ISSN 2787-2947

Broj 5 • septembar 2025. **№ 5** • September 2025.

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Trendovi u **molekularnoj biologiji**
Trends in **Molecular Biology**

26 GODINA • YEARS

Personalizovana medicina
Personalized medicine

Beograd • Belgrade • 2025.
IMGGI • IMGGE • MolBioS

Predgovor

Tematski zbornik „Trendovi u molekularnoj biologiji“ (TMB) je zamišljen kao zbornik revijskih radova sa temama proizašlim iz doktorskih disertacija iz molekularne biologije, sa argumentacijom da se prevaziđe nepopularna statistika koja govori da doktorska teza u proseku ima 1,6, a najviše 7 čitalaca. Pored toga, predviđeni su i revijski radovi naših naučnika kao prikazi tema iz molekularne biologije koje su obeležile prethodnu godinu, i koji svedoče o tome kako su naučnici u Srbiji učestvovali u tim svetskim trendovima. Vremenom je tematski zbornik TMB postao mnogo više od toga. Otvorena su vrata mladim naučnicima, Z-molekularcima za njihove istraživačke radove, a takođe su i naučnici iz inostranstva uzeli učešće sa svojim radovima, pa se od ove godine radovi štampaju na engleskom jeziku. Već pet godina TMB privlače autore i čitaoce što neosporno ukazuje na to da je ovakva publikacija bila potrebna našoj naučnoj zajednici.

Prve godine, u TMB1 je izražena nada da će ovaj Zbornik inspirisati mlade ljude da se opredele za nauku i da će buduće generacije uvideti da naučni rad u Srbiji može dati doprinos svetskoj nauci a pri tome i dovesti do poboljšanja života ljudi u našoj zemlji. Poruka za one koji dolaze je bila: „Hoćemo li na molekularnu?!“

Ove godine je smer Molekularna biologiju na Biološkom fakultetu Univerziteta u Beogradu imao duplo više zainteresovanih studenata nego što je bilo moguće upisati. Zbog toga su TMB5 posvećeni našim studentima i budućim kolegama kao znak vere u budućnost obrazovanja i nauke u Srbiji.

Sonja Pavlović

Preface

The thematic collection "Trends in molecular biology" (TMB) is conceived as a collection of review papers with topics derived from doctoral dissertations in molecular biology, with the argumentation to overcome the unpopular statistics that say that a doctoral thesis has an average of 1.6 and a maximum of 7 readers. In addition, review papers of our scientists are planned as presentations of topics from molecular biology that marked the previous year, and which testify to how scientists in Serbia participated in those world trends. Over time, the thematic collection TMB became much more than that. The door was opened to young scientists, researchers from the Z-generation for their research works and also, scientists from abroad took part with their papers, so from this year the articles are printed in English. For five years, TMB has been attracting authors and readers, which undeniably indicates that this kind of publication was needed by our scientific community.

In the first year, TMB1 expressed the hope that this collection would inspire young people to dedicate themselves to science and that future generations would see that scientific work in Serbia could contribute to world science and at the same time lead to the improvement of people's lives in our country. The message for the future was: "Shall we go to molecular biology?!"

This year, the Molecular Biology department at Faculty of Biology University of Belgrade had twice as many interested students as it was possible to enroll. That is why TMB5 are dedicated to our students and future colleagues as a sign of faith in the future of education and science in Serbia.

Sonja Pavlović

Izvod iz recenzija/ Extracts from reviews

Ovogodišnje izdanje *Trendova u molekularnoj biologiji* posvećeno je mikroRNK i personalizovanoj medicini.

Za otkriće mikroRNK dodeljena je Nobelova nagrada za fiziologiju ili medicinu 2024. godine, a radovi naučnika iz Srbije prikazani u ovom zborniku posmatraju mikroRNK i druge nekodirajuće RNK sa aspekta medicine. Uloga ovih molekula u regulaciji ekspresije gena čini ih ne samo značajnim biomarkerima fizioloških poremećaja, već i molekulima sa terapijskim potencijalom i mogućnostima integracije u personalizovane strategije lečenja.

Personalizovana medicina kao prekretnica u pristupima prevenciji, dijagnostici i terapiji bolesti temelji se na značaju individualnih genetičkih razlika. Ovaj novi pristup u medicini omogućen je razvojem i primenom metoda molekularne biologije i njihovom integracijom sa bioinformatičkim i drugim naprednim metodama. Radovi u ovom zborniku govore o primeni i modelima individualizovanog pristupa bolestima, od neonatalnog genetičkog skrininga, praćenja i modifikovanja toka terapija, do primene indukovanih pluripotentnih matičnih ćelija i farmakoekonomske evaluacije efekata integracije personalizovanog pristupa u zdravstvene sisteme.

