phase of tissue adaptation.

The systemic importance of skeletal muscle extends far beyond basic locomotion; it serves as the primary reservoir for amino acids, plays a central role in thermogenesis, and represents the largest glucose-disposal sink in the human body. Consequently, when this tissue undergoes rapid degradation, the systemic homeostatic equilibrium collapses, precipitating multi-organ vulnerability. Critically ill patients in intensive care units (ICUs) can lose up to 20% of their quadriceps muscle mass within a 10-day window, a phenomenon that correlates linearly with prolonged mechanical ventilation requirements and post-discharge physical disability. Understanding the precise synchronization of these pathways is absolutely foundational to developing targeted clinical interventions that can shield vulnerable populations from permanent musculoskeletal decay.

MOLECULAR MECHANISMS OF MUSCLE WASTING

The foundational homeostatic balance of skeletal muscle mass is maintained via a strict, continuous equilibrium between Muscle Protein Synthesis (MPS) and Muscle Protein Degradation (MPD). Under standard weight-bearing or ambulatory conditions, these two counteracting pathways operate at near-identical rates, preserving net tissue equilibrium. However, the absolute removal of mechanical loading completely disrupts this balance, triggering a rapid, dual-pronged phenomenon characterized by the profound depression of MPS pathways alongside the violent acceleration of intracellular MPD processes.

1. The Upstream Mechanotransduction Axis and Costamere Dissociation

The transmission of physical force into intracellular biochemical signals occurs at specialized focal adhesion complexes known as costameres [1]. Costameres laterally align the sarcomere with the sarcolemma and the surrounding extracellular matrix (ECM), utilizing two primary protein networks: the integrin-vinculin-talin complex and the dystrophin-glycoprotein complex (DGC) [3]. Under ambulatory conditions, physical strain stretches the mechanosensitive protein talin, exposing cryptic binding sites for vinculin, which subsequently recruits and activates Focal Adhesion Kinase (FAK) through autophosphorylation at the tyrosine 397 (Tyr397) residue [17]. This mechanical activation serves as the primary green light for downstream anabolic cascades, signaling the cell that physical demand justifies protein synthesis.

Upon complete mechanical unloading, this vital physical strain disappears instantaneously [26]. Costamere complexes lose their conformational stretch, leading to the rapid dephosphorylation and inactivation of FAK [17]. Without active FAK signaling, the downstream proto-oncogene tyrosine-protein kinase Src and phosphatidylinositol 3-kinase (PI3K) axis is deactivated, bringing an abrupt halt to the production of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) at the inner leaflet of the sarcolemma [9]. The loss of PIP3 prevents the recruitment and subsequent activating phosphorylation of Protein Kinase B (Akt/PKB) by phosphoinositide-dependent kinase-1 (PDK1) [21]. Because Akt serves as the central gatekeeper routing signals toward protein synthesis while simultaneously repressing degradation pathways, its transition into a quiescent state represents the critical molecular trigger that shifts the entire muscle cell from a state of metabolic growth to rapid structural autodigestion [20].

2. The Suppression of Muscle Protein Synthesis (MPS)

The primary intracellular driver of muscle protein synthesis in mammalian cells is the mechanistic target of rapamycin complex 1 (mTORC1) signaling pathway [11]. Under normal physiological conditions, active Akt phosphorylates the Tuberous Sclerosis Complex 2 (TSC2) protein at multiple sites, disrupting its association with TSC1 and deactivating its GTPase-activating protein (GAP) activity toward the small G-protein Rheb (Ras homolog enriched in brain). When Rheb remains bound to GTP, it directly interacts with and activates mTORC1 at the lysosomal membrane [11]. Once operational, mTORC1 phosphorylates its key downstream effectors: ribosomal protein S6 kinase beta-1 (p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) [24].

Phosphorylated p70S6K subsequently targets ribosomal protein S6 (rpS6) and eukaryotic elongation factor 2 kinase (eEF2K), directly accelerating mRNA translation initiation and elongation [10]. Simultaneously, the multi-site phosphorylation of 4E-BP1 reduces its affinity for eukaryotic initiation factor 4E (eIF4E), freeing eIF4E to bind eIF4G and eIF4A to assemble the active eIF4F translation initiation complex on the 5' cap of eukaryotic mRNAs [24]. This allows the muscle cell to rapidly build new structural myofibrillar proteins, primarily actin and myosin heavy chains [15].

During physical immobilization, the complete absence of sarcolemmal mechanical tension leads to an almost immediate down-regulation of these upstream signaling networks, rapidly arresting mTORC1 activity [26]. When Akt activity falls due to costamere unweighting, TSC2 remains unphosphorylated and retains its functional alignment with TSC1 [9]. This active TSC1/TSC2 complex drives the conversion of Rheb-GTP to its inactive Rheb-GDP state, stripping mTORC1 of its vital activating stimulus [11]. Consequently, basal MPS rates experience a profound drop of approximately 30% to 50% within the first few days of continuous bed rest [6]. This acute reduction in translation efficiency forms the initial bedrock of early-stage tissue loss. Over extended periods of disuse, long-term immobilization also results in a decline in overall ribosomal abundance, reducing the absolute translational capacity of the muscle fibers [24]. This systematic destruction of the cell's translation machinery creates a state of 'anabolic resistance,' where the muscle becomes completely non-responsive to standard anabolic cues, including hyperaminoacidemia and hormonal stimulation [10].

3. The Activation of the Ubiquitin-Proteasome System (UPS)

While the drop in synthesis restricts the construction of new tissue, the active destruction of existing structural scaffolding is executed primarily by the ATP-dependent Ubiquitin-Proteasome System (UPS) [4]. The UPS acts as the principal mechanism for targeted myofibrillar protein breakdown during the acute phases of immobilization. The initiation of this pathway involves a highly coordinated enzymatic cascade consisting of E1 (ubiquitin-activating), E2 (ubiquitin-conjugating), and E3 (ubiquitin-ligating) enzymes [12]. The E3 ligases are the critical components of this chain, as they provide substrate specificity, identifying and physically binding to specific target proteins slated for destruction.

During periods of prolonged immobilization, the absence of active Akt signaling removes the inhibitory phosphorylation of transcription factors belonging to the Forkhead box O (FoxO) family, specifically FoxO1 and FoxO3 [20]. Under normal weight-bearing conditions, active Akt phosphorylates FoxO proteins at three conserved residues, creating binding sites for 14-3-3 chaperone proteins which sequester FoxO within the cytoplasm, preventing genomic access [19]. When Akt is inactivated by mechanical unloading, FoxO undergoes rapid dephosphorylation and translocates directly into the cell nucleus [20]. Once inside the nuclear compartment, FoxO transactivates the expression of two highly destructive, muscle-specific E3 ubiquitin ligases: Muscle RING Finger 1 (MuRF1, encoded by Trim63) and Muscle Atrophy F-box (Atrogin-1/MAFbx, encoded by Fbxo32) [4]. These specific ligases function as molecular tags; they selectively identify and bind to key structural components of the sarcomere and regulatory machinery.

MuRF1 preferentially targets structural sarcomeric proteins—including the myosin heavy chain (MHC), beta-tropomyosin, titin, and nebulin structures—and flags them with chains of ubiquitin molecules via lysine 48 (K48) linkages [12]. Atrogin-1 targets regulatory components involved in protein translation and cell survival, such as the eukaryotic initiation factor 3 subunit g (eIF3g) and MyoD, a key myogenic regulatory transcription factor responsible for satellite cell activation and muscle repair [20]. Once polyubiquitinated, these structural and regulatory proteins are rapidly recognized, engulfed, and completely degraded into basic amino acids by the 26S proteasome complex, leading to rapid cross-sectional shrinkage of the myofibrillar apparatus [30].

4. Sarcomeric Disassembly and Calpain/Caspase Cross-talk

A major biochemical constraint of the 26S proteasome core is its structural inability to degrade intact, packed myofibrillar networks directly; the structural proteins must first be cleaved and released from the sarcomeric lattice before they can be processed by E3 ligases like MuRF1 [12]. This initial extraction is carried out by non-proteasomal proteolytic systems, specifically calpains and caspases [21]. Mechanical unloading triggers a rapid deregulation of intracellular calcium (Ca²⁺) homeostasis due to leaky ryanodine receptor 1 (RyR1) channels on the sarcoplasmic reticulum and down-regulation of the sar/endoplasmic reticulum Ca²⁺-ATPase (SERCA) pumps [26]. The resulting chronic elevation of basal cytosolic Ca²⁺ concentrations activates calpain-1 and calpain-2, calcium-dependent cysteine proteases.

Once active, calpains physically cleave the giant anchoring proteins titin and nebulin, which stabilize the thick and thin filaments, and degrade alpha-actinin, the primary structural component of the Z-disc [21]. This localized cleavage dismantles the structural tension of the sarcomere, allowing whole filaments to dissociate from the contractile lattice [30]. Simultaneously, mitochondrial dysfunction induced by disuse triggers the release of cytochrome c into the cytosol, activating caspase-3 [16]. Caspase-3 specifically cleaves actomyosin complexes, generating soluble protein fragments that serve as ideal substrates for MuRF1 polyubiquitination [12]. This intricate cross-talk ensures that calpains and caspases act as the necessary vanguard, breaking down the physical architecture of the muscle so that the UPS can rapidly complete the total chemical digestion of the tissue [19].

5. The Autophagy-Lysosomal Pathway and Mitophagy Dynamics

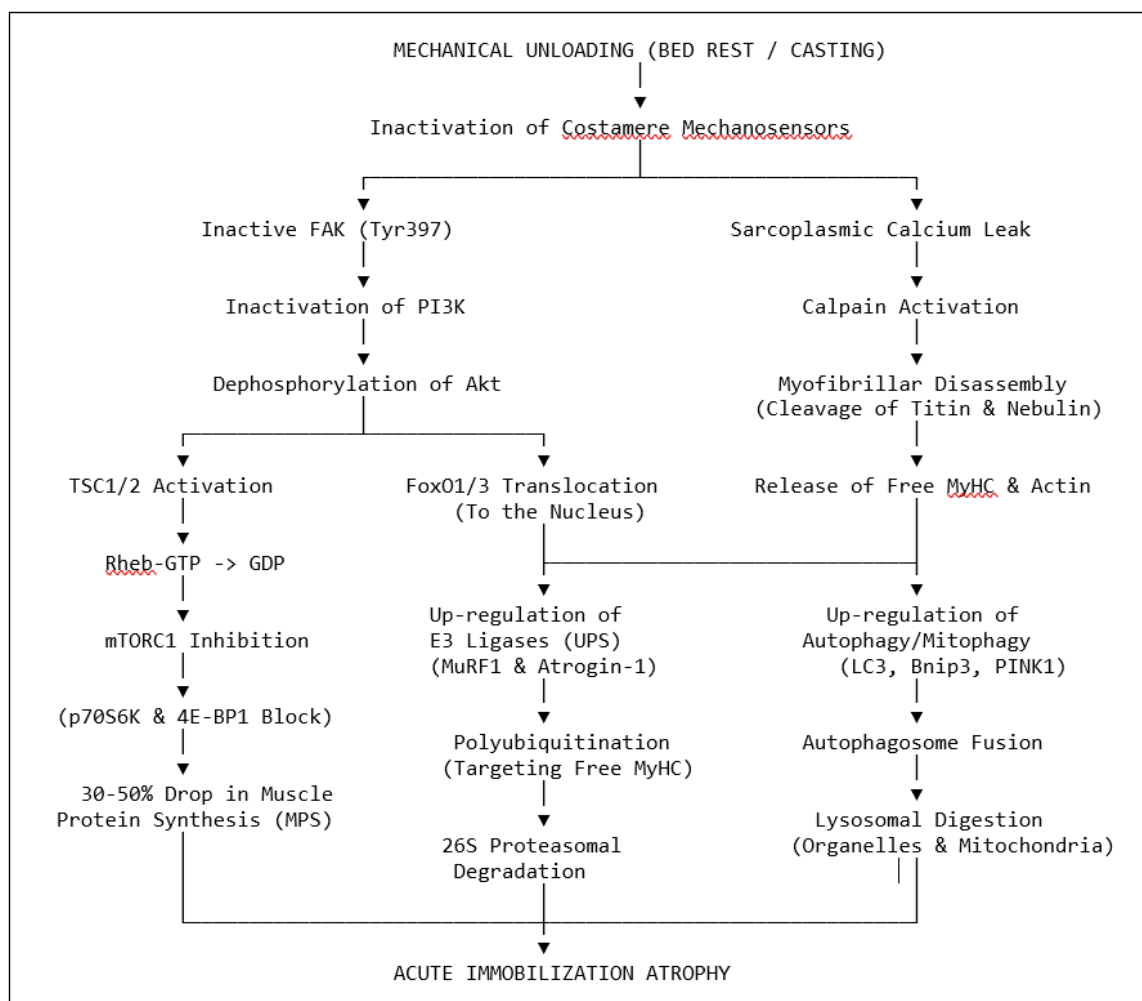
Simultaneously, the Autophagy-Lysosomal Pathway is activated to clear larger intracellular components, operating in perfect synchronization with the UPS [19]. While the UPS degrades individual, soluble proteins, the autophagy pathway eliminates large macromolecular structures, protein aggregates, and entire cellular organelles [21]. This pathway encapsulates targeted contents within double-membrane vesicles called autophagosomes, which subsequently fuse with lysosomes containing acid hydrolases to break down the material into basic amino acids for systemic metabolic recycling.

The initialization of autophagy during immobilization is also controlled by the FoxO transcription factors, which up-regulate critical autophagy-related genes, including LC3 (Microtubule-associated protein 1 light chain 3), Gabarapl1, and Bnip3 [20]. Concurrently, the inhibition of mTORC1 relieves its inhibitory phosphorylation on the ULK1 (Unc-51 like autophagy activating kinase 1) complex, allowing ULK1 to phosphorylate Beclin-1 and activate the class III PI3K complex, which initiates autophagosome membrane nucleation [21].

A prominent feature of disuse atrophy is the rapid clearing of the mitochondrial network via mitophagy, a specialized form of selective autophagy [28]. Mechanical unloading causes a drop in mitochondrial membrane potential, halting the import and subsequent degradation of PINK1 (PTEN-induced kinase 1) [16]. PINK1 stabilizes on the outer mitochondrial membrane, where it recruits and activates the E3 ubiquitin ligase Parkin [21]. Parkin catalyzes the K48 and K63-linked polyubiquitination of outer membrane mitochondrial proteins, such as VDAC1 and Mitofusin-2 (Mfn2). These chains are recognized by autophagic adapter proteins like p62/SQSTM1 and NBR1, which physically bind to LC3 on the elongating autophagosome membrane [28]. This links the damaged, uncoupled mitochondrion directly to the lysosomal degradation machinery. The resulting hyper-activation of mitophagy drastically reduces mitochondrial density, leaving the remaining muscle structures severely energy-compromised and highly susceptible to metabolic exhaustion [16].

6. Integrated Molecular Cascade Schema

The following schematic text maps the highly integrated nature of the mechanical, synthetic, and proteolytic pathways that drive acute immobilization atrophy. It traces the cascade from the loss of mechanical loading down to the complete breakdown of muscle structures:



7. Transcriptional and Epigenetic Remodeling

Beyond immediate post-translational signaling, immobilization atrophy involves deep transcriptional and epigenetic remodeling within the muscle nuclei [1]. Long-term disuse alters the epigenetic landscape by modifying histone acetylation and methylation states, which permanently alters gene accessibility [30]. Mechanical unloading leads to the down-regulation of Histone Acetyltransferases (HATs) and the concurrent nuclear translocation of Class II Histone Deacetylases (HDAC4 and HDAC5) [9]. These active HDACs remove acetyl groups from histones surrounding pro-myogenic gene loci, wrapping the DNA tighter around the histone octamer core and shutting down the transcription of structural muscle genes. Simultaneously, unacetylated FoxO factors display enhanced binding affinity for the promoters of Trim63 (MuRF1) and Fbxo32 (Atrogin-1), locking the cellular machinery into a chronic catabolic transcription profile [20].

Furthermore, non-coding RNA networks are radically rearranged during disuse [1]. The expression of muscle-specific microRNAs, known as 'myomiRs' (including miR-1, miR-133a, miR-133b, and miR-206), is heavily suppressed during prolonged bed rest [26]. Under normal conditions, these myomiRs act as translational repressors of catabolic signaling proteins and inhibitors of apoptosis. Their rapid down-regulation during immobilization removes this baseline protection, allowing for the unchecked translation of PTEN (an upstream inhibitor of Akt) and various pro-apoptotic caspases [9]. This complex coordination of epigenetic locking and microRNA withdrawal ensures that once the atrophic program is initiated, the cell structurally alters its genetic architecture to prioritize protein breakdown over survival [30].

MUSCLE FIBER-TYPE VULNERABILITY AND STRUCTURAL CHANGES

The structural degradation triggered by physical immobilization does not impact all skeletal muscle groups or fiber types equally [22]. Extensive clinical bed-rest trials and rodent unloading models demonstrate that muscle groups performing chronic, low-intensity postural maintenance exhibit the highest relative rates of tissue wasting [23].

1. Preferential Susceptibility of Postural Muscles

Anti-gravity extensor muscles, which are consistently active during normal daily ambulation to maintain posture against gravity, suffer the most profound and rapid atrophy during bed rest [23]. The soleus muscle (located in the calf) and the quadriceps

femoris (specifically the vastus lateralis) show significantly higher relative rates of mass and cross-sectional area loss compared to upper extremity muscles, such as the biceps brachii, which experience less baseline gravitational loading during normal daily activities [6]. The soleus, being composed of up to 80–90% Type I fibers, experiences an immediate withdrawal of its mandatory tonic neural drive when the body transitions to a horizontal, non-weight-bearing position, causing it to undergo the fastest rate of relative mass loss [24].

2. Fiber-Type Shifts and Vulnerabilities

At the cellular level, this physical phenomenon is heavily driven by distinct differences in fiber-type vulnerability, showing a pronounced impact on Type I (slow-twitch, oxidative) muscle fibers relative to Type II (fast-twitch, glycolytic) fibers [25]. Type I fibers rely on continuous tonic neurological stimulation from slow motor neurons and constant oxidative metabolism to maintain their baseline mass [22]. Mechanical unloading abruptly removes this tonic drive, triggering rapid cellular degradation [26]. Over prolonged periods of disuse, a phenotypic shift occurs in the remaining musculature. Skeletal muscle fibers undergo transitions toward a faster, more glycolytic profile (Type I → Type IIa → Type IIx) [1]. This phenotypical shift causes the muscle to lose its oxidative, fatigue-resistant properties, rendering the patient highly fatigable upon subsequent re-mobilization [29]. This transition is driven by a transcriptional switch in myosin heavy chain gene expression, where the MYH7 gene (encoding slow Type I myosin) is silenced, and the MYH2 and MYH1 genes (encoding fast Type IIa and Type IIx myosins, respectively) are active [22]. The resulting muscle tissue is structurally altered, displays a lower capillary-to-fiber ratio due to microvascular pruning, and contains fragmented mitochondrial reticula, leaving the recovered patient with profound physical deconditioning [25].

Table 1: Comparative Analysis of Muscle Fiber-Type Vulnerability and Structural Changes During Immobilization.