U ovom broju *Trendova* dva rada govore o naprednim metodama molekularne biologije (izotermalna strategija i DNK barkodiranje u molekularnoj dijagnostici i forenzici). Generacija Z molekularnih biologa predstavljena je jednim radom. .

Radovi objavljeni u *Trendovima u molekularnoj biologiji 5* svedoče ne samo o praćenju svetskih trendova u biomedicini od strane naših naučnika, već i o značajnom doprinosu razumevanju molekularnih mehanizama nastanka i lečenja bolesti.

Trendovi u molekularnoj biologiji ostaju verni svojoj suštini i nazivu i ne samo da oslikavaju trenutno stanje, već nastavljaju da usmeravaju, podstiću i inspirišu buduća istraživanja u oblasti molekularne biologije.

Dr Svetlana Radović, redovni professor u penziji
Biološki fakultet Univerziteta u Beogradu

This year's edition of Trends in Molecular Biology is dedicated to microRNAs and personalized medicine.

The Nobel Prize in Physiology or Medicine in 2024 was awarded for the discovery of microRNAs, and the works of scientists from Serbia presented in this collection look at microRNAs and other non-coding RNAs from a medical perspective. The role of these molecules in the regulation of gene expression makes them not only important biomarkers of physiological disorders, but also molecules with therapeutic potential and possibilities of integration into personalized treatment strategies.

Personalized medicine as a turning point in approaches to disease prevention, diagnosis and therapy is based on the importance of individual genetic differences. This new approach in medicine is made possible by the development and application of molecular biology methods and their integration with bioinformatics and other advanced methods. The works in this collection talk about the application and models of individualized approach to diseases, from neonatal genetic screening, monitoring and modifying the course of therapies, to the application of induced pluripotent stem cells and pharmacoeconomic evaluation of the effects of integrating a personalized approach into healthcare systems.

In this issue of Trends, two papers talk about advanced methods of molecular biology (isothermal strategy and DNA barcoding in molecular diagnostics and forensics). Generation Z of molecular biologists is represented by one paper.

The papers published in Trends in Molecular Biology 5 testify not only to the monitoring of world trends in biomedicine by our scientists, but also to a significant contribution to the understanding of the molecular mechanisms of the origin and treatment of diseases.

Trends in Molecular Biology remain dedicated to their essence and name and not only reflect the current state, but continue to guide, encourage and inspire future research in the field of molecular biology.

Dr Svetlana Radović, full professor retd.
Faculty of Biology, University of Belgrade

Ove godine je tematski zbornik "Trendovi u molekularnoj biologiji 5" posvećen izuzetno važnim temama za lekare. Od 14 poglavlja, 13 je posvećeno najnovijimj dostignućima moderne medicine.

Prvi deo obuhvata radove koji prikazuju istraživanja naučnika iz Srbije na temu mikroRNK, koje su 2024. godine svojim "očevima" donele Nobelovu nagradu. Zanimljivo je da se već više godina unazad Nobelova nagrada za medicinu ili fiziologiju dodeljuje otkrićima iz oblasti molekularnu biologije. Izuzetno je važno da su ta otkrića zaslužila Nobelovu nagradu zato što su primenjena u medicinskoj praksi i tako doprinela njenom značajnom napretku. Naši naučnici slede u svojim istraživanjima ovaj trend i nastoje da svako njihovo istraživanje sadrži i moguću primenu u medicini.

Drugi deo TMB5 je posvećen personalizovanoj medicini, koja je unela revolucionarni napredak u dijagnostici i lečenju pacijenata. U ovom delu, pored uvodnog dela koji prikazuje istoriju personalizovane medicine i njen doprinos medicini, tu su i radovi naučnika iz Italije i Grčke koji predstavljaju teme koje tek treba da zažive u našoj nauci i praksi.

Posebno postignuće ovogodišnjeg broja TMB je to što su svi radovi napisani na engleskom jeziku, što omogućava da budu dostupni širokoj naučnoj javnosti. Kvalitet radova i zanimljivost tema čine TMB5 izuzetno važnim izdanjem jer prikazuje koliko je biomedicinska nauka snažna u Srbiji. Ovaj broj Tematskog zbornika će svakom našem lekaru biti izuzetno privlačan, a mogao bi da posluži i kao štivo za edukaciju studenata medicine.

Dr Vesna Škodrić Trifunović, redovni professor u penziji
Medicinski fakultet Univerziteta u Beogradu

This year, the thematic collection "Trends in molecular biology 5" is dedicated to extremely important topics for doctors. Out of 14 chapters, 13 are devoted to the latest achievements of modern medicine.