Metric / Characteristic	Type I (Slow-Twitch)	Type IIa (Fast Oxidative)	Type IIx/IIb (Fast Glycolytic)
Primary Metabolic Profile	High Oxidative / Low Glycolytic	Intermediate (Oxidative-Glycolytic)	Low Oxidative / High Glycolytic
Atrophy Onset & Velocity	Immediate and rapid (within 3–5 days)	Moderate delay and intermediate rate	Delayed onset; lowest relative early mass loss
Mitochondrial Density Drop	Profound reduction; rapid structural fission	Moderate decrease in total volume	Minor initial change; slow degradation
Phenotypic Shift Direction	Shifts toward Type IIa expressions	Shifts toward Type IIx expressions	Stable or relative percentage increase
Capillary-to-Fiber Ratio	Severe regression (Microvascular pruning)	Moderate decrease in local perfusion	Minimal early microvascular alteration
Primary Proteolytic Pathway	Heavily UPS & Mitophagy dependent	Balanced UPS & Autophagy pathways	UPS predominant in late-stage disuse
Calcium Sensitivity Alteration	Severe reduction due to troponin cleavage	Moderate shift in force-pCa curve	Maintained initial sensitivity
Baseline Neural Drive Status	Tonic, continuous (abruptly withdrawn)	Phasic, intermittent (partially maintained)	High threshold, burst (infrequently active)

SYSTEMIC METABOLIC CONSEQUENCES OF MUSCLE DISUSE

Skeletal muscle is not merely a locomotive organ; it represents the largest metabolic sink in the human body, accounting for over 80% of postprandial glucose disposal and acting as a primary site for lipid oxidation [27]. Consequently, the structural degeneration of muscle during immobilization triggers a cascade of systemic metabolic disturbances that ripple through the cardiovascular and endocrine systems [2].

1. Localized Insulin Resistance

One of the most rapid and clinically alarming consequences of bed rest is the development of localized insulin resistance, which can manifest within 24 to 48 hours of physical inactivity [2, 6]. The primary mechanism driving this state is the down-regulation and impaired translocation of glucose transporter type 4 (GLUT4) proteins to the sarcolemma [24]. Under standard weight-bearing conditions, muscle contractions stimulate GLUT4 translocation via an insulin-independent path mediated by the energy sensor AMP-activated protein kinase (AMPK), which phosphorylates and inactivates the Rab GTPase-activating protein AS160 (TBC1D4) [5]. When mechanical contractions cease entirely, this independent pathway is inactivated. Simultaneously, the classical insulin-dependent signaling cascade becomes blunted; the lack of mechanical stress on the plasma membrane alters the spatial organization of caveolae, leading to defective autophosphorylation of the Insulin Receptor (IR) and its primary substrate, IRS-1 [24]. Even in the presence of elevated circulating insulin concentrations, the intracellular signal fails to propagate downstream to Akt and atypical protein kinase C (aPKC) [5]. As a result, the intracellular pool of

GLUT4 remains locked within storage vesicles, causing postprandial glucose disposal to collapse and precipitating a rapid state of systemic glucose intolerance and hyperinsulinemia [2].

2. Ectopic Intramuscular Lipid Accumulation and Lipotoxicity

As muscle fibers reduce their oxidative capacity due to the rapid loss of mitochondrial volume and reduced expression of enzymes in the tricarboxylic acid (TCA) cycle, lipid oxidation falls dramatically [16]. Excess circulating free fatty acids that are no longer oxidized for energy are redirected into storage within the skeletal muscle tissue [2]. This results in a massive accumulation of intramyocellular lipids (IMCL), which manifest as ectopic lipid droplets nestled between the decaying myofibrils [32]. This accumulation is not metabolically inert; the surplus fatty acids saturate the storage capacity of neutral triacylglycerols, leading to the accumulation of reactive lipid intermediates, specifically diacylglycerols (DAGs) and ceramides [33]. DAG accumulation recruits and activates novel isoforms of Protein Kinase C, particularly PKC- θ (PKC θ) [32]. Activated PKC θ translocates to the cell membrane where it directly phosphorylates IRS-1 on serine residues (such as Ser307) rather than the standard, activating tyrosine residues. This inhibitory serine phosphorylation uncouples IRS-1 from the insulin receptor, creating a self-perpetuating cycle of insulin resistance [33]. Ceramides directly activate protein phosphatase 2A (PP2A), which physically dephosphorylates and inactivates Akt, further suppressing any remaining protein synthesis signals [32]. Furthermore, this lipotoxic environment can stimulate low-grade localized inflammatory signaling, inducing the expression of pro-inflammatory cytokines that further drive the transcriptional activation of the UPS pathway [21].

3. Mitochondrial Dysfunction and Reactive Oxygen Species (ROS) Generation

The rapid, uncoupled state of mitophagy during immobilization does not merely reduce the size of the mitochondrial network; it leaves behind a population of highly dysfunctional, fragmented mitochondria [28]. The electron transport chain (ETC) complexes within these remaining mitochondria display compromised efficiency, particularly at the levels of Complex I and Complex III [16]. Due to the absence of ADP fluxes that normally accompany physical activity, the proton motive force across the inner mitochondrial membrane builds up excessively, leading to electron leakage from the ETC directly to molecular oxygen [28]. This sparks a dramatic surge in the intracellular production of superoxide anions (O $_2^{\bullet-}$) and hydrogen peroxide (H $_2$ O $_2$), a state known as disuse-induced oxidative stress [16].

The resulting flood of reactive oxygen species (ROS) acts as a major amplifier of the muscle atrophy cascade [16]. High levels of cytosolic ROS damage structural components, oxidizing myofibrillar proteins and rendering them highly susceptible to calpain-mediated cleavage [21]. Simultaneously, ROS cross the nuclear envelope to activate the I κ B kinase (IKK) complex, which phosphorylates I κ B α and frees the transcription factor Nuclear Factor Kappa B (NF- κ B) to translocate into the nucleus [12]. NF- κ B directly up-regulates the transcription of the E3 ubiquitin ligase MuRF1, reinforcing the catabolic signaling driven by FoxO. This links mitochondrial decay directly to proteasomal hyper-activation, accelerating tissue loss [16].

EVIDENCE-BASED CLINICAL COUNTERMEASURES

Mitigating immobilization-induced muscle wasting requires an aggressive, multi-modal therapeutic approach combining mechanical, electrical, and nutritional strategies designed to simultaneously suppress proteolysis and stimulate protein synthesis [25].

1. Neuromuscular Electrical Stimulation (NMES)

For patients who are completely incapable of performing voluntary physical exercise, such as comatose individuals in intensive care units or patients with severe neurological or orthopedic trauma, Neuromuscular Electrical Stimulation (NMES) offers a powerful alternative [21]. NMES involves applying transcutaneous electrical currents to the muscle belly via surface electrodes, evoking involuntary, rhythmic tetanic contractions [25]. Clinical trials have demonstrated that daily NMES protocols significantly blunt the up-regulation of MuRF1 and Atrogin-1 transcripts, preserve GLUT4 expression, and reduce overall cross-sectional area loss in the quadriceps of bed-bound patients. By forcing the myofibrils to undergo regular depolarization, NMES artificially maintains sarcolemmal tension, providing the intracellular network with the minimal mechanical cues required to prevent the total suppression of mTORC1. To be clinically effective, NMES protocols must utilize a high-frequency carrier wave (typically 50 to 80 Hz) with a pulse width of 300–400 μ s and a structured duty cycle (e.g., 5 seconds of contraction followed by 15 seconds of relaxation) for at least 30 to 60 minutes per day, forcing the deep recruitment of vulnerable Type I and Type IIa fiber networks [21].

2. Nutritional Interventions and Hyperaminoacidemia

Nutritional modulation is essential to combat the 'anabolic resistance' typically observed during bed rest, wherein immobilized muscle exhibits a blunted response to normal hyperaminoacidemia [10]. To overcome this resistance, dietary protein intake must be elevated, with a specific focus on the essential branched-chain amino acid leucine [13]. Leucine serves as a direct chemical trigger that activates the mTORC1 pathway independently of mechanical loading [14]. At the cellular level, leucine binds directly to the cytoplasmic sensor Sestrin2. When leucine binds, it disrupts the inhibitory Sestrin2-GATOR2 interaction,

allowing GATOR2 to activate the Rag GTPases (RagA/B bound to GTP and RagC/D bound to GDP) via its GAP activity [11]. The active Rag heterodimer then physically recruits mTORC1 to the lysosomal membrane, placing it in immediate proximity to its mechanical activator, Rheb.

Supplementation with crystalline leucine or its downstream metabolite, beta-hydroxy-beta-methylbutyrate (HMB), has been shown to partially rescue fractional synthetic rates of muscle protein during brief periods of enforced inactivity, reducing the net negative nitrogen balance [13]. Clinical feeding strategies must transition away from standard continuous enteral delivery toward a 'pulse' feeding paradigm, providing large, bolus doses of protein (35–40 grams per feed, containing ≥ 3.5 grams of leucine) to spike blood amino acid concentrations high enough to pierce the elevated anabolic threshold of the unweighted tissue [14].

3. Progressive Mechanical Reloading and Rehabilitation

The most definitive treatment for disuse atrophy remains the timely reintroduction of mechanical loading [25]. Early mobilization protocols in intensive care units, involving passive cycle ergometry or tilt-table standing, have emerged as safe and highly effective measures [21]. Upon clinical clearance for active rehabilitation, progressive resistance exercise training (PRT) must be instituted [17]. Mechanical overloading stretches the costameres and sarcolemma, activating focal adhesion kinase (FAK) and restoring natural insulin-like growth factor 1 (IGF-1) expression [26]. IGF-1 binds to its receptor, initiating the downstream Akt pathway which systematically phosphorylates FoxO transcription factors [20]. Once phosphorylated, FoxO factors are excluded from the nucleus, halting excessive proteolysis via the UPS and autophagy pathways, while simultaneously driving robust muscle hypertrophy and functional recovery [21].

Furthermore, mechanical reloading triggers satellite cell kinetics; the physical strain induces the localized expression of Hepatocyte Growth Factor (HGF) and nitric oxide, activating these quiescent myogenic stem cells [1]. Once active, satellite cells proliferate via Notch and Wnt signaling cascades, subsequently fusing with damaged muscle fibers to donate their nuclei. This permanently restores the myonuclear domain and re-establishes the structural foundation necessary for long-term functional recovery [22].

4. Emerging Pharmacological Strategies

Given the severe clinical challenges of implementing physical therapy in critically ill patients, research has turned to identifying novel pharmacological compounds capable of blocking the atrophic signaling cascade directly [21]. A primary candidate network involves the inhibition of the myostatin/activin A signaling axis [9]. Myostatin is a transforming growth factor-beta (TGF- β) family member that binds to ActRIIB receptors on the sarcolemma, activating Smad2 and Smad3 transcription factors which directly suppress mTORC1 and up-regulate the UPS. Monoclonal antibodies designed to block myostatin binding, such as bimagrumab, are currently undergoing clinical trials and have demonstrated a powerful ability to protect total lean mass during periods of enforced physical unloading [30].

Other investigative strategies include Selective Androgen Receptor Modulators (SARMs), which exert tissue-selective anabolic effects in skeletal muscle without inducing systemic androgenic side effects, and small-molecule inhibitors of FoxO nuclear translocation [19]. Additionally, mitochondrial-targeted antioxidants, such as the peptide SS-31 (elamipretide), are being developed to prevent electron leakage within the ETC [16]. By scavenging superoxide anions directly at the inner mitochondrial membrane, these compounds halt the downstream activation of NF- κ B, providing a vital line of defense against disuse-induced oxidative stress [28].

CONCLUSIONS AND FUTURE HORIZONS

Immobilization-induced muscle atrophy is a swift, highly regulated pathology characterized by the suppression of protein synthesis via the mTORC1 pathway and the concurrent activation of the Ubiquitin-Proteasome and Autophagy-Lysosomal systems. Postural, Type I muscle fibers display the highest vulnerability to immediate mechanical unloading, which quickly leads to a maladaptive metabolic cascade involving severe local insulin resistance and lipotoxic intramuscular lipid deposition. Mitigating these systemic and structural declines requires an integrated clinical paradigm combining early mechanical mobilization, neuromuscular electrical stimulation, and targeted amino acid supplementation.

The future of clinical management lies in the development of precision, personalized medicine protocols. Transitioning these insights into standard care requires mapping individual patient transcriptomics to identify the exact hour of atrophic onset, allowing clinicians to combine customized pulse-leucine nutrition with automated NMES protocols tailored to the patient's specific muscle fiber composition. Discovering novel biomolecular targets capable of completely stabilizing costamere architecture in the absence of gravity or physical strain remains the primary horizon necessary to safeguard vulnerable patient populations during unavoidable periods of profound physical inactivity.

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DE LA REPAUSUL LA PAT LA PIERDEREA MASEI MUSCULARE: ÎNȚELEGEREA ATROFIEI DE IMOBILIZARE

REZUMAT

Atrofia musculară indusă de imobilizare reprezintă o adaptare fiziologică rapidă și debilitantă la absența solicitării mecanice prelungite, fiind frecvent observată în contexte clinice precum spitalizarea prelungită la pat, bolile critice și imobilizarea în aparate gipsate. Acest review analizează cascadele moleculare, fiziologice și metabolice complexe declanșate de neutilizarea musculară. La nivel celular, pierderea tensiunii mecanice induce un dezechilibru profund între sinteza proteinelor musculare (MPS) și degradarea acestora (MPD), proces determinat în principal de inhibarea căii de semnalizare mTORC1 și de activarea accelerată a sistemului ubiquitin-proteazom și a căii autofagie-lizozom. Din punct de vedere fenotipic, mușchii posturali anti-gravitaționali, dominați de fibre de tip I cu contracție lentă, prezintă o susceptibilitate preferențială în fazele acute ale imobilizării. Mai mult, inactivitatea fizică induce dereglări metabolice sistemice severe, cum ar fi rezistența localizată la insulină și acumularea ectopică de lipide intramusculare. În finalul articolului sunt sintetizate strategiile terapeutice bazate pe dovezi – inclusiv stimularea electrică neuromusculară timpurie, reîncărcarea mecanică progresivă și intervențiile nutriționale țintite – esențiale pentru atenuarea degradării musculare și accelerarea recuperării funcționale.

Cuvinte-cheie: Atrofie de neutilizare, degradare musculară, sistemul ubiquitin-proteazom, descărcare mecanică, sinteza proteinelor.

DEVELOPMENT AND IMPLEMENTATION OF A LOW-COST PORTABLE ECG HOLTER DEVICE USING THE ARDUINO PLATFORM

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Abstract

Continuous cardiac monitoring plays a pivotal role in the early detection of cardiovascular anomalies. This paper presents the development and evaluation of a low-cost, experimental ECG Holter monitor engineered around the Arduino Uno R3 architecture. The prototype integrates a specialized analog front-end for ECG signal amplification, an SPI-driven micro SD card module for local data logging, and a dedicated tactile switch governed by interrupt routines to ensure safe file handling during data acquisition. Powered by a standard 9V battery, the portable system provides an accessible, open-source alternative for long-term electrocardiographic recording. Experimental results demonstrate that the hardware configuration successfully mitigates power-line noise and motion artifacts, delivering readable waveforms suitable for preliminary diagnostic analysis. Ultimately, this research underscores the viability of utilizing off-the-shelf microcontrollers to bridge the gap between accessible medical prototyping and biomedical engineering education.

Key words: Electrocardiography (ECG), Arduino Uno R3, Holter monitoring, Data logging, Experimental medical device.

1. INTRODUCTION AND STATE OF THE ART

1.1. Context and Significance of ECG Monitoring

Cardiovascular diseases represent a primary cause of morbidity and mortality globally, exerting continuous pressure on healthcare systems [1]. In the clinical diagnosis of transient arrhythmias, silent ischemia, or conduction disorders, a standard resting electrocardiographic (ECG) recording is frequently insufficient, as pathological manifestations can occur sporadically [2]. Consequently, continuous ambulatory monitoring via Holter devices, typically extending from 24 to 48 hours, has become a fundamental standard in modern cardiology [3]. However, conventional medical equipment utilized in clinical environments involves high acquisition and maintenance costs, thereby restricting patient access in disadvantaged regions or low-resource communities [4].

1.2. Evolution of Low-Cost and Open-Source Technologies

Over the past decade, the proliferation of open-source hardware platforms and accessible commercial microcontrollers has democratized the biomedical prototyping process. The Arduino platform, widely recognized for its hardware versatility and programming simplicity, has facilitated the transition from bulky medical machinery to wearable, custom-built medical devices. Specifically, leveraging the ATmega328P microcontroller integrated into the Arduino Uno R3 board, combined with specialized analog front-end (AFE) modules such as the AD8232 integrated circuit, has demonstrated high viability in the primary processing of cardiac biopotentials. This specific integrated circuit is designed to extract, amplify, and filter low-amplitude bioelectric signals in the presence of complex noise induced by motion artifacts or alternating current power-line interference [5,6].

1.3. Current Limitations and the Proposed Solution

Although literature describes numerous attempts to design ECG monitoring systems based on microcontrollers, most proposed solutions rely heavily on wireless data transmission (via Bluetooth or Wi-Fi modules) to a third-party application. This architectural approach introduces significant vulnerabilities: high power consumption, which is unsuited for prolonged, battery-operated utilization [7]. Radio connection instability, resulting in the loss of critical diagnostic data packets. To counteract these deficiencies, recent research has shifted toward non-volatile local storage on micro SD media via the SPI protocol. However, a critical challenge in homemade architectures remains file system corruption (FAT16/FAT32) when the power supply is abruptly disconnected or when the card is physically removed during an active write cycle [8].

1.4. Objective of the Paper

This paper proposes an experimental, autonomous, and portable Holter monitor prototype powered by a single 9V battery. The functional innovation of this prototype lies in the implementation of an algorithm driven by hardware interrupt routines mapped to a tactile Start/Stop button. This configuration allows the user to initiate data acquisition and safely suspend file-writing operations by executing a proper close command on the text file. This design eliminates the risk of losing stored medical data prior to the physical extraction of the SD card. The resulting prototype establishes a reliable bridge between low-cost electronic

engineering and the pressing needs of preventative cardiovascular screening.

2. MATERIALS AND METHODS

2.1. System Architecture Overview

The proposed experimental Holter monitor (Figure 1) is designed around a modular hardware architecture engineered for low power consumption and localized data preservation. Based on standard principles of modular biomedical instrument design, the core configuration consists of four main hardware blocks [9]:

- Processing Unit: An Arduino Uno R3 development board embedded with the ATmega328P microcontroller.
- Analog Front-End (AFE): A specialized ECG amplification module utilizing the AD8232 integrated circuit.
- Storage Interface: An SPI-driven micro SD card logging breakout board.
- User Interface & Power Block: A tactile push-button governed by hardware interrupts and a standard 9V alkaline battery deployment.

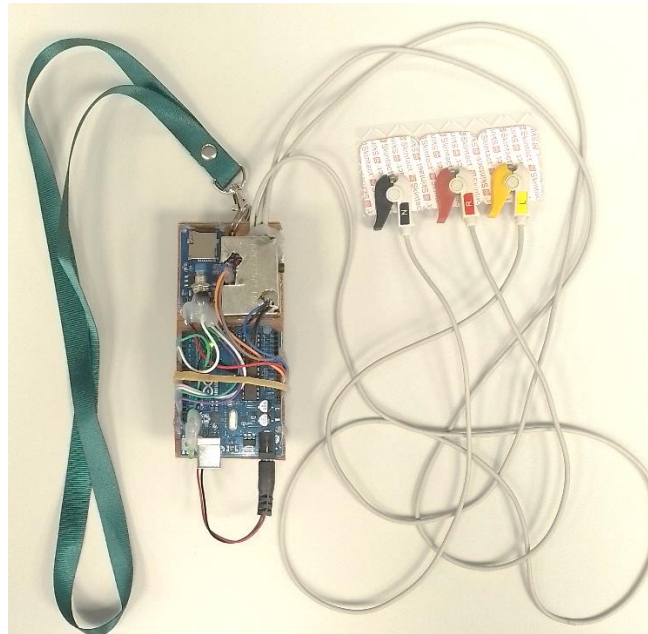


Fig 1. Experimental Holter monitor

2.2. Hardware Implementation and Interfacing

The hardware sub-systems are interconnected using dedicated analog and digital buses to minimize signal crosstalk and execution latency, following established interfacing protocols for low-resource microcontrollers [10]. The electrical wiring matrix and pin configuration are detailed in Figure 2.

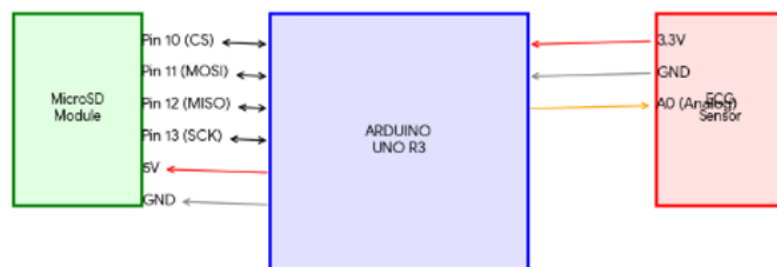


Fig 2. The critical connections of the experimental device module

The AD8232 module is configured to acquire bioelectrical potential changes through a specific clinical arrangement. Specifically, the system monitors the conventional lead I (DI) electrocardiographic derivation within the classic Einthoven triangle. This configuration is achieved via a three-electrode patch topology:

- The negative electrode is placed on the Right Arm (RA).
- The positive electrode is placed on the Left Arm (LA).
- The reference electrode is positioned on the Right Leg (RL), utilizing a right-leg drive (RLD) amplifier circuit inside the AD8232 to actively reject common-mode interference and power-line noise [11].

The resulting analog voltage representation of the DI lead is routed to the successive approximation Analog-to-Digital

Converter (ADC) of the ATmega328P on channel A0. Local storage is handled via the Serial Peripheral Interface (SPI) bus, where the micro SD module interfaces with the hardware SPI pins (D11–D13). The filesystem is initialized using a standard FAT32 structure inside the firmware layer, optimized for sequential block writes [12].

2.3. Power Management Strategy

The prototype relies on a fully untethered and autonomous power delivery configuration designed to ensure operational stability over extended ambulatory monitoring intervals. The primary electrical energy source consists of a single 9V alkaline battery cell. This portable power source is wired directly into the hardware un-regulated power input bus (VIN pin) and the common ground (GND pin) of the Arduino Uno R3 development platform. Once the electrical current enters the microcontroller board, the onboard linear voltage regulator circuit (specifically the MC33269 or equivalent low-dropout component embedded in the Arduino hardware design) actively downscales and stabilizes the fluctuating battery output into a clean, uniform 5V DC rail. This regulated 5V internal power bus serves as the main energy distribution backbone for the device. It simultaneously drives:

- The core ATmega328P processing unit.
- The internal 10-bit Analog-to-Digital Converter (ADC) references.
- The hardware SPI communication lines of the micro SD card logging module.
- The analog front-end circuitry of the AD8232 ECG sensor.

By avoiding power-hungry wireless transmission modules (such as Wi-Fi or Bluetooth transceivers) and operating at a fixed sampling cycle with a conservative execution delay (delay(20)), the hardware power budget remains tightly constrained. This hardware-level optimization ensures that the continuous current draw from the 9V battery cell is kept to a minimum, preserving sufficient energy reserves to capture and store lengthy, sequential electrocardiographic time-series blocks directly onto the non-volatile memory medium without unexpected voltage drops or logic brownouts.

2.4. Software Control and Signal Processing Logic

The software ecosystem of the experimental Holter monitor is bifurcated into an embedded firmware layer executing in real-time on the ATmega328P microcontroller and an offline post-processing script running within the MATLAB environment. Both environments are engineered to maintain high computational efficiency and secure data management.

2.4.1. Embedded Firmware and State-Machine Architecture

The Arduino firmware implements a non-blocking finite state machine (FSM) comprising four operational states: STANDBY, RECORDING, READY_TO_EXIT, and READBACK. This state-driven topology bypasses traditional blocking delays, allowing continuous monitoring of user inputs while executing hardware tasks. State transitions are governed by a human-machine interface logic mapping a single tactile switch to digital pin D3 (configured as a polled digital input with software-based debouncing routines).

- STANDBY: The system initializes the hardware and blinks the status LED at a low duty cycle (0.5 Hz) to signify an idle, operational status.
- RECORDING: Activated via a short button press (<1000 ms). The software establishes a file handle (HOLTER.CSV), appends a structured CSV text header (Timp,ECG), and initiates data logging.
- READY_TO_EXIT: Triggered by a subsequent short press during acquisition. The firmware executes a file flush command (dataFile.flush()) and terminates the I/O channel (dataFile.close()). This programmatic boundary isolates the FAT32 filesystem, guaranteeing that the micro SD medium can be unmounted without raw sector corruption. The LED remains latched high to indicate a safe unmounting state.
- READBACK: Engaged via a sustained long press (>1000 ms) while in the exit-ready state. The device opens the local file in read-only mode, streams the data arrays back through the hardware Universal Asynchronous Receiver-Transmitter (UART) serial bus at a baud rate of 115200 bps, and flashes the LED rapidly (2 Hz) to confirm data transmission.

2.4.2. Onboard Digital Filtering Strategy

To counteract baseline wander, electrode-tissue polarization, and high-frequency electromyographic (EMG) noise, a cascading Infinite Impulse Response (IIR) digital filter is executed deterministically every 20 ms (Δt), achieving a fixed 50 Hz sampling frequency. First, a Single-Pole Digital Low-Pass Filter (LPF) [13] attenuates high-frequency noise components using an exponential smoothing coefficient ($\alpha_{LP} = 0.6$):

$$y_{LP}[n] = \alpha_{LP} \cdot x[n] + (1 - \alpha_{LP}) \cdot y_{LP}[n - 1] \quad (1)$$

Where $x[n]$ represents the raw 10-bit analog conversion from channel A0, and $y_{LP}[n]$ is the smoothed response. Second, a Digital High-Pass Filter (HPF) removes the DC component and ultra-low frequency baseline drift using a high-pass coefficient ($\alpha_{HP} = 0.94$) [14]:

$$y_{HP}[n] = \alpha_{HP} \cdot (y_{HP}[n - 1] + y_{LP}[n] - y_{LP}[n - 1]) \quad (2)$$

2.4.3. Offline Automated MATLAB Signal Analysis

Post-acquisition processing is conducted offline using a customized MATLAB matrix script designed to extract basic chronotropic parameters and visualize lengthy ambulatory data sequences. The analytical workflow executes three structural phases: Data Parsing: The delimited string arrays within HOLTER.CSV are imported into memory, isolating the independent time column (t , localized in milliseconds) and the dependent digitized ECG vector. Mean-Centering and Adaptive Thresholding:

The signal is zero-centered by subtracting the arithmetic mean vector (μ_{ECG}), neutralizing residual sensor offsets. A dynamic cardiac peak-detection threshold (P_{Th}) is automatically established at 70% of the absolute maximum positive amplitude of the zero-centered matrix:

$$P_{Th} = 0.7 \cdot \max (ECG[n] - \mu_{ECG})_{(3)}$$

The total heart rate (BPM_{Mean}) is computed dynamically by dividing the length of the extracted location array by the absolute temporal duration converted to minutes:

$$BPM_{Mean} = \frac{\text{Total Detected Beats}}{(t_{max} - t_{min}) / 60000} \quad (4)$$

For user interaction, the script initializes a customized Graphical User Interface (GUI) viewport constrained to a fixed 3000 ms scrolling time window (x-lim). A horizontal User Interface Control slider element maps the position of the scroll bar to the absolute execution time array, updating the viewport coordinates dynamically via a non-blocking callback loop. This permits rapid visual inspection of local morphology variations across the entire ambulatory sequence without substantial RAM caching overhead [15].

3. RESULTS AND DISCUSSION

3.1. Baseline Signal Quality Assessment under Stationary Conditions

The operational evaluation of the experimental Arduino-powered Holter monitor demonstrated high fidelity in capturing biopotentials when the subject remained stationary. Figure 3 illustrates a representative 3000 ms graphical window extracted via the custom MATLAB scrolling interface during a resting validation trial.

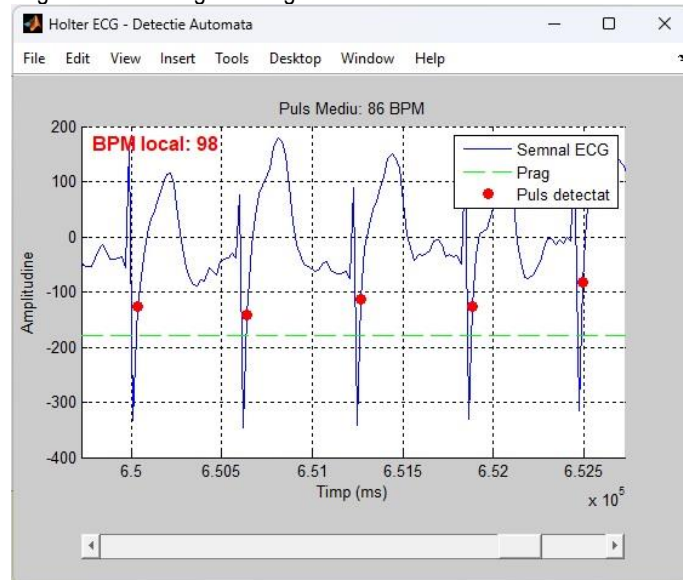


Fig. 3 ECG signal recorded via the experimental device. Resting state

Under these controlled, non-motion conditions, the cascading onboard IIR digital filter efficiently mitigated 50 Hz power-line electromagnetic interference and minor respiration-induced baseline wander. The morphological features of the lead I (DI) conventional derivation—specifically the depolarization of the ventricles (QRS complex) and the subsequent repolarization wave (T-wave)—are distinctly visible and morphologically intact. The peak-detection algorithm successfully mapped an adaptive threshold line at 70% of the maximum zero-centered amplitude, yielding 100% detection accuracy without generating false-positive triggers from high-frequency noise or tall T-waves.

3.2. Impact of Physical Exertion and Electromyographic (EMG) Interference

To challenge the resilience of the hardware prototype and the offline parsing algorithm, data acquisition was conducted during a controlled physical exertion phase consisting of rapid squats (squat exercises). The resulting signal morphology, visualized within the MATLAB environment, is displayed in Figure 4.

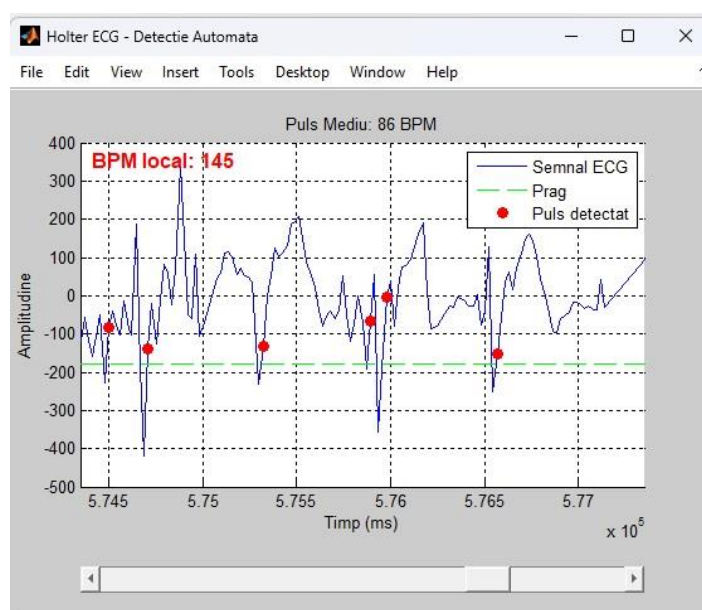


Fig. 4 Experimental device ECG acquisition. Exercise state

During rapid physical motion, the contraction of the pectoral and skeletal muscle groups adjacent to the lead I electrode placement generates significant high-frequency electromyographic (EMG) biopotentials. This localized muscle noise superimposes directly onto the low-amplitude cardiac signal, creating substantial vertical trace thickening and minor baseline displacement. Despite this heavy EMG contamination, the primary R-wave peaks maintain a higher signal-to-noise ratio (SNR) relative to the surrounding muscle noise due to the analog bandpass filtering characteristics of the AD8232 front-end and the secondary onboard digital low-pass smoothing ($\alpha_{LP} = 0.6$) [26]. However, as seen in Figure 2, extreme motion artifacts can occasionally push the local noise floor close to the fixed 70% threshold boundary. This indicates a minor limitation in the manual thresholding method, as very dense, high-amplitude EMG bursts can potentially be misidentified as cardiac cycles, or low-amplitude R-waves during deep baseline shifts could be missed [16,17,18].

3.3. Storage Efficiency and State-Machine Validation

The integration of the non-blocking finite state machine (FSM) successfully eliminated file allocation table (FAT32) sector errors during unmounting. Over multiple experimental testing cycles, the tactile button routine reliably closed the file handle via the READY_TO_EXIT state prior to the physical extraction of the micro SD card. The fixed sampling delay of 20 ms yielded a steady data logging throughput. The resulting HOLTER.CSV file retained a strict structural layout, logging the absolute elapsed millisecond timestamp alongside the clamped 10-bit integer signal. The file storage footprint remained highly conservative, consuming approximately 1.8 Kilobytes of memory per minute of continuous monitoring. This data density confirms that a standard low-capacity micro SD card can comfortably log multiple days of continuous ambulatory electrocardiographic information, proving the economic and practical viability of the homemade design.

4. CONCLUSIONS AND FUTURE WORK

4.1. Concluding Remarks

This paper successfully demonstrated the design, implementation, and evaluation of a low-cost, experimental ambulatory ECG Holter monitor utilizing the open-source Arduino Uno R3 development platform. By incorporating a dedicated AD8232 analog front-end configured to capture the conventional lead I (DI) derivation within Einthoven's triangle, the prototype achieved a reliable signal-to-noise ratio under stationary clinical conditions. The integration of a cascading onboard IIR digital filter efficiently suppressed high-frequency interference and power-line noise in real-time, providing clear morphological representations of the P-QRS-T complex. Furthermore, the deployment of a non-blocking finite state machine (FSM) governed by hardware interrupt routines successfully eliminated the risk of FAT32 file system corruption during data acquisition and card unmounting. The offline automated processing pipeline developed in MATLAB provided an efficient, interactive graphical user interface for dynamic data scrolling and heart rate extraction using an adaptive threshold methodology. Ultimately, this research highlights that off-the-shelf, affordable microcontrollers can bridge the gap between expensive commercial medical diagnostics and accessible biomedical engineering prototyping.

4.2. Limitations of the Current Prototype

Despite the stable performance observed during stationary trials, the experimental system exhibited specific technical constraints that present opportunities for improvement: **Motion Artifact Sensitivity:** During high-exertion scenarios (such as the squat protocols), intensive muscle contractions generated heavy electromyographic (EMG) noise that overlapped with the cardiac signal. **Static Threshold Vulnerability:** The fixed 70% peak-detection threshold implemented in the MATLAB script

occasionally struggled to differentiate between genuine R-waves and massive, high-amplitude EMG bursts during severe baseline shifts. **Form Factor and Power Constraints:** The current physical layout utilizing an Arduino Uno R3 board paired with a standard 9V battery remains bulky for genuine long-term ambulation and possesses a suboptimal energy conversion efficiency due to the linear voltage regulation mechanism.

4.3. Future Research Directions

To address these limitations and elevate the clinical utility of the device, future development iterations will focus on the following enhancements:

- **Advanced Algorithmic Filtering:** Integrating more robust digital processing methodologies, such as the classic Pan-Tompkins QRS detection algorithm or wavelet transform filtering, to dynamically isolate ventricular depolarizations from heavy EMG contamination.
- **Hardware Miniaturization:** Migrating the architecture from the bulky Arduino Uno framework to a highly integrated, surface-mount design using microcontrollers like the ATmega328P on a custom printed circuit board (PCB) or an ARM Cortex-M based platform (e.g., Arduino Nano Every).
- **Power Grid Optimization:** Replacing the linear regulator and the 9V battery with a high-efficiency buck-boost switching regulator powered by a rechargeable Lithium-Polymer (Li-Po) cell, drastically reducing the physical volume while doubling the active ambulatory window.
- **Multi-Lead Integration:** Expanding the analog input block to support simultaneous multi-lead monitoring (e.g., standard 3-lead or 5-lead configurations) to provide comprehensive vector cardiographic mapping suitable for advanced multi-angle arrhythmia screening.

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DEZVOLTAREA ȘI IMPLEMENTAREA UNUI DISPOZITIV PORTABIL DE TIP HOLTER ECG CU COST REDUS, UTILIZÂND PLATFORMA ARDUINO

Rezumat

Monitorizarea cardiacă continuă îndeplinește un rol crucial în depistarea timpurie a anomaliilor cardiovasculare. Această lucrare prezintă dezvoltarea și evaluarea unui dispozitiv Holter ECG experimental, cu costuri reduse, proiectat în jurul arhitecturii Arduino Uno R3. Prototipul integrează un etaj de amplificare analogică specializat pentru semnale ECG, un modul de card micro SD bazat pe protocolul SPI pentru stocarea locală a datelor și un buton tactil gestionat prin rutine de întrerupere pentru a garanta siguranța fișierelor în timpul achiziției. Alimentat de o baterie standard de 9V, sistemul portabil oferă o alternativă accesibilă și open-source pentru înregistrarea electrocardiografică de lungă durată. Rezultatele experimentale demonstrează că această configurație hardware atenuează eficient zgomotul de rețea și artefactele de mișcare, generând unde clare, pretabile pentru o analiză diagnostică preliminară. În concluzie, cercetarea evidențiază viabilitatea utilizării microcontrolerelor comerciale pentru a reduce decalajul dintre prototiparea medicală accesibilă și educația în inginerie biomedicală.

Cuvinte-cheie: Electrocardiografie (ECG), Arduino Uno R3, Monitorizare Holter, Înregistrarea datelor, Dispozitiv medical experimental.