The first part includes papers that show the research of scientists from Serbia on the topic of microRNA, which in 2024 brought their "fathers" the Nobel Prize. It is interesting that for several years now, the Nobel Prize for Medicine or Physiology has been awarded to discoveries in the field of molecular biology. It is extremely important that these discoveries deserved the Nobel Prize because they were applied in medical practice and thus contributed to its significant progress. Our scientists follow this trend in their research and strive to ensure that each of their research contains a possible application in medicine.

The second part of TMB5 is dedicated to personalized medicine, which has introduced revolutionary advances in the diagnosis and treatment of patients. In this part, in addition to the introductory part that shows the history of personalized medicine and its contribution to medicine, there are also works by scientists from Italy and Greece that represent topics that have yet to come to life in our science and practice.

A special achievement of this year's issue of TMB is that all papers are written in English, which makes them accessible to a wide scientific public. The quality of the papers and the interesting topics make TMB5 an extremely important issue because it shows how strong biomedical science is in Serbia. This issue of the Thematic collection will be extremely attractive to each of our doctors, and it could also serve as reading material for the education of medical students.

Dr Vesna Škodrić Trifunović, full professor retd.
University of Belgrade-Faculty of Medicine

Tematski zbornik „Trendovi u molekularnoj biologiji 5“ nastavlja tradiciju prikazivanja nekih od najznačajnijih tema u molekularnoj biologiji koje su obeležile prethodnu godinu, uz značajan pomak da su u ovogodišnjem izdanju svi radovi napisani na engleskom jeziku.

Prvi deo Zbornika je posvećen mikroRNK i ulozi koje ove i druge male nekodirajuće RNK imaju u nastanku različitih oboljenja. Ovo je izuzetno aktuelna tema, s obzirom na to da je za otkriće mikroRNK i njihovu ulogu u posttranskripcionoj regulaciji gena dodeljena Nobelova nagrada za fiziologiju ili medicinu u 2024. godini. U okviru ove teme prikazani su najnoviji rezultati naših istraživača koji se odnose na ulogu mikroRNK u nastanku urođenih anomalija bubrega i urinarnog trakta, oralnog planocelularnog karcinoma i gestacijskog dijabetes melitusa. Istaknuta je značajna uloga ove klase molekula, kao i drugih nekodirajućih RNK, za koje je pokazano da mogu biti pokretači i modifikatori ispitivanih bolesti, sa velikim potencijalom da kao biomarkeri budu iskorišćeni za dijagnozu, prognozu i praćenje bolesti, a sve više i u terapijske svrhe.

Najveći broj radova u Zborniku je posvećen personalizovanoj medicini - od osvrta na značaj ove oblasti uz prikaz dosadašnjih rezultata i ukazivanje na prespektive za budućnost, preko farmakoekonomije, gde se naglašava važnost optimizacije terapijskog izbora i kreiranja zdravstvene politike, do prikaza pojedinačnih model sistema na kojima se ispituju i razvijaju najnoviji dijagnostički i terapijski pristupi u preciznoj i personalizovanoj medicini. Naime, u revijskim i eksperimentalnim radovima u ovom delu Zbornika prikazani su rezultati koji se odnose na: korišćenje tehnologije indukovanih pluripotentnih stem ćelija, neonatalni skrining za spinalnu mišićnu atrofiju, genetička ispitivanja i terapiju amiotrofične lateralne skleroze, frontotemporalne demencije i Pompeove bolesti kasnog početka, kao i korišćenje prirodnih jedinjenja u terapiji tumora i mikrobiote creva za unapređenje efekata antitumorskih i antiinflamatornih terapija. Prikazani rezultati daju veliku nadu da će personalizovana terapija u bliskoj budućnosti biti integrisana u nacionalne zdravstvene sisteme.

Aktuelnosti u vezi naprednih metoda molekularne biologije date su u pregledu metodologija izotermalne amplifikacije, sa fokusom na njihovu upotrebu u dijagnostičke svrhe u biomedicini, procenu njihove integracije sa novim molekularnim alatima, uz evaluaciju primene u dijagnostici, praćenju bolesti i personalizovanim terapijama. Takođe, opisano je i DNK barkodiranje u forenzici životinja, čime je ukazano na značaj savremenih molekularnih metoda u ovoj oblasti, kojima se unapređuje preciznost u identifikaciji vrsta, doprinosi očuvanju biodiverziteta i zaštiti javnog zdravlja.

Treba istaći da se novitet iz prethodne godine, učešće predstavnika iz tzv. Generacije Z, realizovao i ove godine i to prikazom rezultata iz odbranjenog master rada koji se odnose na prediktivne markere za neodjuvantnu hemioradioterapiju u karcinomu rektuma.