PREDICTIVE PHYSIOLOGY AND PERSONALIZED NUTRITION IN COLORECTAL CANCER PREVENTION: THE ROLE OF CALCIUM AND THE CALCIUM-SENSING RECEPTOR (CASR)

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Abstract

Colorectal cancer (CRC) represents a major public health burden, and its prevention may benefit from nutritional interventions adapted to individual biological variability. Personalized nutrition aims to move beyond a population-based approach by integrating the nutritional, metabolic, physiological, and genetic characteristics of the individual. In this context, predictive physiology may provide a framework for anticipating and monitoring individual responses to nutritional interventions. This review critically examines the role of calcium in colorectal epithelial homeostasis and the relevance of the calcium-sensing receptor (CaSR) as an interface between Ca^{2+} availability, cellular signaling, and the colorectal microenvironment. The mechanisms involved in the regulation of cellular proliferation and differentiation, epithelial barrier integrity, interactions with the intestinal microbiome, and inflammatory responses are discussed, together with the influence of vitamin D status, parathyroid hormone (PTH), and Mg^{2+} on calcium homeostasis. Evidence regarding genetic variability, including the potential roles of CaSR, vitamin D receptor (VDR), and transient receptor potential melastatin 7 (TRPM7), in modulating responses to calcium intake is also reviewed. Based on these findings, a conceptual predictive physiology model is proposed that integrates dietary intake, physiological biomarkers, and individual biological characteristics, followed by reassessment of the biological response and adaptation of the nutritional intervention. A didactic case illustrates the application of this reasoning. At present, the proposed model should be regarded as conceptual and research-oriented, as prospective and interventional studies are required to validate the biomarkers and establish the clinical utility of individualized nutritional interventions for CRC prevention.

Keywords: Colorectal cancer, personalized nutrition, predictive physiology, calcium, calcium-sensing receptor (CaSR)

1. INTRODUCTION

Colorectal cancer (CRC) represents one of the leading causes of cancer-related morbidity and mortality worldwide, with a substantial impact on both patient survival and quality of life and on healthcare systems [2].

The development and progression of CRC result from a complex interaction between genetic predisposition, environmental factors, lifestyle, and metabolic status. Among modifiable factors, diet has a particular role, as it simultaneously influences energy metabolism, intestinal homeostasis, inflammation, the microbiome, and the processes of proliferation and differentiation of the colorectal epithelium.

However, the relationship between nutrition and CRC risk is more complex than a simple association between the consumption of a particular food or nutrient and disease occurrence. Although numerous epidemiological and experimental studies support the role of specific dietary components in modulating colorectal risk, their effects are not uniform at the individual level. The same nutritional intervention may induce different physiological responses depending on metabolic, hormonal, inflammatory, genetic, and intestinal microbiome characteristics. Consequently, nutritional recommendations based exclusively on population mean values may not always reflect an individual's requirements or biological response. This interindividual variability represents one of the premises underlying the development of personalized nutrition [15].

Personalized nutrition aims to adapt dietary interventions to the biological characteristics of the individual by integrating information on metabolic, nutritional, and genetic status and, increasingly, on the characteristics of the intestinal microbiome. Within this paradigm, the objective is not only to correct an overt nutritional deficiency but also to identify early physiological changes that may precede the development of metabolic imbalance or disease. Thus, diet may be viewed not only as a means of meeting energy and nutrient requirements but also as a factor capable of modulating homeostasis and individual disease risk [15].

In this context, the concept of predictive physiology is emerging as an approach in which measurable physiological and biological parameters are used to anticipate the body's response to different stimuli, including nutritional interventions. Unlike predominantly descriptive physiology, which focuses on characterizing physiological functions under given conditions, the predictive approach seeks to identify relationships between an individual's physiological profile and their subsequent response to an intervention. In nutrition, this approach may integrate information on metabolic rate, insulin sensitivity, hormonal profile, micronutrient status, systemic inflammation, intestinal function, and microbiome characteristics. Therefore, predictive physiology may provide a bridge between individual biological assessment and the development of nutritional interventions adapted to individual risk.

Calcium represents a relevant example of this perspective, as it is an essential nutrient whose relationship with colorectal

health extends beyond its well-established role in mineral homeostasis and bone metabolism. Calcium participates in cellular processes involved in maintaining intestinal epithelial homeostasis, and its effects on the colon are related, at least in part, to its interaction with the calcium-sensing receptor (CaSR) [10, 11]. Activation of CaSR in colonic epithelial cells has been associated with the promotion of cellular differentiation, limitation of excessive proliferation, regulation of the Wnt/ β -catenin pathway, and maintenance of intercellular junction integrity [3, 11]. At the same time, reduced or absent CaSR expression has been observed in numerous colorectal tumors, suggesting that the functionality of this signaling axis may influence the response of the colonic mucosa to calcium availability [11].

This relationship highlights an important aspect of personalized nutrition: the biological effect of a nutrient depends not only on the amount ingested but also on the physiological context in which it acts [11]. In the case of calcium, the response may be influenced by vitamin D status, calcium-phosphate homeostasis, the availability of other micronutrients, characteristics of the intestinal microbiome, and, potentially, individual variation in receptors involved in calcium metabolism and signaling. Therefore, simply establishing a universal dietary intake does not fully capture the complexity of the relationship between calcium and colorectal physiology.

From this perspective, calcium and CaSR may represent a relevant model for applying predictive physiology to personalized nutrition, as they allow consideration of the relationship between nutrient intake, individual physiological status, molecular mechanisms, and potential disease risk. This approach does not imply that calcium supplementation represents a universal strategy for CRC prevention; rather, it raises the broader question of identifying individuals in whom a nutritional intervention may provide a relevant physiological benefit and determining the biological conditions required to achieve such a response.

The aim of this review is to critically analyze the relationship between predictive physiology, personalized nutrition, and CRC prevention, with a particular focus on calcium metabolism and the Ca^{2+} /CaSR axis. The roles of physiological and nutritional biomarkers, the potential contribution of genetic factors and metabolic status to variability in the response to calcium, and the possibility of integrating this information into a proactive, prevention-oriented nutritional model are discussed.

2. METHODS

2.1. Type of review

This article represents a critical narrative review of the literature.

2.2. Literature search strategy

The literature search was structured to identify relevant publications addressing the relationship between colorectal cancer, nutritional factors, and the physiological mechanisms involved in individual responses to nutrients. The main biomedical and multidisciplinary databases considered were PubMed/MEDLINE, Scopus, and Web of Science.

The search strategy included combinations of terms related to colorectal cancer ("colorectal cancer," "colon cancer," "colorectal neoplasms"), nutrition and micronutrients ("nutrition," "diet," "calcium," "vitamin D," "folate," "magnesium"), as well as terms related to individual response mechanisms ("calcium-sensing receptor," "CaSR," "VDR," "TRPM7," "genetic polymorphism," "nutrigenetics," "biomarkers," "personalized nutrition," "precision nutrition").

The terms were combined using the Boolean operators AND/OR to identify both studies focusing on the relationship between calcium and CRC and those investigating the physiological, genetic, and molecular mechanisms relevant to the individualization of nutritional intake.

2.3. Inclusion criteria

Publications were considered eligible for the narrative analysis if they met one or more of the following criteria: clinical studies and prospective observational studies addressing calcium intake and the risk of CRC or precancerous colorectal lesions; meta-analyses and systematic reviews relevant to the relationship between nutrients and CRC; epidemiological studies investigating the association between micronutrient intake and CRC burden; experimental studies investigating the mechanisms through which calcium may influence proliferation, differentiation, and homeostasis of the colonic epithelium; studies addressing CaSR, VDR, TRPM7, and other molecular mechanisms relevant to calcium metabolism; and publications addressing physiological, metabolic, or genetic biomarkers with potential applications in personalized nutrition and CRC risk assessment.

2.4. Exclusion criteria

Publications that did not present a relevant relationship with the topic of the review, studies providing insufficient information on nutritional exposure or oncological outcomes, and duplicate publications were excluded. Sources with limited relevance to the physiological objective of the article were also excluded, particularly those addressing CRC treatment exclusively, without implications for prevention or nutrition.

For statements concerning physiological and molecular mechanisms, preference was given to original studies and review articles published in specialized scientific journals.

2.5. Evidence analysis and integration

The identified information was comparatively analyzed and thematically integrated according to the physiological level investigated: (1) dietary intake and nutritional status, (2) mineral and metabolic homeostasis, (3) physiological and molecular biomarkers, (4) cellular mechanisms involved in colorectal carcinogenesis, and (5) implications for personalized nutrition and predictive physiology.

Particular attention was given to studies that enable a transition from the epidemiological association between calcium intake

and CRC risk toward the identification of biological mechanisms that may help explain interindividual variability in the response to calcium intake.

3. BIOMARKERS AND PREDICTIVE CRITERIA IN CALCIUM METABOLISM

3.1. Calcium Homeostasis – From Serum Calcium to a Predictive Physiological Network

Calcium represents a relevant example of how a nutrient can be evaluated from the perspective of predictive physiology. Although serum calcium is maintained within a relatively narrow range through complex homeostatic mechanisms, serum calcium concentration, considered in isolation, provides limited information about calcium nutritional status and about how the body responds to dietary calcium intake.

Calcium homeostasis results from the interaction among the intestine, bone, kidneys, and parathyroid glands, predominantly coordinated by parathyroid hormone (PTH), vitamin D, and the calcium-sensing receptor (CaSR). These mechanisms ensure the maintenance of extracellular Ca^{2+} concentration within a range compatible with neuromuscular function, bone mineralization, and cellular signaling. Therefore, the physiological assessment of calcium metabolism should go beyond the interpretation of a single serum value and consider the relationship between intake, absorption, hormonal regulation, and tissue utilization of the mineral [10, 19].

In this context, serum calcium should be regarded primarily as an indicator of systemic homeostasis rather than as a direct indicator of dietary calcium intake. Maintenance of serum calcium through endocrine mechanisms may temporarily mask variations in calcium intake or intestinal absorption by modifying calcium exchange among the intestine, bone, and kidneys. From this perspective, a serum calcium value within the physiological range does not exclude insufficient dietary calcium intake or alterations in calcium metabolism.

3.2. Vitamin D and PTH – Integrated Assessment of Calcium Status

Assessment of calcium status can be improved by integrating serum calcium with the main regulators of mineral homeostasis, particularly PTH and vitamin D. PTH is one of the principal regulators of extracellular Ca^{2+} concentration, contributing to increased renal calcium reabsorption, stimulation of calcium mobilization from bone, and renal activation of vitamin D. In turn, vitamin D increases intestinal calcium absorption and contributes to the maintenance of calcium-phosphate homeostasis.

From a physiological perspective, therefore, combining serum calcium with PTH and vitamin D status provides more comprehensive information than the isolated measurement of serum calcium. This approach allows differentiation between situations in which serum calcium is maintained through compensatory mechanisms and those in which a more evident disturbance of mineral homeostasis is present [10].

3.3. The Role of Magnesium and the Relevance of the Ca:Mg Ratio

Magnesium should be considered when interpreting calcium metabolism, as it participates in numerous enzymatic processes and signaling mechanisms involved in mineral homeostasis. Severe magnesium deficiency may impair both PTH secretion and action, thereby indirectly affecting Ca^{2+} homeostasis. Therefore, assessment of calcium outside the metabolic context of magnesium may provide an incomplete picture of individual mineral status [5].

From this perspective, the calcium-to-magnesium ratio represents an interesting hypothesis for the development of integrated indicators of mineral status. However, unlike serum calcium, PTH, or 25(OH)D, the use of the Ca:Mg ratio as a predictive biomarker of colorectal neoplasia risk requires further clinical validation before it can be proposed as a routine screening tool.

3.4. Assessment of Dietary Intake and Integration of Nutritional Data with Biological Biomarkers

Assessment of physiological status cannot be separated from the assessment of dietary intake. Food Frequency Questionnaires (FFQs) and other dietary assessment tools allow estimation of habitual calcium intake and of the main nutrients that influence calcium metabolism. However, dietary intake assessment and the measurement of biological biomarkers provide different and complementary information [18].

Within a predictive physiology approach, dietary intake data should be correlated with available biological parameters to identify situations in which the estimated intake is not adequately reflected in the individual's physiological status. This integration may help differentiate between insufficient intake, reduced absorption, increased requirements, or a different individual physiological response.

3.5. From an Individual Biomarker to a Predictive Physiological Profile

Consequently, predictive physiology does not involve identifying a single biomarker capable of anticipating individual risk, but rather the construction of an integrated physiological profile in which nutritional, hormonal, metabolic, and, subsequently, genetic information are interpreted together [16].

For calcium metabolism, such a profile may start with dietary calcium intake, complemented by serum calcium, PTH, and 25(OH)D, and may be extended, depending on the clinical question, to magnesium, phosphate, markers of bone metabolism, and molecular assessments. In the context of colorectal cancer, the predictive value of such a profile should be evaluated not by a single parameter, but by its ability to identify relevant individual differences in calcium absorption, utilization, and tissue response.

This perspective opens the way to the nutrigenetic analysis of calcium metabolism, in which genetic differences in proteins involved in Ca^{2+} sensing, transport, and signaling may contribute to explaining individual variability in the response to dietary intake [12].

4. CALCIUM NUTRIGENETICS: INDIVIDUALIZING THE RESPONSE TO DIETARY INTAKE

4.1. Genetic Variability in Calcium Metabolism

The body's response to dietary calcium intake is influenced by complex mechanisms involving absorption, transport, hormonal regulation, and cellular signaling. Although nutritional requirements are generally expressed as reference values established at the population level, these values do not fully capture interindividual variability in calcium metabolism. Genetic differences affecting proteins involved in Ca^{2+} homeostasis and signaling may contribute to this heterogeneity and represent one of the premises underlying the nutrigenetic approach [12].

In the context of CRC prevention, interest in calcium nutrigenetics arises from the possibility that genetic variation may influence not only systemic calcium availability but also the cellular response of the intestinal epithelium to this nutrient. Thus, the biological effect of a similar calcium intake may differ between individuals depending on the genetic characteristics of pathways involved in Ca^{2+} sensing and utilization.

4.2. CaSR – A Relevant Nutrigenetic Interface

A particular candidate of interest is the calcium-sensing receptor (CaSR), a membrane receptor with a central role in sensing variations in extracellular Ca^{2+} concentration and regulating mineral homeostasis. CaSR is expressed not only in the parathyroid glands and kidneys but also throughout the gastrointestinal tract, including the colonic epithelium [11].

In the colon, CaSR activation may influence fundamental processes involved in maintaining tissue homeostasis, including cellular proliferation, differentiation, and apoptosis. In addition, CaSR signaling interacts with molecular pathways involved in epithelial homeostasis and colorectal carcinogenesis, including the Wnt/ β -catenin pathway. These observations provide a physiological basis for investigating CaSR as a potential mediator of the relationship between calcium availability and the individual response of the colonic mucosa [3, 11].

From a nutrigenetic perspective, genetic variation in the CASR gene may influence receptor expression or function and, consequently, the way tissues respond to changes in Ca^{2+} availability. However, the existence of a biologically plausible association does not, in itself, demonstrate that a particular polymorphism determines different nutritional requirements or that it can currently be used to guide individualized recommendations for calcium intake.

4.3. VDR and TRPM7 – Components of the Calcium Response Network

In addition to CaSR, other components of the network involved in calcium metabolism and signaling may exhibit genetic variation with potential nutrigenetic relevance.

The vitamin D receptor (VDR) mediates the biological effects of the active form of vitamin D and contributes to the regulation of intestinal calcium absorption. Genetic variants of the VDR gene have been investigated in relation to mineral metabolism, bone mineral density, and various diseases, including colorectal cancer. However, the available findings are heterogeneous, and the clinical significance of these variants for individualizing calcium intake remains insufficiently defined [12].

TRPM7 (Transient Receptor Potential Melastatin 7) represents another protein of interest in this context, with roles in ion transport and cellular signaling and implications for both Ca^{2+} and Mg^{2+} . Experimental and genetic studies suggest that functional variation in components of this network may contribute to individual differences in cellular responses to mineral availability [5].

Therefore, CaSR, VDR, and TRPM7 should be regarded as components of an interconnected physiological network rather than as independent biomarkers currently capable of determining individual calcium requirements.

4.4. From Nutrigenetics to the “Responder” and “Non-responder” Concept

The concepts of “responder” and “non-responder” can therefore be used as research models to describe the heterogeneity of responses to nutritional interventions. However, this classification should be distinguished from an individualized clinical recommendation: identifying a polymorphism statistically associated with a particular response is not equivalent to demonstrating that modifying calcium intake on the basis of that genotype improves clinical outcomes [16].

At present, the available evidence supports the research potential of the nutrigenetic approach rather than the routine use of genotyping to determine individual calcium intake for the purpose of CRC prevention.

4.5. Implications for Predictive Physiology

Integrating genetic information with physiological biomarkers and dietary intake data may represent a further step in the development of the predictive physiology model proposed in this review. Rather than relying on a single indicator, the individual profile could incorporate dietary Ca^{2+} intake, serum calcium, PTH, 25(OH)D, Mg^{2+} , genetic factors, and the mechanisms involved in CaSR/VDR/TRPM7 signaling.

Such a profile could, in the future, contribute to the identification of individuals with different physiological responses to calcium intake and to the formulation of nutritional interventions better adapted to individual characteristics. However, clinical validation of such models is an essential prerequisite before nutrigenetic information can be used to guide individualized recommendations regarding calcium intake for CRC prevention [16].

4.6. Calcium Nutrigenetics: The Gene–Nutrient Interaction Between TRPM7 and Calcium and Magnesium Intake

The effects of a nutrient are not determined exclusively by the amount consumed but may depend on individual biological characteristics, including genetic variation in proteins involved in nutrient transport, absorption, and homeostasis. In the case of calcium, TRPM7 represents a relevant example, as it is an ion channel involved in cellular magnesium and calcium homeostasis and may therefore be relevant to how the body responds to the intake of these minerals.

An example of a gene-nutrient interaction comes from the Tennessee Colorectal Polyp Study, a case-control epidemiological study that investigated the relationship between calcium and magnesium intake, the Ca:Mg ratio, and the Thr1482Ile polymorphism of the TRPM7 gene in relation to the risk of colorectal neoplasia. The study included 688 cases with colorectal adenomas, 210 cases with hyperplastic polyps, and 1,306 polyp-free controls [5].

The results suggested that the effects of calcium and magnesium intake on colorectal neoplasia risk are not uniform across the population but may be modified by genetic characteristics of TRPM7 and by the ratio between calcium and magnesium intake. In particular, carriers of the Ile1482 variant allele exhibited a higher risk of adenoma in the context of a higher dietary Ca:Mg ratio compared with individuals without this variant [5].

These observations are important not because they support the notion that higher calcium intake is “good” for some individuals and “bad” for others in an absolute sense, but because they illustrate that the relationship between nutritional exposure and biological response may depend on genetic and metabolic context. Moreover, the study authors interpreted these findings as a possible explanation for inconsistencies previously observed in studies investigating the relationship between calcium, magnesium, and colorectal neoplasia and emphasized the need for confirmation of these observations [5].

From the perspective of personalized nutrition, the implication is conceptually important: the same nutritional intervention should not automatically be assumed to have the same benefit–risk relationship in all individuals. A recommendation regarding calcium intake should be interpreted in relation to individual requirements, dietary sources, mineral status, the Ca:Mg ratio, metabolic characteristics, and, when supported by sufficient evidence, relevant genetic susceptibility.

Therefore, nutrigenetics does not aim to replace general nutritional recommendations with recommendations based exclusively on genotype, but rather to identify situations in which individual biological variability may modify the response to a nutritional intervention. In this context, the TRPM7–calcium interaction represents an example of the transition from the question “How much calcium should a person consume?” to a more complex question: “What is this person's response to a given level of calcium intake, and which biological factors may modify its benefits and risks?”

4.7. From Nutrigenetics to Predictive Physiology

Nutrigenetics represents one of the levels through which interindividual variability in responses to nutrients may be explained. However, genotype is not the sole determinant of the physiological response. The effect of a nutrient results from the interaction among the individual's genetic characteristics, nutritional and metabolic status, physiological characteristics, intestinal microbiome, lifestyle, and environmental factors [12, 16].

In this context, the identification of a genetic variant alone is insufficient to formulate an individualized nutritional recommendation. The same genetic variant may have different consequences depending on the biological context in which it is expressed. In the case of calcium, for example, the body's response depends not only on the amount consumed but also on vitamin D status, calcium-phosphate metabolism, renal function, magnesium intake, physiological requirements, and cellular transport and signaling mechanisms.

Therefore, personalized nutrition involves a shift from an approach based predominantly on population-level recommended intake values toward an approach that takes into account the individual's biological response. From this perspective, biomarkers can complement genetic information by providing data on the current physiological state and on how the body responds to a given nutritional intake [12].

The concept of predictive physiology thus extends the nutrigenetic paradigm. Whereas nutrigenetics seeks to identify how genetic variation may influence the response to nutrients, predictive physiology aims to integrate multiple levels of biological information in order to estimate an individual's probable response to a nutritional intervention before or during its implementation.

In the context of colorectal cancer prevention, this approach can be conceptualized through the integration of multiple levels:



Figure 1. From nutritional intake to individualized adaptation of the intervention: principles of predictive physiology

This perspective also modifies the fundamental question addressed by nutrition. Instead of asking, “What is the optimal calcium intake for the population?”, predictive physiology proposes a question that is closer to individual biological reality: “What is this individual's probable response to a given level of calcium intake, and what is the anticipated balance between benefits and risks?”

Consequently, population-based recommendations do not lose their value but rather represent the starting point for nutritional intervention. Personalized nutrition seeks to identify situations in which individual characteristics justify an adaptation of these recommendations. In this sense, predictive physiology does not represent an alternative to classical physiology, but rather an extension of it toward the prediction of individual responses.

4.8. Biomarkers as Tools of Predictive Physiology

If nutrigenetics provides information about the individual's potential response to a nutrient, biomarkers can provide information about the individual's current biological state. In personalized nutrition, this distinction is important because the same genetic variant may have different physiological consequences depending on the person's nutritional, metabolic, and hormonal status. Biomarkers can be used at different levels, ranging from the assessment of dietary intake and nutritional status to the characterization of metabolism, inflammation, and cellular responses. In nutritional research, a biomarker relevant to an intervention should have a biologically plausible relationship with the process under investigation and, ideally, should change reproducibly in response to the nutritional intervention [7].

Consequently, interpretation of the response to calcium intake requires the integration of serum calcium with indicators of mineral regulation and metabolism, such as PTH, 25(OH)D, and magnesium.

This perspective is particularly relevant to vitamin D and calcium, as their homeostasis is closely interdependent. Vitamin D status may influence intestinal calcium absorption, whereas PTH contributes to the maintenance of extracellular calcium concentration through coordinated actions at the bone, renal, and, indirectly, intestinal levels. Therefore, an identical calcium intake may occur within a different physiological context in two individuals with different vitamin D status or calcium-phosphate metabolism [10].

In addition, biomarkers can extend beyond the simple assessment of nutritional status and contribute to the characterization of an individual's metabolic phenotype. Circulating metabolic profiles, inflammatory biomarkers, metabolomics, and other markers derived from host–intestinal microbiome interactions represent areas of research in the development of precision nutrition. The concept of metabotyping specifically aims to identify subgroups of individuals who exhibit different metabolic responses to the same nutritional intervention [16].

In colorectal cancer prevention, interest in biomarkers is even greater because the effect of a nutrient can be investigated at intermediate biological levels, before changes manifest as a clinical event. For calcium and vitamin D, for example, tissue and circulating biomarkers associated with proliferation and differentiation of the colonic epithelium have been studied, including TGF- α , TGF- β 1, APC, β -catenin, and E-cadherin. These data support the potential use of intermediate markers to investigate the mechanisms through which nutritional interventions may influence colorectal carcinogenesis, although their value as predictive clinical biomarkers still requires validation [1].

Thus, biomarkers may occupy an intermediate position between genotype and phenotype, contributing to the description of how individual characteristics translate into a measurable physiological state. Integrating genetic information with nutritional and metabolic biomarkers may allow a more precise characterization of the individual than the isolated use of any one of these types of data.

From this perspective, predictive physiology can be represented as a dynamic process:

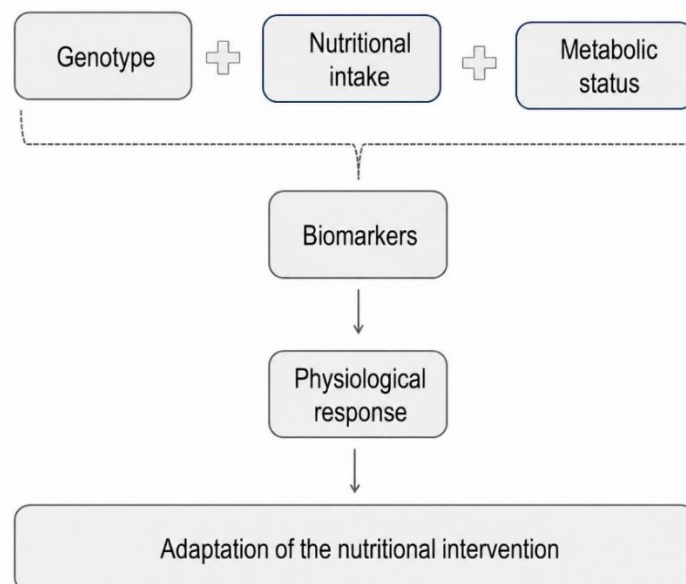


Figure 2. Integration of genetic, nutritional, and metabolic information in the prediction of the physiological response

However, it is important that this approach not be confused with the notion that any available biomarker can immediately be used to prescribe a personalized diet. Biomarker validation requires demonstration of its relationship with the biological process of interest, measurement reproducibility, and the ability to effectively contribute to the prediction of a response or a clinically relevant outcome. At present, many of these tools remain at the research stage and cannot be used to establish standardized individualized recommendations regarding calcium intake for CRC prevention [7].

5. Predictive Physiology Model for Colorectal Cancer Prevention

The model proposed in this paper is conceptual and is derived from the integration of physiological mechanisms, biomarkers, and individual variability factors. In its current form, it does not represent a clinically validated tool for calculating individual CRC risk or for prescribing a personalized calcium dose.

5.1. From Population-Based Recommendations to the Individual Physiological Profile

Current nutritional recommendations are necessarily established at the population level. They provide useful reference values for most individuals but cannot capture the full extent of biological variability within the population.

In CRC prevention, a predictive physiology approach could start from this general recommendation and progressively integrate individual information regarding dietary intake, nutritional status, mineral metabolism, metabolic and inflammatory factors, genetic characteristics, and biomarkers relevant to the response to the nutritional intervention.

From this perspective, the population-based recommendation is not abandoned but becomes the starting point for an individualized assessment.

5.2. Conceptual Architecture of the Model

The predictive physiology model for colorectal cancer prevention can be represented as a sequence of biological levels, namely nutritional exposure, nutritional and metabolic status, physiological biomarkers, genetic and molecular variability, cellular and tissue responses, assessment of biological conditions associated with carcinogenesis, and, ultimately, adaptation of the nutritional intervention. In the case of calcium, this sequence can be further specified as follows:

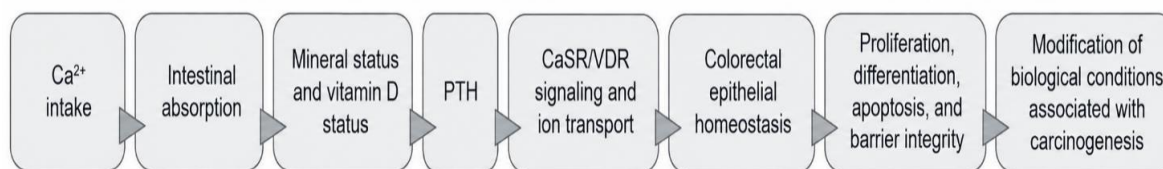


Figure 3. Integrative physiological model of the relationship between calcium intake and colorectal epithelial homeostasis

5.3. Calcium as a Model for Application

Calcium represents a suitable example for illustrating this model because it acts simultaneously at multiple levels of physiology. At the systemic level, Ca^{2+} homeostasis is maintained through interactions among the intestine, bone, and kidneys and through hormonal regulation. At the intestinal level, calcium availability is influenced by absorption, vitamin D status, and interactions with other nutrients and minerals [10].

At the level of the colorectal epithelium, Ca^{2+} may also act as a cellular signal, including through CaSR, influencing processes such as cellular proliferation and differentiation. Thus, the biological effect of calcium cannot be reduced to the amount absorbed or to serum concentration but should be analyzed in relation to the physiological context in which the nutrient is available and utilized [10, 11].

This perspective provides an explanation for why epidemiological studies of calcium and CRC may yield heterogeneous results [20]: the populations investigated are not biologically homogeneous, and the observed effect may reflect interactions among intake, nutritional status, genetic factors, metabolism, and characteristics of the intestinal environment.

5.4. Predictive Objective of the Model

The model aims to estimate the probable physiological response to a nutritional intervention and to identify individual factors that may modify the balance between its potential benefits and risks.

Thus, prediction may refer to:

- a) the probability of a favorable physiological response;
- b) the identification of substantial variability in the response;
- c) the identification of situations in which the intervention may have limited benefits;
- d) the identification of biological contexts in which an intervention that appears beneficial at the population level may require caution or individualization.

Predictive physiology is not equivalent to predicting an oncological diagnosis.

5.5. The Dynamic Model

The predictive physiology model for CRC prevention should not be conceived as a sequence of genotype followed by a fixed recommendation, but rather as a dynamic and iterative process consisting of initial assessment, nutritional intervention, measurement of the biological response, reassessment, and, ultimately, adaptation of the intervention.

In other words, predictive physiology should enable a transition from personalized nutrition based exclusively on the initial prediction to an approach based on both prediction and verification of the individual response.

This concept is particularly important because a genetic profile may suggest a certain probability of response, whereas the measured physiological phenotype remains the integrated expression of genes, environment, and dietary behavior [15, 16].

6. CALCIUM AND COLORECTAL EPITHELIAL HOMEOSTASIS: FROM Ca^{2+} SIGNALING TO THE CARCINOGENESIS-ASSOCIATED MICROENVIRONMENT

The relationship between calcium intake and colorectal cancer prevention cannot be understood exclusively by relating the amount of calcium consumed to the epidemiological risk of CRC. Calcium available at the intestinal level interacts with cellular and tissue regulatory mechanisms that contribute to the maintenance of colorectal epithelial homeostasis. In this context, CaSR and Ca^{2+} -dependent signaling pathways represent a potential interface between the nutritional environment and epithelial cell behavior [10, 11].

To understand this relationship, the mechanisms can be followed from the availability of Ca^{2+} in the intestinal lumen, through cellular sensing and signaling, to the processes that maintain the integrity and homeostasis of the colorectal epithelium.

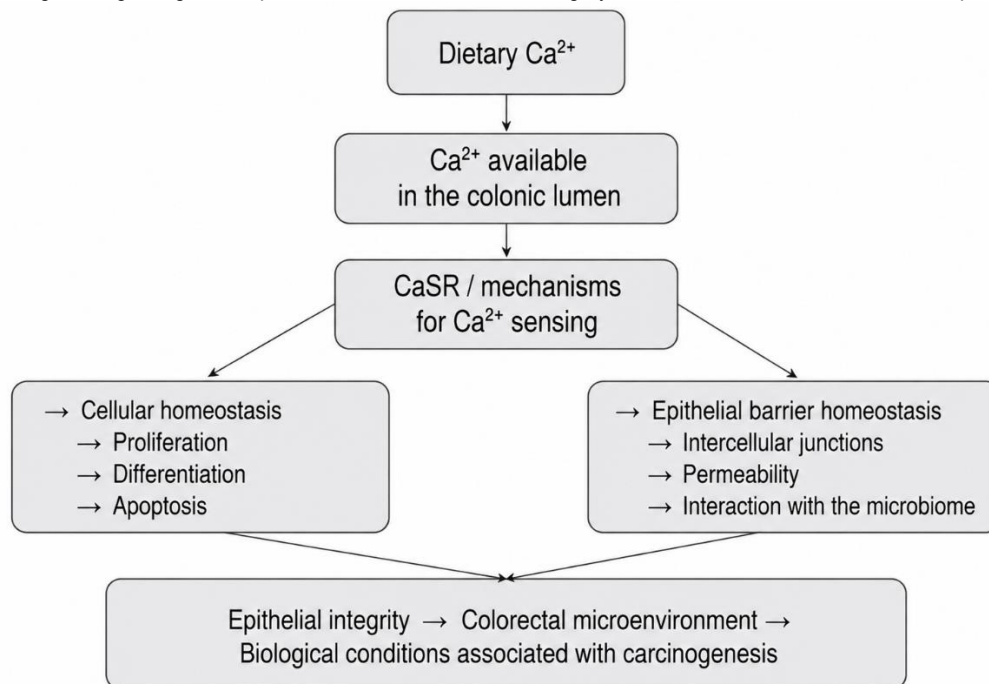


Figure 4. From luminal Ca^{2+} availability to colorectal epithelial homeostasis and biological conditions associated with carcinogenesis

6.1. Calcium as a Nutrient and as a Biological Signal

Calcium should not be regarded exclusively as a mineral involved in systemic homeostasis. At the level of the intestinal epithelium, Ca^{2+} also participates in cellular signaling processes, and changes in the extracellular availability of the ion may influence epithelial cell function. This dual perspective—calcium as a nutrient and Ca^{2+} as a cellular signal—is essential for understanding its potential role in colorectal epithelial physiology [10, 11].

At the level of the colon, epithelial homeostasis involves a dynamic balance among the proliferation of stem and progenitor cells, cellular differentiation, migration and maturation of epithelial cells from the crypt base toward the luminal surface, and the elimination of senescent or damaged cells through apoptosis. Disruption of this balance represents one of the early characteristics of processes that may favor neoplastic transformation [6].

In this context, Ca^{2+} -dependent signaling may represent one of the interfaces through which the nutritional environment interacts with the physiology of the colorectal epithelium [10, 11].

6.2. CaSR – The Interface Between Calcium and the Epithelial Cell

The calcium-sensing receptor (CaSR) is a membrane receptor capable of sensing changes in extracellular Ca^{2+} concentration. Although it is primarily known for its role in regulating systemic calcium homeostasis, CaSR is also expressed throughout the gastrointestinal tract, including in the colorectal epithelium [10, 11].

Activation of CaSR generates intracellular signals that may influence processes such as cellular proliferation, differentiation, and apoptosis. In this way, the receptor may function as an interface between extracellular Ca^{2+} availability and epithelial cell behavior [11].

This perspective is important for CRC prevention because neoplastic transformation is associated with loss of control over these processes [11]. Maintaining an appropriate balance between proliferation and cellular elimination is therefore a fundamental component of colorectal epithelial homeostasis [6].

6.3. CaSR and the Wnt/ β -catenin Pathway

The Wnt/ β -catenin pathway represents a central mechanism in the regulation of intestinal epithelial homeostasis and plays a

major role in colorectal carcinogenesis. Aberrant activation of this pathway promotes the maintenance of a proliferative phenotype and represents a molecular event characteristic of the development of many colorectal neoplasms [6]. Experimental data suggest that CaSR signaling may interact with this molecular network and contribute to the modulation of β -catenin activity. Consequently, CaSR may represent a potential link through which Ca^{2+} availability influences the processes of proliferation and differentiation in the colorectal epithelium [3, 11].

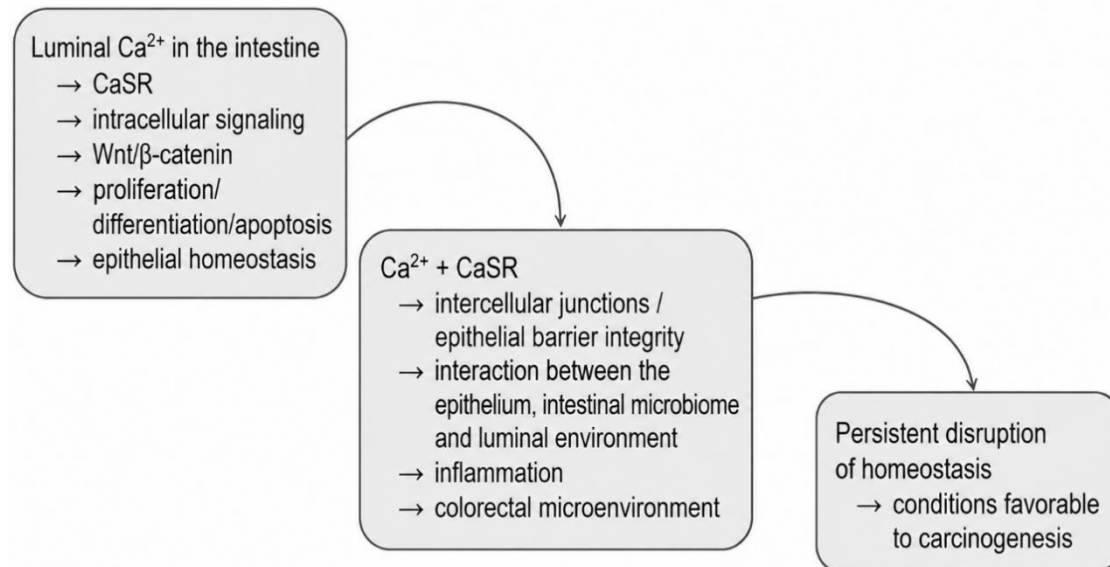


Figure 5. Interaction between Ca^{2+} /CaSR signaling, the epithelial barrier, and the colorectal microenvironment

6.4. Calcium, Intercellular Junctions, and Epithelial Barrier Integrity

The colorectal epithelium represents a dynamic interface between the organism and the intestinal luminal environment and simultaneously performs functions of absorption, transport, protection, and communication with the intestinal microbiome. The integrity of this barrier depends on the structural organization of epithelial cells and strict control of paracellular permeability. A key role is played by intercellular junctional complexes, particularly tight junctions and adherens junctions. These structures regulate the paracellular passage of water, ions, and molecules and contribute to the maintenance of epithelial polarity and cohesion. Alteration of junctional proteins may increase epithelial permeability and allow greater exposure of the subepithelial compartment to components of the luminal environment [13].