U realizaciji ovogodišnjeg Zbornika učestvovali su istraživači iz naše zemlje i to iz četiri naučna instituta i četiri fakulteta, Univerziteta u Beogradu i Novom Sadu, a ono što je veoma bitno doprinos su dali i istraživači iz inostranstva, sa fakulteta iz Italije i Grčke. Zbornik tako osim edukativnog značaja ima i veliki potencijal da obezbedi dodatno povezivanje istraživača i uspostavljanje novih naučnih saradnji. Sve navedene karakteristike svrstavaju ovogodišnji Zbornik „Trendovi u molekularnoj biologiji 5“ u kategoriju tematskog zbornika vodećeg nacionalnog značaja.

Dr Gordana Nikčević, naučni savetnik u penziji
Institut za molekularnu genetiku i genetičko inženjerstvo Univerzitet u Beogradu (IMGGI)

Thematic collection "Trends in molecular biology 5" continues the tradition of presenting some of the most significant topics in molecular biology that marked the previous year, with a significant change that in this year's edition all papers are written in English.

The first part of the Thematic collection is dedicated to microRNAs and the role that these and other small non-coding RNAs play in the development of various diseases. This is particularly actual topic, given that for the discovery of microRNAs and their role in post-transcriptional gene regulation has been awarded the Nobel Prize in Physiology or Medicine in 2024. Within this topic, the latest results of researchers from our country related to the role of microRNA in the development of congenital anomalies of the kidney and urinary tract, oral squamous cell carcinoma and gestational diabetes mellitus are presented here. The significant role of this class of molecules, as well as other non-coding RNAs, was highlighted, which were shown to have ability to act as both initiators and modifiers of the studied diseases, with great potential to be used as biomarkers for diagnosis, prognosis and monitoring of diseases, and increasingly for therapeutic purposes.

The majority of papers in the Thematic collection is dedicated to personalized medicine - from a review related to the significance of this field with presentation of the results achieved so far and prospects for the future, through pharmacoeconomics, where the importance of optimizing the therapeutic choices and healthcare policy making is emphasized, to the presentation of individual model systems on which the latest diagnostic and therapeutic approaches in precision and personalized medicine are tested and developed. Namely, in the review and experimental manuscripts in this part of the Thematic collection, the results related to: the use of induced pluripotent stem cell technology, neonatal screening for spinal muscular atrophy, genetic testing and therapy of amyotrophic lateral sclerosis, frontotemporal dementia and late-onset Pompe disease, as well as the use of natural compounds in cancer therapy and the use of gut microbiota to improve the effects of anti-tumor and anti-inflammatory therapies are presented. These results raise the hope that personalized therapy will be integrated into national health systems in the near future.

In the section related to advanced methods in molecular biology a comprehensive review of isothermal amplification methodologies is presented, focusing on their use in biomedicine, assessing their integration with emerging molecular tools, along with evaluation of their potential applications in diagnostics, disease monitoring and personalized therapies. In addition, DNA barcoding in animal forensics is described, pointing to the importance of application of modern molecular methods in this field, which enhance species resolution, support both biodiversity and public health protection.

It should be noted that the novelty from the previous year, the participation of representatives from the so-called Generation Z, took place this year as well, by presenting the results from the defended master's thesis related to predictive markers for neoadjuvant chemoradiotherapy in rectal cancer.

Researchers from Serbia, from four scientific institutes and four faculties, University of Belgrade and University of Novi Sad participated in the realization of this year's Thematic collection, and it is important to point out that contribution has also been made by researchers from abroad, from faculties from Italy and Greece. Thus, in addition to educational role, the Thematic collection also has a great potential to enable additional links between researchers and help in formation of new scientific collaborations. All the above-mentioned characteristics place this year's Thematic collection "Trends in Molecular Biology 5" in the category of thematic collections of leading national importance.

Dr Gordana Nikčević, full reserach professor retd.
IMGGE

NOBELOVA NAGRADA ZA FIZIOLOGIJU ILI MEDICINU 2024:

Otkriće mikroRNK i njihova uloga u posttranskripcionoj regulaciji gena

THE NOBEL PRIZE IN PHYSIOLOGY OR MEDICINE 2024:

Discovery of microRNA and its role in post-transcriptional gene regulation

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Integrative approaches in the study of microRNAs: from transcriptomics and structural genomics to functional validation in CAKUT

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Abstract

Congenital Anomalies of the Kidney and Urinary Tract (CAKUT) are the most common developmental malformations and the leading cause of chronic kidney disease in children. Although numerous point mutations and structural variants in genes associated with CAKUT have been discovered in previous research, they can explain only 20% of cases. This points to the existence of a more complex control of gene function through regulatory molecules and networks of gene interactions in the development of the urinary system. Of special note are microRNAs, small non-coding RNAs that perform fine tuning of gene expression and whose changes in expression level have the potential to significantly affect penetrability and expressivity of CAKUT.