In this context, the relationship between Ca^{2+} and epithelial integrity is relevant from two perspectives. On the one hand, extracellular calcium participates in processes of cell adhesion and intercellular organization; on the other hand, CaSR expressed in the intestinal epithelium may contribute to the regulation of barrier function and local homeostasis. Experimental studies have shown that CaSR deficiency in the intestinal epithelium is associated with impaired barrier integrity, alterations in the microbiota, and a shift in the immune response toward a proinflammatory profile [4].

Consequently, CaSR can be viewed not only as a sensor of Ca^{2+} availability but also as a component of the communication system linking the nutrient, epithelium, intestinal microbiome, and immune system. This perspective extends the role of calcium beyond mineral homeostasis and provides a potential mechanism through which the nutritional environment may influence colorectal mucosal homeostasis.

A particularly interesting aspect is the interaction between CaSR and E-cadherin/ β -catenin. In colorectal epithelial cells, Ca^{2+} - and CaSR-dependent signaling has been associated with increased E-cadherin expression and reduced β -catenin/TCF activation, mechanisms compatible with the maintenance of a more differentiated cellular phenotype [3].

6.5. The Epithelial Barrier and Its Interaction with the Intestinal Microbiome

The surface of the colorectal epithelium is in continuous contact with a complex microbial ecosystem. Under physiological conditions, this relationship is characterized by a dynamic balance among the microbiome, the mucus layer, the intestinal epithelium, and components of mucosal immunity.

The intestinal barrier is not an inert structure. It selectively regulates exchange between the lumen and the organism, allowing the absorption of nutrients and microbial metabolites while limiting the access of microorganisms and their proinflammatory components to the tissue compartment. Alterations of the mucus layer, intercellular junctions, or microbiota composition may disrupt this balance [13].

In the context of CRC, dysbiosis and epithelial barrier disruption may potentiate each other. Changes in the microbiota may affect the mucus layer and junctional integrity, whereas barrier impairment facilitates the proximity of microorganisms and microbial products to the epithelium and mucosal immune cells. This interaction may contribute to the maintenance of a

proinflammatory microenvironment [8].

Experimental and observational data suggest that calcium intake may influence microbiota composition and intestinal integrity, but the relationship is complex and dependent on dietary pattern, calcium form, and metabolic context [9]. Therefore, in the proposed model, the microbiome should be regarded as an intermediate component of the biological response rather than as a direct and uniform target of calcium supplementation.

6.6. Endotoxin (LPS), Intestinal Permeability, and Low-Grade Inflammation

One of the potential consequences of intestinal barrier disruption is increased exposure of the organism to microbial components. Among these, lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, represents an important ligand for innate immune receptors, particularly TLR4, whose MyD88-dependent signaling contributes to the activation of the proinflammatory response [17].

Under conditions of increased intestinal permeability, translocation of microbial products may stimulate epithelial and immune cells and promote the activation of proinflammatory pathways. Repeated or persistent activation of these mechanisms may contribute to the maintenance of a low-grade inflammatory state, with potential effects on the colorectal microenvironment [17]. In colorectal carcinogenesis, chronic inflammation may promote cellular proliferation, oxidative stress, impairment of repair mechanisms, and the survival of cell populations with neoplastic potential. However, the relationship among inflammation, the intestinal microbiome, and carcinogenesis is not linear and cannot be attributed to a single microbial product. Rather, it results from interactions among dysbiosis, barrier disruption, microbial metabolites, and the host immune response [8].

6.7. Integrating Calcium into the Epithelial Barrier–Microbiome–Inflammation Axis

The available data support a model in which Ca^{2+} /CaSR signaling, epithelial barrier integrity, the microbiome, and intestinal inflammation form an interdependent physiological network. Experimentally, disruption of CaSR signaling may compromise barrier function, alter the microbiome, and promote inflammatory responses, while the intestinal environment and microbial components may, in turn, modify epithelial homeostasis [11, 14].

From this perspective, the potential effect of calcium on colorectal cancer risk should not be reduced to a single molecular pathway. Rather, it may result from the convergence of multiple mechanisms acting at the level of the epithelial cell, the intestinal barrier, the microbial environment, and the inflammatory response.

The initial physiological chain illustrated in Figure 5 can therefore be extended as follows:

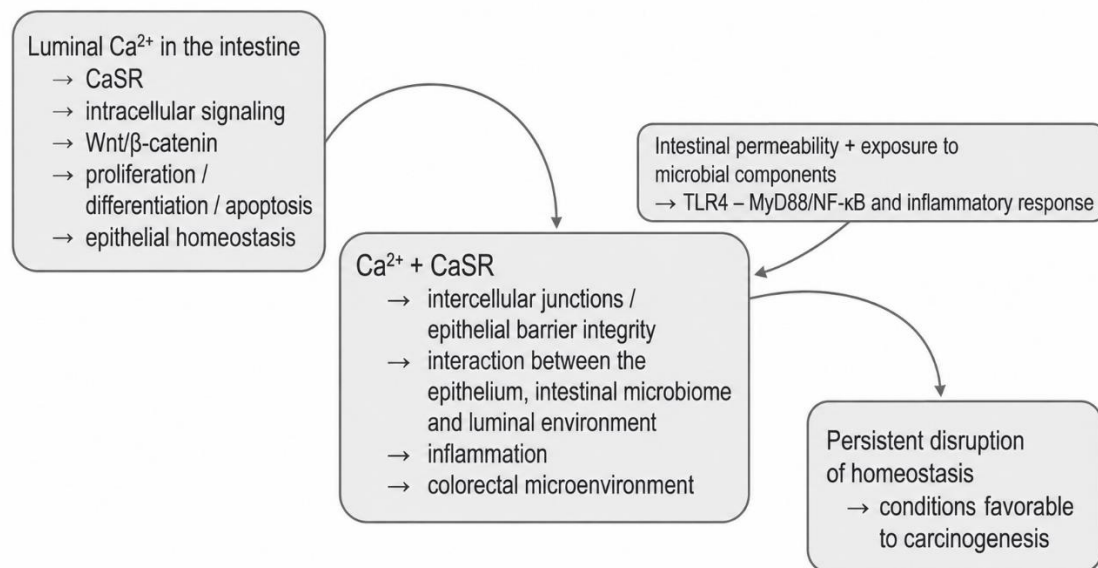


Figure 6. Integration of the epithelial barrier, microbiota, and inflammation in the relationship between Ca^{2+} /CaSR and the colorectal microenvironment

Calcium and CaSR signaling may contribute to the maintenance of epithelial homeostasis and intestinal barrier integrity, while the integrity of this barrier may influence interactions among the intestinal microbiome, microbial products, and the host inflammatory response [3, 11].

Interesting epidemiological data indicate that the association between calcium intake and CRC risk may vary according to tumor CaSR expression. In a large prospective study, total calcium intake was inversely associated with CRC risk, and the authors specifically investigated this relationship according to CaSR status [20].

6.8. Integration of Mechanisms: From Epithelial Homeostasis to the Colorectal Microenvironment

The potential effects of calcium on colorectal carcinogenesis cannot be attributed to a single molecular pathway. Rather, the available evidence supports the existence of an interconnected network in which Ca^{2+} /CaSR signaling, the regulation of cellular proliferation and differentiation, epithelial barrier integrity, the intestinal microbiome, and the inflammatory response influence

one another. CaSR is expressed in the colonic epithelium and has been implicated in the regulation of proliferation, differentiation, barrier function, and inflammation, while experimental data support its interaction with the Wnt/ β -catenin pathway [11].

Under physiological conditions, Ca^{2+} availability and CaSR activation are integrated into mechanisms that maintain the balance among epithelial cell proliferation, differentiation, and cellular elimination. At the same time, the organization of intercellular junctions and the integrity of the epithelial barrier limit the exposure of the epithelium and the subepithelial immune compartment to components of the luminal environment. Disruption of this barrier may promote changes in the interaction between the intestinal microbiome and the epithelium, increased permeability, and activation of inflammatory pathways, thereby contributing to alterations in the colorectal microenvironment [11].

Therefore, the epithelial barrier represents a link between cellular homeostasis and the intestinal microenvironment. Its integrity conditions the interaction between the epithelium and the intestinal microbiome, whereas dysbiosis and barrier disruption may potentiate each other and contribute to the development of a proinflammatory microenvironment. Persistent inflammation may, in turn, influence cellular proliferation, oxidative stress, and tissue repair mechanisms, contributing to conditions favorable to carcinogenesis. This epithelial barrier–microbiome–inflammation axis is currently considered relevant to the initiation and progression of CRC [8].

From this perspective, the relationship between calcium intake and CRC risk should be analyzed in the context of the individual's biological status and the characteristics of the target tissue. The question is no longer simply whether calcium intake influences CRC risk, but rather in what biological context, through which mechanisms, and in which individuals this intervention is likely to produce a favorable response [11].

Thus, the effect of a nutrient is not an isolated property of the nutrient itself but the result of interactions among dose, bioavailability, the individual's biological status, cellular sensing and signaling mechanisms, target-tissue integrity, and characteristics of the intestinal environment. This perspective supports a shift from the isolated assessment of dietary intake toward an integrative approach in which nutritional intervention is correlated with the physiological response and with individual biological characteristics.

7. APPLICATION OF THE PREDICTIVE PHYSIOLOGY MODEL: A DIDACTIC CASE FOR INDIVIDUALIZING CALCIUM INTAKE

7.1. Didactic Case: Individualizing Calcium Intake

To illustrate the practical application of the predictive physiology model, a hypothetical didactic case can be used in which two individuals have a similar dietary calcium intake but different physiological characteristics. The purpose of this example is not to establish an individualized calcium dose, but to highlight how the integration of nutritional, physiological, and, when available, genetic information may modify the interpretation of an identical dietary intake.

Consider two adult individuals, conventionally designated as Individual A and Individual B, with a similar estimated dietary Ca^{2+} intake but different biological profiles. In Individual A, dietary Ca^{2+} intake is relatively low, whereas serum calcium, PTH, 25(OH)D, and Mg^{2+} are within physiological ranges. In Individual B, calcium intake is comparable, but the biological profile reveals particularities in vitamin D status and the regulation of mineral homeostasis. In a traditional model, both individuals might receive the same recommendation based exclusively on dietary intake. In a predictive physiology approach, however, the initial data are interpreted in an integrated manner, and the intervention is considered in relation to the individual physiological profile.

Table I. Comparative physiological profile in the didactic case of individualized calcium intake

Parameter	Individual A	Individual B	Physiological interpretation
Dietary Ca^{2+} intake	↓	↓	Similar nutritional exposure
Serum calcium	N	N	Does not by itself reveal the difference
PTH	N	↑	Indicates differences in the regulation of mineral homeostasis
25(OH)D	Adequate	↓	May modify Ca^{2+} metabolism
Mg^{2+}	N	Altered	Factor associated with mineral homeostasis
Genetic factors	No relevant information	Potentially relevant genetic variant	Additional information that cannot be used in isolation
Approach	Increase dietary calcium intake and monitor the response	Clarify the biological profile before intervention	Individualized intervention

N = within the physiological range.

Note: The values and profiles presented are hypothetical and are intended exclusively for illustrative purposes.

For Individual A, insufficient dietary intake in the absence of evident alterations in biomarkers may support an intervention aimed at increasing calcium intake through dietary sources, with subsequent monitoring of the physiological response. For Individual B, the same intervention should not be implemented automatically before clarifying the factors that may influence calcium homeostasis. In this case, assessment of vitamin D status, PTH, and other relevant parameters may be more important than simply increasing the amount of calcium consumed.

The difference between the two situations illustrates the central principle of predictive physiology: the same nutritional intake does not necessarily result in the same physiological context. The body's response depends on the interaction among intake, bioavailability, metabolic and mineral status, endocrine regulation, and individual biological characteristics. When genetic information is available and relevant, it may complement the physiological profile without replacing the assessment of biomarkers and nutritional context (Table I).

Therefore, in this model, the nutritional decision is not formulated as a direct consequence of a single biological value or genotype, but rather as the result of integrating multiple levels of information. The initial intervention thus represents a nutritional hypothesis adapted to the individual profile, which should subsequently be tested through reassessment of the physiological response. This reassessment stage is the element that distinguishes a static nutritional recommendation from a dynamic predictive physiology model.

7.2. Reassessment of the Physiological Response and Adaptation of the Intervention

In the proposed predictive physiology model, the nutritional intervention does not represent the final stage of the process but rather the starting point for a new assessment of biological status. Following modification of Ca^{2+} intake, the individual response can be monitored through reassessment of relevant physiological parameters, in correlation with changes in dietary intake and the individual's metabolic context. This stage allows verification of the concordance between the response anticipated on the basis of the initial profile and the observed physiological response.

If the nutritional intervention is associated with maintenance of an adequate physiological profile and is well tolerated, it may be continued, with periodic monitoring of relevant parameters. Conversely, the occurrence of unexpected changes in biomarkers or the identification of factors that modify the biological status may lead to reconsideration of the initial intervention. Thus, reassessment does not automatically imply increasing or reducing calcium intake, but rather allows identification of the reasons why the observed response differs from the anticipated response.

This approach is particularly important in the context of individual biological variability. A physiological response that differs from the anticipated response should not be interpreted exclusively as a "failure" of the intervention, but may provide additional information about the interaction among dietary intake, metabolic status, endocrine regulation, and individual characteristics. In this regard, biomarkers acquire a dynamic function, being used not only for the initial characterization of the individual but also for monitoring changes resulting from the intervention.

Therefore, the model can be represented as a cycle of assessment–intervention–response–reassessment–adaptation, in which each stage provides information for the subsequent stage. This characteristic distinguishes predictive physiology from the application of a fixed nutritional recommendation and allows the intervention to be conceived as a dynamic process based on the observed biological response. In CRC prevention, such a model may have conceptual value for identifying conditions under which a nutritional intervention is biologically plausible and for defining research strategies aimed at individualizing the response. However, it should not be interpreted as a validated clinical protocol for CRC prevention or as a method for predicting an individual's development of cancer.

7.3. Current Limitations and Applicative Perspectives

The clinical application of the predictive physiology model proposed in this review for colorectal cancer prevention currently presents important limitations. Although findings from the fields of nutrigenetics and physiological biomarkers support the existence of interindividual variability in responses to nutrients, the use of this information to formulate individualized clinical recommendations still requires further validation through prospective and interventional studies [15].

At present, there are no sufficiently validated biomarkers that allow the precise identification of individuals who will benefit from a specific nutritional intervention based on calcium intake. The observed associations among dietary intake, genetic variants, and colorectal neoplasia risk should be interpreted with caution, as most derive from observational studies or gene–nutrient interaction analyses and, in isolation, do not demonstrate a causal relationship sufficient to support individualized recommendations [5, 15].

The complexity of the relationships among nutrients, metabolism, the intestinal microbiome, genetic factors, and the tissue microenvironment suggests that the biological response to calcium intake results from an integrated physiological network rather than from a single determinant. In addition, the estimation of dietary intake using epidemiological methods remains distinct from the measurement of the body's actual biological response [15, 18].

Prospective and interventional studies are needed to simultaneously evaluate dietary intake, metabolic biomarkers, the expression of receptors involved in calcium homeostasis, and long-term clinical outcomes. Such an approach would allow validation of predictive models and identification of individuals who respond differently to the same nutritional intervention [15]. The development of metabotypes and the integration of multi-omics approaches represent promising directions for transforming predictive physiology into a tool applicable to clinical practice. However, before such models can provide a basis for individualized recommendations regarding calcium intake for CRC prevention, they must demonstrate reproducibility,

independent validation, and clinical utility [15, 16].

8. CONCLUSIONS

Colorectal cancer prevention represents a field in which nutritional interventions may benefit from an approach focused not only on dietary intake but also on variability in the individual biological response. The analysis presented in this review supports the concept of predictive physiology as an integrative framework in which dietary information is correlated with mineral and metabolic status, physiological biomarkers, and, when sufficient evidence is available, the individual's genetic and molecular characteristics.

Calcium represents a relevant example of this approach because its biological role extends beyond its function as a nutrient involved in mineral homeostasis. Ca^{2+} availability and CaSR signaling lie at the interface among cellular homeostasis, proliferation and differentiation of the colorectal epithelium, epithelial barrier integrity, and interactions among the epithelium, intestinal microbiome, and inflammatory response. These mechanisms provide a physiological basis for understanding how the same dietary intake may have different biological consequences in distinct individual contexts.

Integrating dietary intake with serum calcium, PTH, 25(OH)D, Mg^{2+} and, prospectively, genetic and molecular information may contribute to the characterization of an individual physiological profile and to the formulation of hypotheses regarding the response to a nutritional intervention. Reassessment of this profile after the intervention introduces a dynamic component, allowing progressive adaptation of the nutritional strategy according to the observed biological response.

At present, this approach should be considered a conceptual, research-oriented model, as the proposed biomarkers and combinations of factors have not yet been validated for individualized prescription of calcium intake in CRC prevention. Prospective and interventional studies are needed to establish the predictive value and clinical utility of these biological profiles and to determine whether individualization of nutritional interventions leads to demonstrable health benefits.

In this context, predictive physiology may represent a direction for the development of personalized nutrition by moving from predominantly population-based recommendations toward interventions adapted to biological status and individual response, while maintaining a physiological foundation and the principles of evidence-based medicine.

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FIZIOLOGIA PREDICTIVA ȘI NUTRIȚIA PERSONALIZATA IN PREVENIREA CANCERULUI COLORECTAL: ROLUL CALCIULUI ȘI AL RECEPTORULUI SENSIBIL LA CALCIU (CASR)

Rezumat

Cancerul colorectal (CCR) reprezintă o problemă majoră de sănătate publică, iar prevenția sa poate beneficia de intervenții nutriționale adaptate variabilității biologice individuale. Nutriția personalizată urmărește depășirea abordării exclusiv populaționale prin integrarea caracteristicilor nutriționale, metabolice, fiziologice și genetice ale individului. În acest context, fiziologia predictivă poate constitui un cadru pentru anticiparea și monitorizarea răspunsului individual la intervențiile nutriționale. Prezentul review analizează critic rolul calciului în homeostazia epiteliului colorectal și relevanța receptorului sensibil la calciu (CaSR) ca interfață între aportul de Ca^{2+} , semnalizarea celulară și micromediul colorectal. Sunt discutate mecanismele implicate în reglarea proliferării și diferențierii celulare, integritatea barierei epiteliale, interacțiunea cu microbiomul intestinal și răspunsul inflamator, precum și influența statusului vitaminei D, PTH și Mg^{2+} asupra homeostaziei calciului. De asemenea, sunt analizate dovezile privind variabilitatea genetică, inclusiv rolul potențial al CaSR, VDR și TRPM7 în modularea răspunsului la aportul de calciu. Pe baza acestor date este propus un model conceptual de fiziologie predictivă care integrează aportul alimentar, biomarkerii fiziologici și particularitățile biologice individuale, urmat de reevaluarea răspunsului și adaptarea intervenției. Un caz didactic ilustrează aplicarea acestui raționament. În prezent, modelul are valoare conceptuală și orientată către cercetare, fiind necesare studii prospective și intervenționale pentru validarea biomarkerilor și a utilității clinice a individualizării intervențiilor nutriționale în prevenția CCR.