This paper describes a journey in which CAKUT research became a model for understanding the potential of microRNAs in the development of congenital anomalies. From transcriptomic discoveries and bioinformatic integrations, through mapping of DNA regions with Copy Number Variation, to functional experiments, a new perspective of understanding inheritance and phenotypic variability has been shaped. Today, CAKUT is defined as a complex network of regulatory signals in which microRNAs have a potential role as both initiators and modifiers of the disease.

The developed integrative approach shows that the integration of molecular data better explains the molecular basis of CAKUT and opens up space for translational methods. This defines the foundations for more precise risk stratification, monitoring the course of the disease and the development of personalized therapeutic strategies. This framework lays the foundation for the future application of advanced molecular methods and multi-omics analysis, as well as machine learning algorithms, in order to create predictive models for use in the diagnosis and treatment of CAKUT.

Keywords: CAKUT, transcriptomics, microRNA, bioinformatics, structural variation, CRISPR-Cas9

Integrativni pristupi u proučavanju mikroRNK: od transkriptomike i strukturne genomike do funkcionalne validacije u CAKUT-u

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Apstrakt

Urođene anomalije bubrega i urinarnog trakta (engl. *Congenital Anomalies of the Kidney and Urinary Tract – CAKUT*) predstavljaju najčešće razvojne malformacije i vodeći uzrok hronične bubrežne insuficijencije kod dece. Iako su dosadašnjim istraživanjima otkrivene brojne pojedinačne mutacije i strukturne varijante u genima koji su povezani sa sa CAKUT-om, one mogu objasniti samo 20% slučajeva. To upućuje na postojanje složenije kontrole genske funkcije preko regulatornih molekula i mreža genskih interakcija u razvoju urinarnog sistema. Posebno se izdvajaju mikroRNK, male nekodirajuće RNK koje fino regulišu gensku ekspresiju i čije promene u nivou ekspresije imaju potencijal da značajno utiču na penetrabilnost i ekspresivnost CAKUT-a.

U ovom radu prikazan je put kojim je istraživanje CAKUT-a postalo model za razumevanje potencijala mikroRNK u nastanku urođenih anomalija. Od transkriptomskih otkrića i bioinformatičkih integracija, preko mapiranja regiona DNK sa varijabilnim brojem kopija, pa sve do funkcionalnih eksperimenata, oblikovana je nova perspektiva razumevanja nasleđivanja i fenotipske varijabilnosti. CAKUT se danas definiše kao složena mreža regulatornih signala u kojoj mikroRNK imaju potencijalnu ulogu i kao pokretači i kao modifikatori bolesti.

Razvijeni integrativni pristup pokazuje da integracija molekularnih podataka bolje objašnjava molekularnu osnovu CAKUT-a i otvara prostor translacionim metodama. Time se definišu temelji za precizniju stratifikaciju rizika, praćenje toka bolesti i razvoj personalizovanih terapijskih strategija. Ovakav okvir postavlja osnovu za buduću primenu naprednih molekularnih metoda i multi-omics analiza, kao i algoritama mašinskog učenja, u cilju kreiranja prediktivnih modela za primenu u dijagnostici i lečenju CAKUT-a.

Ključne reči: CAKUT, transkriptomika, mirko RNK, bioinformatika, strukturna varijanta, CRISPR-Cas9

Introduction: CAKUT as a model for exploring the molecular basis of congenital disorders

Congenital anomalies of the kidney and urinary tract (CAKUT) are among the most frequent developmental malformations in humans, occurring in approximately 1 in 500 live births (1,2). Clinically, CAKUT encompasses a wide spectrum of phenotypes, ranging from posterior urethral valves, vesicoureteral reflux and obstructive megaureter to multicystic dysplastic kidney and renal agenesis (3–5). Despite their heterogeneity, CAKUT anomalies share a unifying feature: they constitute the leading cause of chronic kidney disease and pediatric end-stage renal failure (4). The high incidence and frequent lifelong morbidity make CAKUT not only a major clinical problem but also a paradigm for understanding complex congenital disorders.

The genetic underpinnings of CAKUT have been intensively investigated over the past two decades. Monogenic mutations in genes such as *HNF1B*, *PAX2*, and *DSTYK* were identified in syndromic or familial cases (6–8), while large chromosomal aberrations and structural variants explained a proportion of severe syndromic phenotypes (9,10). Yet, for the majority of sporadic CAKUT patients, the molecular cause remained unresolved. Current estimates suggest that rare copy number variants (CNVs) and point mutations could describe 20–25% of cases (6,11,12). This gap between clinical incidence and genetic explanation underscores a broader challenge in developmental biology: the incomplete penetrance and variable expressivity of complex congenital traits (13, 14).

These limitations prompted a shift away from a purely mutation-centric approach toward a systemic view of gene regulation in CAKUT. The disease offered an exceptional opportunity to apply high-throughput transcriptomics and integrative bioinformatics, enabling the exploration of global gene expression changes in affected tissues. By focusing on transcriptional signatures and post-transcriptional regulation mediated by non-coding RNAs, particularly microRNAs (miRNAs), CAKUT emerged as a model system for dissecting how regulatory layers beyond coding genes shape developmental outcomes.

The rationale for investigating miRNAs in CAKUT is grounded in both experimental and theoretical considerations. miRNAs are small non-coding RNAs that repress gene expression post-transcriptionally, thereby controlling key developmental processes such as cell proliferation, differentiation, apoptosis, and morphogenesis (15). Studies in mice demonstrated that conditional deletion of *Dicer*, the enzyme required for miRNA processing, results in severe renal malformations consistent with CAKUT phenotypes (16). These findings highlighted the importance of miRNA-dependent gene regulation during nephrogenesis and raised the hypothesis that miRNA dysregulation could represent a missing link in the unresolved heritability of human CAKUT.

Taken together, the following body of work positioned CAKUT at the forefront of translational miRNA research in human congenital disease. By combining transcriptome profiling, integrative bioinformatics, CNV analysis, and functional validation, we have pioneered a comprehensive framework for dissecting how miRNAs contribute to developmental disorders. The CAKUT model illustrates how regulatory RNAs can bridge the gap between genotype and phenotype, offering both mechanistic insights and potential avenues for clinical translation.

High-throughput Transcriptomics and Bioinformatic Integration

The first transcriptome-wide analysis of human ureter tissue from pediatric CAKUT patients and healthy controls was done by our group and has revealed more than 70 differentially expressed genes (DEGs) (Figure 1) (17). This dataset provided the earliest insights into human CAKUT molecular pathology, highlighting major dysregulated processes covering neurological processes, inflammation, immunomodulation and developmental signaling. Among the significantly altered transcripts were *LCN2*, *PROM1*, *SOSTDC1*, *INA*, *RASD1*, and *TAC3*, which emerged as notable nodes in bioinformatic network analysis and were subsequently validated at the expression level on independent group of CAKUT patients, aligning with processes relevant to obstructive uropathy, tissue remodeling, and neuro-immune crosstalk. (17).