Cuvinte-cheie: Cancer colorectal, nutriție personalizată, fiziologie predictivă, calciu, receptor sensibil la calciu (CaSR)

BIOFILM IN ORTHOPAEDIC IMPLANT-ASSOCIATED INFECTIONS: PREVENTION AND TREATMENT

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ABSTRACT

Background: Orthopedic implant-associated infections represent a major complication of arthroplasty and other implant-based procedures. Biofilm formation on implant surfaces plays a central role in microbial persistence, antimicrobial tolerance, treatment failure, and infection recurrence.

Main findings: This review summarizes current evidence on the formation and clinical significance of biofilms in orthopedic implant-associated infections, with particular emphasis on periprosthetic joint infection. The mechanisms underlying microbial adhesion, biofilm maturation, persistence, and antimicrobial tolerance are discussed. Current diagnostic approaches, including conventional culture, implant sonication, and molecular techniques, are reviewed, highlighting their respective advantages and limitations. Treatment strategies are considered from an integrated perspective, combining surgical source control with appropriate antimicrobial therapy. Particular attention is given to the relationship between antimicrobial susceptibility, biofilm-associated tolerance, pharmacokinetic/pharmacodynamic considerations, and clinical response. Emerging approaches, including antimicrobial implant coatings, nanoparticles, antimicrobial peptides, bacteriophages, and advanced local drug-delivery systems, are also discussed.

Conclusions: Biofilm-associated infections require a multidisciplinary and multimodal approach integrating microbiological diagnosis, biofilm characterization, antimicrobial therapy, surgical management, and patient-related factors. Improved understanding of the interaction between biofilm phenotype, antimicrobial exposure, surgical source control, and clinical outcome may facilitate more individualized treatment strategies and improve infection control and implant survival. Future research should focus on translating experimental anti-biofilm approaches into clinically validated diagnostic and therapeutic strategies.

Keywords: orthopedic implant-associated infections, biofilm, implant sonication, antimicrobial tolerance, surgical treatment, biofilm prevention

INTRODUCTION

Orthopedic implant-associated infections are among the most challenging complications following arthroplasty and other implant-based procedures. Although relatively uncommon, they are associated with substantial morbidity, prolonged antimicrobial therapy, repeated surgery, increased healthcare costs, and potential implant failure. The growing number of orthopedic implant procedures worldwide further increases their clinical importance [1, 4].

A central feature of these infections is the ability of microorganisms to adhere to implant surfaces and develop **biofilms**. Following adhesion, bacteria undergo phenotypic and metabolic changes and become embedded within an extracellular matrix, which promotes persistence and protects them from host defenses and antimicrobial exposure. Biofilm-associated bacteria may therefore survive despite antimicrobial concentrations that are effective against planktonic organisms [3, 4].

This phenomenon should be distinguished from conventional antimicrobial resistance. Biofilm-associated bacteria may exhibit reduced metabolic activity and increased **antimicrobial tolerance**, while remaining susceptible according to standard planktonic susceptibility testing. Consequently, conventional susceptibility results may not fully predict the response of implant-associated microorganisms to treatment [1, 3, 4].

Biofilm formation also complicates microbiological diagnosis. Conventional intraoperative tissue culture remains essential, but its sensitivity may be limited in chronic or low-grade infections. **Implant sonication** provides a complementary approach by disrupting biofilm on the explanted prosthesis and improving recovery of implant-associated microorganisms. Molecular methods, including targeted PCR and metagenomic sequencing, may provide additional information, particularly in culture-negative cases. However, all microbiological findings must be interpreted in the context of clinical, laboratory, surgical, and histopathological data [15].

Prevention focuses on reducing bacterial adhesion and biofilm development through appropriate perioperative management, implant surface modification, antimicrobial coatings, and local antimicrobial delivery. Although several anti-biofilm technologies have shown promising experimental results, their clinical translation remains limited [1, 3, 4].

Once an established biofilm is present, successful treatment generally requires **surgical source control combined with appropriate antimicrobial therapy**. Depending on the infection characteristics, surgical strategies include debridement with implant retention, one-stage or two-stage revision, or implant removal. Antimicrobial treatment should be individualized according to the causative microorganism, susceptibility profile, biofilm-associated characteristics, surgical strategy, and patient factors [1, 3].

Despite substantial progress, important gaps remain between experimental biofilm research and clinical practice. The clinical relevance of in vitro biofilm-forming capacity, its relationship with antimicrobial tolerance and treatment response, and the optimal integration of sonication and other diagnostic approaches remain incompletely defined. Low-virulence organisms such as *Cutibacterium acnes* further illustrate the difficulty of distinguishing contamination, colonization, and true infection [15].

The aim of this review is to summarize current knowledge on **biofilm formation, diagnosis, prevention, and treatment of orthopedic implant-associated infections**, with particular emphasis on implant sonication, antimicrobial susceptibility and tolerance, surgical management, and emerging anti-biofilm strategies. We also highlight current knowledge gaps and research priorities relevant to individualized management of these infections [15].

BIOFILM FORMATION ON ORTHOPEDIC IMPLANTS

The development of a biofilm on an orthopedic implant is a dynamic and multistep process involving microbial adhesion, colonization, extracellular matrix production, maturation, and dispersion. Following implantation, the surface of an orthopedic device is rapidly conditioned by host-derived proteins, including fibrinogen, fibronectin, collagen, and other extracellular matrix components. This newly formed biological interface substantially modifies the physicochemical properties of the implant surface and creates an environment to which microorganisms can attach. The interaction between the implant, host proteins, and microorganisms therefore represents a critical determinant of subsequent biofilm development [6, 8, 9].

1. Initial bacterial adhesion

Microbial adhesion to the implant surface is the first critical step in biofilm formation. Initially, bacteria are transported towards the implant by processes such as fluid flow, diffusion, and direct contact during surgery. Early interactions between bacterial cells and the conditioned implant surface are influenced by electrostatic forces, hydrophobic interactions, van der Waals forces, and specific molecular interactions between microbial adhesins and host-derived proteins [8, 9].

The physicochemical characteristics of the implant surface also influence bacterial attachment. Surface roughness, hydrophobicity, chemical composition, charge, and topography may affect both the probability of bacterial adhesion and the subsequent organization of the biofilm. Although surface modification and advanced implant materials have been investigated as potential strategies to reduce bacterial attachment, increasing surface complexity does not necessarily translate into reduced infection risk, and the relationship between surface characteristics and clinical infection remains highly dependent on the specific material and biological context [6, 8].

Different microorganisms exhibit distinct mechanisms of adhesion. *Staphylococcus aureus* and coagulase-negative staphylococci are particularly important in orthopedic implant infections and possess multiple surface-associated adhesins capable of interacting with host proteins deposited on implant surfaces. These interactions facilitate stable attachment and contribute to the transition from transient contamination to persistent colonization [6, 9].

2. Biofilm maturation

Following initial attachment, microorganisms undergo phenotypic and metabolic changes that promote irreversible adhesion and development of a structured microbial community. Bacteria begin to produce an extracellular polymeric substance (EPS) matrix composed of polysaccharides, proteins, extracellular DNA, lipids, and other macromolecules. This matrix provides structural integrity and facilitates microbial aggregation while also creating a protective microenvironment [12, 13].

Biofilm maturation is characterized by increasing structural organization and the formation of heterogeneous microbial populations. Bacteria located within different regions of the biofilm may experience substantial differences in nutrient availability, oxygen concentration, pH, and metabolic activity. Consequently, a biofilm should not be considered a homogeneous bacterial population but rather a complex and spatially organized microbial ecosystem [12, 13].

Quorum sensing and other cell-to-cell communication mechanisms may contribute to biofilm development and regulation of bacterial behavior. In addition, extracellular DNA and matrix-associated proteins can contribute to biofilm architecture and stability. These mechanisms allow microorganisms to adapt to environmental stress and maintain a persistent population on the implant surface [6, 9].

The mature biofilm therefore represents a biologically distinct state compared with free-floating, or planktonic, bacteria. This distinction is important when interpreting antimicrobial susceptibility data and designing therapeutic strategies [8, 9].

3. Biofilm-associated antimicrobial tolerance

One of the most clinically relevant characteristics of biofilms is their increased tolerance to antimicrobial treatment. Biofilm-associated microorganisms may survive antimicrobial concentrations that would effectively inhibit or kill their planktonic counterparts. This phenomenon should be distinguished from classical antimicrobial resistance, which is generally associated with stable genetic or phenotypic mechanisms that increase the minimum inhibitory concentration of an organism [12, 13].

Several mechanisms contribute to biofilm-associated tolerance. The extracellular matrix may limit the penetration or diffusion of certain antimicrobial agents, although this mechanism alone does not explain the full extent of biofilm tolerance. More importantly, biofilms contain metabolically inactive or slow-growing bacterial populations, commonly referred to as persister cells, which are less susceptible to antibiotics targeting actively dividing bacteria. Changes in metabolic activity, stress responses, altered gene expression, and the presence of nutrient and oxygen gradients further contribute to antimicrobial tolerance [12, 13].

This phenomenon creates an important therapeutic discrepancy: an isolate may be classified as susceptible by conventional

antimicrobial susceptibility testing while the same microorganism, when embedded in a mature biofilm on an implant surface, may persist despite apparently appropriate antimicrobial exposure. Consequently, conventional susceptibility testing provides essential information for antimicrobial selection but may not fully capture the biological behavior of implant-associated microorganisms [6, 9].

4. Host immune response and biofilm persistence

The interaction between the biofilm and the host immune system is another major determinant of implant-associated infection. Biofilm-associated microorganisms can persist despite the recruitment of neutrophils, macrophages, and other immune cells to the site of infection. The extracellular matrix and altered bacterial phenotype may reduce effective immune-mediated clearance, while chronic immune activation can contribute to local tissue damage and inflammation [6].

Importantly, biofilm-associated infection does not always produce a pronounced systemic inflammatory response. This is particularly relevant in chronic periprosthetic joint infections, where clinical manifestations may be subtle and laboratory inflammatory markers may be only mildly elevated or even within normal limits. Low-virulence organisms such as coagulase-negative staphylococci and *Cutibacterium acnes* may therefore establish persistent implant-associated infections that are difficult to distinguish from contamination or colonization [8, 9].

The relationship between biofilm and host immunity is consequently bidirectional. The implant provides a non-physiological surface that facilitates microbial persistence, while the host response influences the architecture and evolution of the biofilm. Understanding this interaction is essential for the development of therapeutic strategies that go beyond antimicrobial activity alone [6, 9].

5. Detachment, dissemination, and chronic infection

Biofilm development is not a static process. Portions of the biofilm may detach or disperse, allowing microorganisms to colonize adjacent tissues or other surfaces. This dynamic process can contribute to episodic bacteremia, local dissemination, and recurrent infection. In the context of arthroplasty, microorganisms may persist on the implant while simultaneously being present in surrounding periprosthetic tissues [6, 9].

The capacity of a biofilm to remain attached to an implant while releasing individual bacterial cells provides a biological explanation for the recurrent nature of some implant-associated infections. It also highlights why antimicrobial therapy alone may be insufficient when an established biofilm is present. If the infected implant remains a persistent microbial reservoir, microorganisms may survive treatment and subsequently re-establish infection after antimicrobial therapy is discontinued [8].

6. Factors influencing biofilm formation in orthopedic implants

Biofilm formation is determined by the interaction of microbial, implant-related, and host factors. Microbial factors include species and strain characteristics, adhesin expression, extracellular matrix production, metabolic state, and antimicrobial tolerance. Implant-related factors include material composition, surface roughness, geometry, porosity, and the presence of surface modifications or coatings. Host factors include immune status, tissue integrity, local vascularity, previous surgery, comorbidities, and exposure to antimicrobial agents [6, 8].

The timing and clinical context of infection may also influence biofilm development. Early postoperative infections may involve actively growing microorganisms and may present with more prominent inflammatory manifestations, whereas chronic infections may be dominated by mature biofilm-associated populations and relatively subtle clinical signs. Nevertheless, biofilm formation can occur across the entire spectrum of implant-associated infections, and the distinction between acute and chronic infection should not be regarded as an absolute biological separation [8, 9].

From a clinical perspective, these mechanisms suggest that successful management of orthopedic implant-associated infection requires an integrated approach. Prevention should aim to reduce microbial contamination and initial adhesion, whereas established infection requires strategies capable of controlling or eliminating microorganisms associated with the implant surface. Surgical intervention, appropriate antimicrobial therapy, and, increasingly, biofilm-targeted strategies must therefore be considered as interconnected components of treatment rather than independent interventions [6, 8, 9].

MICROBIOLOGY AND PATHOPHYSIOLOGY

Orthopedic implant-associated infections are caused by a relatively limited spectrum of microorganisms, although their clinical behavior varies considerably. The ability to adhere to implant surfaces, form biofilms, evade host immunity, and persist despite antimicrobial therapy differs among bacterial species and strains. Understanding these characteristics is essential for interpreting microbiological findings and selecting appropriate treatment [6, 8, 11].

1. Major Microbial Pathogens

Staphylococcus aureus is one of the most important pathogens in orthopedic implant-associated infections. Its virulence is related to surface adhesins, extracellular matrix production, immune evasion, and biofilm formation. It may cause both acute infections with marked inflammation and chronic infections dominated by biofilm-associated persistence. Methicillin-resistant *S. aureus* (MRSA) further complicates treatment because of limited antimicrobial options [6, 11].

Coagulase-negative staphylococci, particularly *Staphylococcus epidermidis*, are also important pathogens. Despite relatively low virulence, they have a strong ability to adhere to foreign materials and produce biofilm. Their infections are frequently indolent and may present with limited systemic inflammation, making them particularly relevant in chronic periprosthetic joint infection [6, 8].

Streptococci, enterococci, and Gram-negative organisms such as Enterobacterales and *Pseudomonas aeruginosa* account for a smaller but clinically important proportion of infections. Antimicrobial resistance and previous antimicrobial exposure may further complicate their management [3, 6, 7].

The microbial spectrum also varies according to implant location. **Cutibacterium acnes** is particularly relevant in shoulder arthroplasty. Its slow growth, subtle clinical presentation, and limited inflammatory response make it difficult to distinguish contamination or colonization from true infection [55, 56].

2. From Contamination to Persistent Infection

The detection of bacteria on or around an implant does not necessarily indicate clinically relevant infection. A continuum may exist between contamination, colonization, biofilm-associated persistence, and established infection. This distinction is particularly important for low-virulence organisms isolated from a limited number of specimens [6, 8, 11].

Following microbial inoculation, bacteria may adhere to the conditioned implant surface, produce extracellular matrix, and develop a mature biofilm. The resulting microbial community provides protection from host defenses and antimicrobial exposure, facilitating long-term persistence [6, 11].

The clinical significance of microbiological findings therefore depends on the microorganism, bacterial phenotype, inoculum, implant characteristics, timing of infection, and host factors. Microbiological results should consequently be interpreted together with clinical findings, inflammatory markers, imaging, histology, intraoperative findings, and the number of positive cultures [3, 6, 7].

3. Host Response and Immune Dysregulation

The host response involves activation of innate immune pathways and recruitment of neutrophils, macrophages, and other inflammatory cells. However, the presence of an implant modifies this response and may limit effective microbial clearance [3, 6].

Biofilm-associated bacteria can persist because the extracellular matrix, altered metabolic activity, and spatial organization of the biofilm reduce immune-mediated elimination. Persistent inflammation may simultaneously contribute to tissue damage, bone resorption, implant loosening, and failure of osseointegration [12, 13].

This interaction helps explain why chronic implant-associated infections may present with relatively mild systemic manifestations despite persistent infection [7].

4. Biofilm, Antimicrobial Resistance, and Tolerance

An important distinction is that between **antimicrobial resistance** and **biofilm-associated tolerance**. Resistance involves a stable reduction in antimicrobial susceptibility, whereas tolerance refers to the ability of bacteria to survive antimicrobial exposure without necessarily showing increased susceptibility thresholds in conventional testing [12, 13].

Within biofilms, reduced metabolic activity, altered gene expression, and heterogeneous bacterial populations can decrease antimicrobial efficacy. **Persister cells** represent a particularly important subpopulation capable of surviving antimicrobial exposure and contributing to recurrence [13].

Standard susceptibility testing is generally performed using planktonic bacteria and therefore may not fully reflect the conditions encountered within a mature biofilm. Consequently, an organism classified as susceptible in vitro may still persist clinically [6, 8, 11].

This distinction has direct pharmacological implications. Antimicrobial therapy should consider not only the susceptibility profile but also tissue penetration, achievable antimicrobial exposure, and activity against biofilm-associated populations [6, 11].

5. Microbial and Host Factors Determining Clinical Outcome

Clinical outcome results from the interaction between microbial characteristics, biofilm phenotype, host susceptibility, implant properties, and treatment strategy. Host factors such as immunosuppression, diabetes, obesity, poor tissue perfusion, and previous surgery may impair infection control, while microbial biofilm-forming capacity, resistance, and persistence mechanisms may influence treatment response and recurrence [6, 8, 11].

Microorganism	Biofilm potential	Typical presentation	Diagnostic challenge	Therapeutic challenge
<i>S. aureus</i>	High	Acute/chronic	-	Resistance + persistence
CoNS	High	Indolent/chronic	Contamination vs infection	Biofilm/tolerance
<i>C. acnes</i>	High	Often indolent	Slow growth, contamination vs infection	Delayed diagnosis
Streptococci	Variable	Acute/subacute	-	Species-dependent
Enterococci	Variable	Acute/chronic	-	Limited options/resistance

Overall, orthopedic implant-associated infection should be viewed as a dynamic interaction between the **microorganism, implant surface, and host**. Biofilm formation represents a central mechanism linking microbial persistence, immune evasion, and reduced antimicrobial efficacy. Understanding these interactions may support more accurate diagnosis and more individualized antimicrobial and surgical treatment [8, 11].

DIAGNOSIS OF BIOFILM-ASSOCIATED INFECTIONS

Diagnosis of orthopedic implant-associated infection remains challenging because biofilm-associated microorganisms may be present in low numbers, grow slowly, or behave differently from planktonic bacteria. In addition, detection of a microorganism does not necessarily establish clinically relevant infection. Diagnosis therefore requires integration of clinical, laboratory, microbiological, histopathological, and intraoperative findings [1, 2].

1. Conventional Microbiological Diagnosis

Multiple intraoperative tissue samples remain essential for microbiological diagnosis. Recovery of the same microorganism from several independent specimens increases the likelihood of true infection. However, conventional tissue culture may have reduced sensitivity in chronic infections, low-grade infections, infections caused by slow-growing organisms, and patients previously exposed to antibiotics.

Biofilm-associated microorganisms may also be predominantly attached to the implant surface rather than freely present in surrounding tissues, creating an important limitation of tissue sampling alone [1, 2, 15].

2. Implant Sonication

Implant sonication is an important complementary technique for detecting biofilm-associated microorganisms. After prosthesis removal, controlled ultrasonic energy is used to disrupt the biofilm attached to the implant surface and release microorganisms into a sterile fluid. The resulting sonication fluid can then be cultured and, when appropriate, analyzed using molecular methods [15, 17].

The main advantage of sonication is that it directly targets the **implant-associated microbial reservoir**, rather than relying exclusively on periprosthetic tissue samples. This may be particularly valuable in chronic or low-grade infections, where bacterial burden in surrounding tissues may be limited [18].