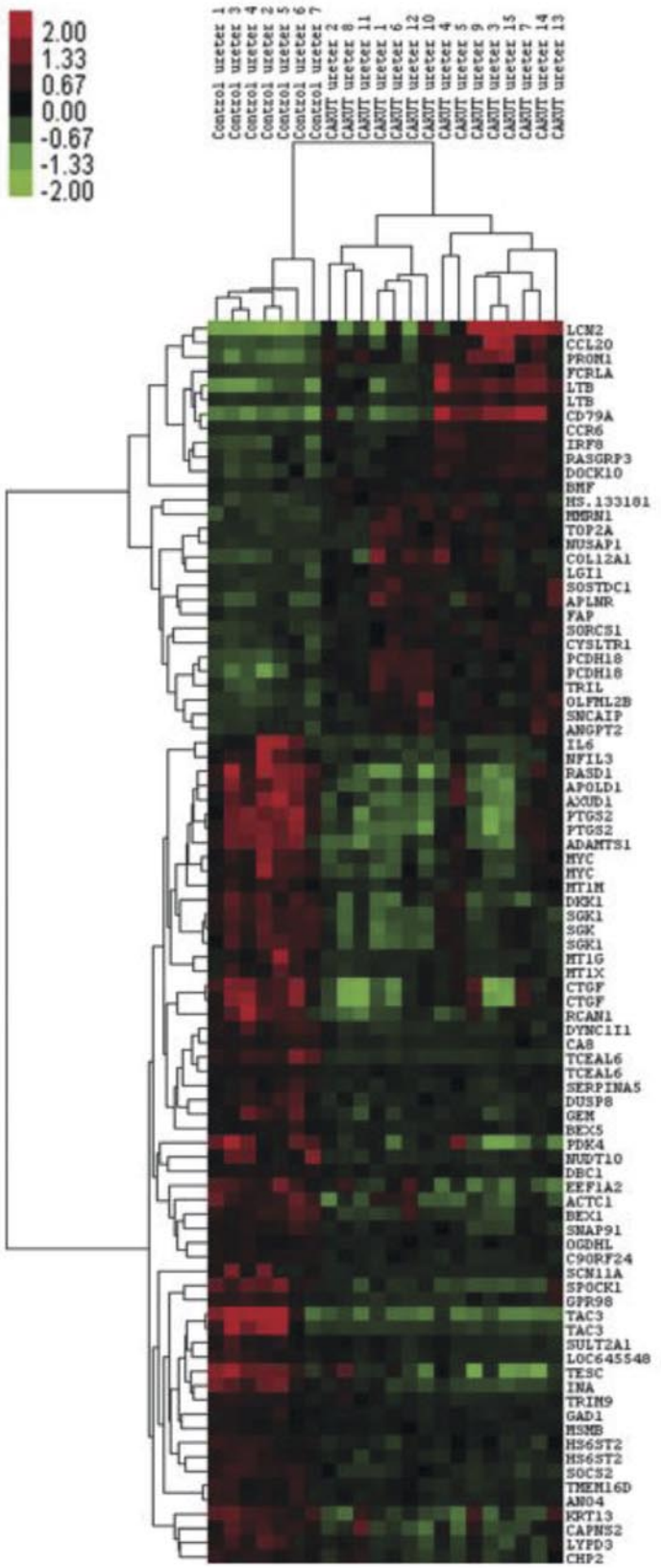

Figure 1. Heatmap of relative gene expression of DEGs in ureter tissue of CAKUT patients and controls. Red color indicates overexpression while green color depicts downregulation. Published in (17).

While these results offered candidate genes and pathways, their interpretative power increased significantly when coupled with integrative bioinformatics. To connect mRNA-level changes with potential upstream miRNA regulators, an analytical pipeline co-inertia analysis (CIA) was employed (Figure 2) (18). CIA allows the integration of gene expression data with in silico miRNA–mRNA target predictions, generating a ranked list of miRNAs most likely to explain the observed transcriptional changes. By applying this approach, a small set of miRNAs emerged as putative regulators of CAKUT transcriptomes.