Importantly, sonication and tissue culture provide complementary information. Tissue cultures identify microorganisms present in periprosthetic tissues, whereas sonication improves recovery of microorganisms associated with the implant surface. Therefore, combining both approaches may increase diagnostic yield [15, 18].

However, a positive sonication result does not automatically establish infection. Low-virulence organisms such as coagulase-negative staphylococci or *Cutibacterium acnes* may represent contamination or colonization. Sonication results should therefore be interpreted together with the clinical presentation, number of positive specimens, inflammatory markers, histology, and other established diagnostic criteria [17, 18].

3. Molecular Diagnostics

Molecular techniques, including 16S rDNA PCR, multiplex PCR, and metagenomic next-generation sequencing, may provide additional diagnostic information, particularly in culture-negative or previously treated infections. Their main advantages include rapid or broad microbial detection [23, 24, 25].

However, detection of microbial DNA does not necessarily indicate viable bacteria or establish causality. Differences in sample preparation, sequencing platforms, and interpretation criteria also limit direct comparison between studies. Molecular methods should therefore currently be considered **complementary tools rather than replacements for culture and sonication**. [15, 17]

4. Contamination, Colonization, and True Infection

Distinguishing contamination, colonization, and true infection remains one of the most difficult aspects of implant-associated infection. This is particularly relevant for *C. acnes* and coagulase-negative staphylococci [57, 59].

The clinical significance of an isolated microorganism should be assessed according to the number and consistency of positive cultures, organism identity, clinical findings, inflammatory markers, histology, and intraoperative appearance. Biofilm-associated infection may produce limited systemic inflammation despite the presence of a persistent microbial reservoir [1, 2, 15].

5. Integrated Diagnostic Approach

No single diagnostic method can reliably identify all biofilm-associated infections. An integrated approach combining **multiple tissue cultures, implant sonication, and selective molecular diagnostics**, together with clinical and histopathological assessment, is therefore preferable [17, 18].

From a therapeutic perspective, recovery of a viable microorganism remains particularly important because it allows antimicrobial susceptibility testing and supports individualized antimicrobial therapy. In this context, sonication may provide clinically relevant microbiological information that is not obtained from tissue culture alone [15, 18].

Ultimately, the diagnostic goal is not simply to detect microorganisms but to establish a clinically meaningful relationship between the **microorganism, biofilm, implant, host response, and clinical infection** [1, 2, 15].

PREVENTION STRATEGIES

Prevention remains the most effective strategy for reducing the burden of orthopedic implant-associated infections. Because biofilm formation begins with microbial contamination and adhesion to the implant surface, preventive interventions should aim to minimize bacterial inoculation, inhibit initial adhesion, and reduce the ability of microorganisms to establish a mature biofilm [46, 47].

1. Perioperative infection prevention

The prevention of implant-associated infection begins with optimization of the surgical and host environment. Appropriate skin antisepsis, perioperative antimicrobial prophylaxis, maintenance of aseptic surgical technique, adequate operating-room conditions, and optimization of modifiable patient-related risk factors are fundamental components of infection prevention [7]. Antimicrobial prophylaxis should be appropriately selected and timed to ensure adequate tissue concentrations at the moment of incision. Prolonged or inappropriate prophylaxis does not necessarily provide additional protection and may contribute to antimicrobial selection pressure [7].

Patient-related factors, including diabetes, obesity, smoking, immunosuppression, malnutrition, and other conditions associated with impaired wound healing, should be identified and optimized whenever possible before elective arthroplasty [7, 46, 47].

2. Implant surface modification

Because bacterial adhesion to the implant is an early and critical step in biofilm development, modification of implant surfaces represents an important area of research. Changes in surface chemistry, roughness, hydrophobicity, and topography may influence bacterial adhesion and biofilm formation [7, 46].

Antimicrobial coatings have received particular attention. These may incorporate antibiotics, antiseptics, metallic ions, antimicrobial peptides, or other compounds capable of inhibiting bacterial adhesion and growth. However, many technologies remain experimental, and concerns regarding durability, toxicity, antimicrobial resistance, and long-term effectiveness continue to limit widespread clinical adoption [47, 48].

3. Local antimicrobial strategies

Local delivery of antimicrobial agents can provide high concentrations at the surgical site while potentially limiting systemic exposure. Antibiotic-loaded bone cement is an established example, particularly in selected revision procedures and two-stage treatment strategies [7, 47].

Other approaches include antibiotic-loaded carriers, hydrogels, biodegradable materials, and implant coatings designed to provide controlled antimicrobial release. These technologies are attractive because they directly target the anatomical site at highest risk for bacterial colonization. Nevertheless, their clinical effectiveness depends on the antimicrobial agent, release kinetics, implant material, microbial spectrum, and specific surgical context [46, 48].

4. Emerging anti-biofilm approaches

New preventive strategies increasingly focus not only on killing bacteria but also on interfering with the biological processes required for biofilm formation. These approaches include anti-adhesive surfaces, quorum-sensing inhibitors, enzymes capable of degrading the extracellular matrix, antimicrobial peptides, bacteriophages, and stimuli-responsive antimicrobial materials [54].

Despite promising experimental results, translation into clinical practice remains challenging. Many emerging technologies have been evaluated predominantly *in vitro* or in animal models, while high-quality clinical evidence remains limited [7, 46].

Overall, prevention of orthopedic implant-associated infection requires a multimodal strategy combining optimized perioperative care, appropriate antimicrobial prophylaxis, careful surgical technique, management of patient-related risk factors, and, where evidence supports their use, implant- or locally delivered antimicrobial technologies. Future preventive strategies are likely to shift from conventional antimicrobial approaches toward targeted interference with bacterial adhesion and biofilm development [46, 47].

TREATMENT STRATEGIES

The treatment of orthopedic implant-associated infection is complex and generally requires a combination of surgical intervention and targeted antimicrobial therapy. The presence of a mature biofilm represents a major therapeutic challenge because microorganisms embedded within the biofilm may persist despite antimicrobial concentrations that are effective against planktonic bacteria. Consequently, successful treatment requires not only suppression of bacterial growth but, whenever possible, elimination of the microbial reservoir associated with the implant [22, 32, 33].

1. Surgical management

Surgical strategy is determined by the timing and duration of infection, implant stability, soft-tissue condition, pathogen characteristics, and patient-related factors. The principal approaches include debridement, antibiotics and implant retention (DAIR), one-stage revision, two-stage revision, and implant removal [34].

DAIR may be considered in selected patients with an acute infection, a stable and well-functioning implant, and adequate soft-tissue conditions. Its principal advantage is preservation of the existing implant. However, its effectiveness may be limited when a mature biofilm has developed, particularly in chronic infection. Patient selection, timing of intervention, quality of debridement, and the susceptibility of the causative organism are therefore critical determinants of outcome [39, 33].

In chronic or established infection, implant exchange may be necessary to achieve eradication. One-stage revision involves removal and replacement of the infected implant during the same surgical procedure, whereas two-stage revision separates implant removal and definitive reimplantation. The choice between these strategies depends on the clinical scenario, pathogen, soft-tissue condition, bone stock, and institutional expertise [32, 40].

From a biofilm perspective, implant removal or exchange has an important theoretical advantage: it physically eliminates a major microbial reservoir that cannot be reliably sterilized by systemic antimicrobial therapy alone [22, 33].

2. Systemic antimicrobial therapy

Antimicrobial therapy should be individualized according to the identified pathogen, antimicrobial susceptibility, infection characteristics, surgical strategy, and patient-specific factors. Whenever possible, treatment should be guided by microbiological results obtained from appropriately collected clinical specimens [22, 33].

A major challenge is the discrepancy between conventional antimicrobial susceptibility testing and the biological behavior of microorganisms within biofilms. Standard susceptibility testing is generally performed using planktonic organisms and therefore may not fully predict antimicrobial activity against biofilm-associated bacteria [32, 33].

The pharmacological management of implant-associated infection should consequently consider not only antimicrobial susceptibility but also tissue penetration, bioavailability, protein binding, pharmacokinetic variability, and the ability to achieve adequate exposure at the site of infection. These considerations provide an important rationale for integrating clinical pharmacology into the management and investigation of implant-associated infections [22, 32].

3. Biofilm-active antimicrobial therapy

Some antimicrobial agents have demonstrated greater activity against biofilm-associated microorganisms than others. The best-established example is rifampicin-based therapy for susceptible staphylococcal implant infections. Rifampicin has substantial intracellular and biofilm activity but should generally not be used as monotherapy because resistance can emerge rapidly [35, 36].

Other antimicrobial agents may be selected according to the causative microorganism and its susceptibility profile. The clinical benefit of a particular antimicrobial regimen depends on multiple factors, including bacterial species, biofilm maturity, surgical intervention, antimicrobial exposure, and host characteristics [22, 32, 33].

Importantly, the concept of a “biofilm-active” antibiotic should not be interpreted as an absolute property. Activity demonstrated in experimental biofilm models does not necessarily translate into improved clinical outcomes. The effectiveness of an antimicrobial regimen is determined by the interaction between pharmacology, microbiology, surgery, and host factors [22, 32, 33].

4. Antimicrobial tolerance and pharmacokinetic/pharmacodynamic considerations

Biofilm-associated tolerance represents an important challenge for antimicrobial treatment. Bacteria within mature biofilms may survive antimicrobial exposure without demonstrating conventional resistance. This phenomenon raises the possibility that conventional susceptibility results may underestimate the antimicrobial exposure required for effective treatment [32, 33].

Pharmacokinetic/pharmacodynamic (PK/PD) principles may therefore provide an important framework for understanding treatment response. Factors such as the ratio between antimicrobial exposure and the pathogen's susceptibility, duration of exposure, tissue penetration, and patient-specific pharmacokinetic variability may influence therapeutic success [22].

In patients with complex infections, altered renal function, obesity, critical illness, or prolonged antimicrobial treatment, individual pharmacokinetic characteristics may differ substantially from standard assumptions. Therapeutic drug monitoring, where appropriate and supported by evidence, may help optimize exposure for selected antimicrobial agents [22, 32].

The integration of microbiological characterization with pharmacological exposure represents a potentially important area for future research. In particular, the relationship between standard antimicrobial susceptibility, biofilm-associated tolerance, antimicrobial exposure, and clinical outcome remains incompletely defined [22, 32, 33].

5. Emerging anti-biofilm therapies

The limitations of conventional antimicrobial therapy have stimulated interest in therapies specifically targeting biofilm biology. Experimental strategies include antimicrobial coatings, biofilm-disrupting enzymes, quorum-sensing inhibitors, antimicrobial peptides, bacteriophages, nanoparticles, and agents designed to interfere with the extracellular polymeric matrix [33].

Bacteriophage therapy has attracted particular attention because of its ability to target specific bacterial populations and, in some settings, interact with biofilm structures. However, clinical evidence remains limited, and questions regarding phage susceptibility, delivery, immune responses, manufacturing, and regulatory implementation remain important barriers [22, 32, 33].

Other emerging strategies aim to combine antimicrobial agents with biofilm-disrupting technologies. Such approaches may theoretically improve antimicrobial penetration and expose bacteria that are otherwise protected within the biofilm. However, most currently available evidence remains experimental, and high-quality comparative clinical studies are needed before widespread clinical adoption [22, 32].

6. The role of combined surgical and antimicrobial treatment

The most important principle in the management of implant-associated infection is that surgery and antimicrobial therapy should be regarded as complementary rather than competing strategies. Antimicrobial therapy may suppress or eradicate

susceptible microorganisms, but an established biofilm attached to an implant may represent a persistent reservoir that cannot be reliably eliminated pharmacologically [22, 32, 33].

Conversely, surgery without appropriate antimicrobial therapy may fail to eradicate microorganisms that remain in surrounding tissues or inaccessible anatomical compartments. Successful treatment therefore requires an individualized combination of adequate surgical source control and appropriate antimicrobial exposure [32].

The future of treatment is likely to move toward increasingly personalized strategies based on the interaction between microbial phenotype, biofilm characteristics, antimicrobial susceptibility, pharmacokinetic exposure, surgical approach, and host factors. Such an approach may help explain why patients with apparently similar infections can experience markedly different outcomes and may provide opportunities to reduce treatment failure and recurrence [22, 33].

Stage	Component	What is assessed	Clinical relevance
1	Microorganism	Species, virulence, antimicrobial resistance mechanisms	Determines the type and severity of infection
2	Biofilm phenotype	Adhesion, biofilm-forming capacity, maturation, persistence	Influences microbial persistence on the implant surface
3	Antimicrobial susceptibility and tolerance	Standard susceptibility testing, planktonic susceptibility, biofilm-associated tolerance	Explains why conventional susceptibility results may not fully predict clinical response
4	PK/PD and antimicrobial exposure	Dose, concentration, tissue penetration, antimicrobial exposure	Determines whether effective antimicrobial exposure is achieved at the site of infection
5	Surgical strategy	DAIR, one-stage revision, two-stage revision, implant removal	Provides source control and/or eliminates the implant-associated microbial reservoir
6	Treatment response	Microbiological eradication, persistence, recurrence, need for reoperation	Reflects the effectiveness of the combined antimicrobial and surgical strategy
7	Clinical outcome	Infection control, reinfection, reoperation, implant survival, functional outcome	Represents the final patient-centered outcome

EMERGING THERAPIES AND FUTURE DIRECTIONS

The increasing recognition of biofilm as a major determinant of persistence and treatment failure in orthopedic implant-associated infections has stimulated the development of novel therapeutic strategies. These approaches aim not only to eradicate planktonic bacteria but also to prevent biofilm formation, disrupt established biofilms, or enhance antimicrobial activity at the implant–tissue interface [44, 46].

Several emerging strategies are currently being investigated, including **antimicrobial implant coatings, nanoparticles, antimicrobial peptides, bacteriophages, and biofilm-disrupting agents**. Antimicrobial coatings may provide local protection against bacterial adhesion during the early postoperative period, while nanoparticle-based systems can facilitate targeted antimicrobial delivery. Antimicrobial peptides and enzymes targeting the biofilm matrix represent additional approaches with potential activity against organisms that are difficult to eradicate using conventional antibiotics [46, 47, 48].

Bacteriophage therapy is of particular interest because of its ability to target specific bacterial species and, in some cases, biofilm-associated bacteria. However, clinical evidence in orthopedic implant-associated infections remains limited, and issues such as phage selection, bacterial resistance, manufacturing, and regulatory requirements need to be addressed before widespread clinical implementation [54].

Another promising direction is the development of **smart and stimuli-responsive delivery systems**, capable of releasing antimicrobials in response to local environmental conditions associated with infection. Such approaches could potentially increase drug concentrations at the infected implant while reducing systemic exposure [44, 45, 46].

Despite encouraging experimental results, most emerging anti-biofilm strategies remain at the preclinical or early clinical stage. A major challenge is the translation of in vitro biofilm models into clinically meaningful outcomes. Future research should therefore prioritize standardized biofilm assessment, clinically relevant experimental models, and prospective studies correlating biofilm characteristics with antimicrobial exposure, surgical strategy, and patient outcomes [44, 46].

Ultimately, the future management of implant-associated infections is likely to involve a **multimodal and personalized approach**, integrating accurate microbial identification, characterization of biofilm-associated phenotypes, optimized antimicrobial therapy, effective surgical source control, and novel local anti-biofilm technologies [45, 46].

CONCLUSIONS

Biofilm formation is a central mechanism underlying the persistence, recurrence, and treatment difficulty of orthopedic implant-associated infections. The ability of microorganisms to adhere to implant surfaces and develop a mature biofilm phenotype can substantially modify their antimicrobial susceptibility and tolerance, making conventional diagnostic and therapeutic approaches less effective [1, 2, 3].

Accurate diagnosis requires the integration of clinical findings, microbiological investigations, and appropriate intraoperative sampling. Conventional cultures remain essential, while techniques such as implant sonication and molecular diagnostics may improve microbial detection, particularly in culture-negative or low-grade infections. However, these methods should be interpreted within the clinical context and should not replace careful clinical assessment [15].

Successful treatment generally requires a combination of **effective surgical source control and appropriately selected antimicrobial therapy**. The choice of surgical strategy, antimicrobial agent, duration of treatment, and potential role of biofilm-active therapies should be individualized according to the infecting microorganism, infection characteristics, implant status, and patient-related factors [6].

Future research should focus on translating experimental knowledge of biofilm biology into clinically applicable diagnostic and therapeutic strategies. In particular, integrating **microbial characteristics, biofilm phenotype, antimicrobial susceptibility and tolerance, pharmacokinetic/pharmacodynamic considerations, surgical management, and clinical outcomes** may provide a more comprehensive framework for understanding treatment failure and recurrence [1, 3].

A better understanding of these interactions may ultimately support more precise and individualized management of orthopedic implant-associated infections and contribute to improved infection control, implant survival, and patient outcomes [1, 2, 3].

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BIOFILMUL ÎN INFECȚIILE ASOCIATE IMPLANTURILOR ORTOPEDICE: PREVENȚIE ȘI TRATAMENT

REZUMAT

Context: Infecțiile asociate implanturilor ortopedice reprezintă o complicație majoră a artroplastiei și a altor proceduri bazate pe implanturi. Formarea biofilmului pe suprafețele implanturilor joacă un rol central în persistența microbiană, toleranța antimicrobiană, eșecul tratamentului și recurența infecției.

Principalele constatări: Această review sintetizează dovezile actuale privind formarea și semnificația clinică a biofilmelor în infecțiile asociate implanturilor ortopedice, cu un accent deosebit pe infecția periprotetică articulară. Sunt discutate mecanismele care stau la baza adezivității microbiene, maturării biofilmului, persistenței și toleranței la antimicrobiene. Sunt revizuite abordările diagnostice actuale, inclusiv cultura convențională, sonicarea implantului și tehnicile moleculare, evidențiind avantajele și limitările lor respective. Strategiile de tratament sunt analizate dintr-o perspectivă integrată, combinând controlul chirurgical al sursei cu terapia antimicrobiană adecvată. O atenție deosebită este acordată relației dintre susceptibilitatea antimicrobiană, toleranța asociată biofilmului, considerentele farmacocinetice/farmacodinamice și răspunsul clinic. De asemenea, sunt discutate abordările emergente, inclusiv învelișurile antimicrobiene pentru implanturi, nanoparticulele, peptidele antimicrobiene, bacteriofagii și sistemele avansate de administrare locală a medicamentelor.

Concluzii: Infecțiile asociate biofilmului necesită o abordare multidisciplinară și multimodală care integrează diagnosticul microbiologic, caracterizarea biofilmului, terapia antimicrobiană, managementul chirurgical și factorii legați de pacient. O mai bună înțelegere a interacțiunii dintre fenotipul biofilmului, expunerea la antimicrobiene, controlul chirurgical al sursei și rezultatul clinic poate facilita strategii de tratament individualizate și poate îmbunătăți controlul infecției și supraviețuirea implantului. Cercetările viitoare ar trebui să se concentreze pe transpunerea abordărilor experimentale anti-biofilm în strategii diagnostice și terapeutice validate clinic.

Cuvinte-cheie: infecții asociate implanturilor ortopedice, biofilm, sonicarea implantului, toleranță la antimicrobiene, tratament chirurgical, prevenirea biofilmului