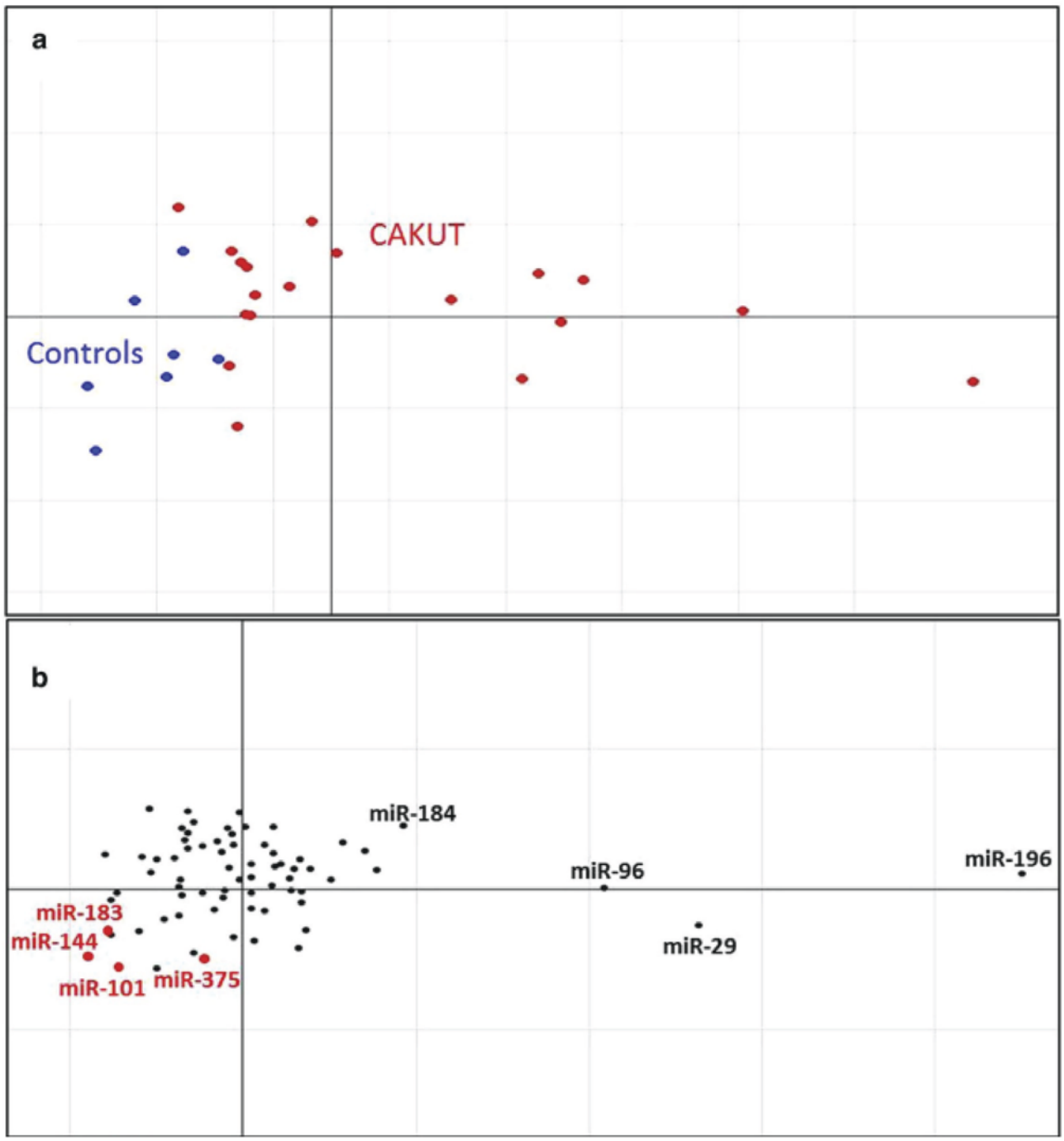

Figure 2. The plot shows the axes of the unsupervised CIA performed on the whole genome gene expression data in ureter tissue of CAKUT patients and controls. The gene/miR frequency table generated with TargetScanS was used to make this figure. A) Shows the projection of CAKUT and control samples. B) Shows the projection of the miRNAs. Motifs in the opposite orientation relative to the origin are associated with that group of samples. Therefore, by the observation of the plots, miRs hsa-miR-144, hsa-miR-101, hsa-miR-183 and hsa-miR-375 are potentially associated with CAKUT according to their position. Published in (18).

Among these, miR-144 was consistently prioritized and subsequently validated as significantly upregulated in ureter tissue of CAKUT patients compared to controls (18). Its increased expression, approximately 5.7-fold in validation cohort, was the first demonstration that specific miRNAs are dysregulated in human CAKUT tissue. Functional enrichment analysis of predicted miR-144 targets revealed processes involved in urinary system development, neural development and signal transduction, all aligning with the pathophysiology of CAKUT (18). This discovery provided both a mechanistic candidate and a proof of principle: miRNAs represent an important layer of regulation in CAKUT, previously invisible to mutation-based genetic screens.

Beyond miR-144, the integrative approach also nominated other candidates such as members of the miR-200 and miR-183 families, though these were not differentially expressed in patient tissue (18). This discrepancy underscored the importance of experimental validation in separating computational predictions from biologically relevant signals. Nevertheless, the analytical framework, systematic transcriptome profiling, followed by CIA-based integration with miRNA target predictions established a replicable strategy for moving from unbiased discovery toward mechanistic hypotheses.

Importantly, the finding that miR-144 is located within a CNV-rich region on chromosome 17q11.2 offered a bridge between transcriptomics and structural genomics (25). The possibility that dosage-sensitive regulatory elements such as miRNAs might explain unexplained CNV associations with CAKUT was a conceptual turning point. This observation set the stage for subsequent investigations into CNV–miRNA interactions as potential drivers of phenotypic variability.

CNVs and Functional Validation of miRNA Roles

The discovery of miR-144 upregulation in CAKUT tissue coincided with an important realization: many of the recurrent copy number variants (CNVs) reported in CAKUT lacked obvious protein-coding gene drivers. This observation raised the possibility that structural variation may affect non-coding regulatory elements, particularly miRNA genes, thereby shaping disease penetrance and expressivity. Given the established role of miRNAs in renal development, mapping CNVs against the human miRNome offered a new dimension for exploring the genetic architecture of CAKUT.

The first comprehensive effort to catalogue CAKUT-associated rare CNVs involved the systematic mining of published datasets, generating a curated database of pathogenic and likely pathogenic CNV regions (20). When miRNA loci were overlaid on this landscape, it became clear that the majority of CAKUT patients harbouring rare CNVs also carried at least one disrupted miRNA gene. Among these, *MIR484* and *MIR185* were particularly prominent, frequently intersecting with CNVs (20) while bioinformatic network analysis highlighted these miRNAs as central regulators of genes implicated in processes associated with CAKUT (Figure 3) (20). The enrichment of miR-548 family members in CAKUT-associated CNVs further suggested the notion that dosage of non-coding RNAs may act as a genetic driver, even in the absence of protein-coding disruptions. Interestingly, while the members of this miRNA family are relatively highly affected in CAKUT, it is almost completely unaffected by polymorphic CNVs found in controls, regardless of their high number (73 precursors) and chromosomal dispersion, which suggest evolutionary pressure on CNVs encompassing hsa-miR-548 family genes. This speculation could also be supported by the functional enrichment analysis of the hsa-miR-548 family target genes, depicting key developmental processes (20).

Functional research of rare CNVs was pursued using CRISPR/Cas9-engineered HEK293 models depicting heterozygous (*MIR484*+/–) and homozygous (*MIR185*–/–) deletions (22). These models revealed dosage-sensitive reductions in the corresponding miRNAs, which translated into dysregulation of critical target genes. The *MIR484*+/– model, for instance, showed reduced miR-484 expression with concomitant MDM2

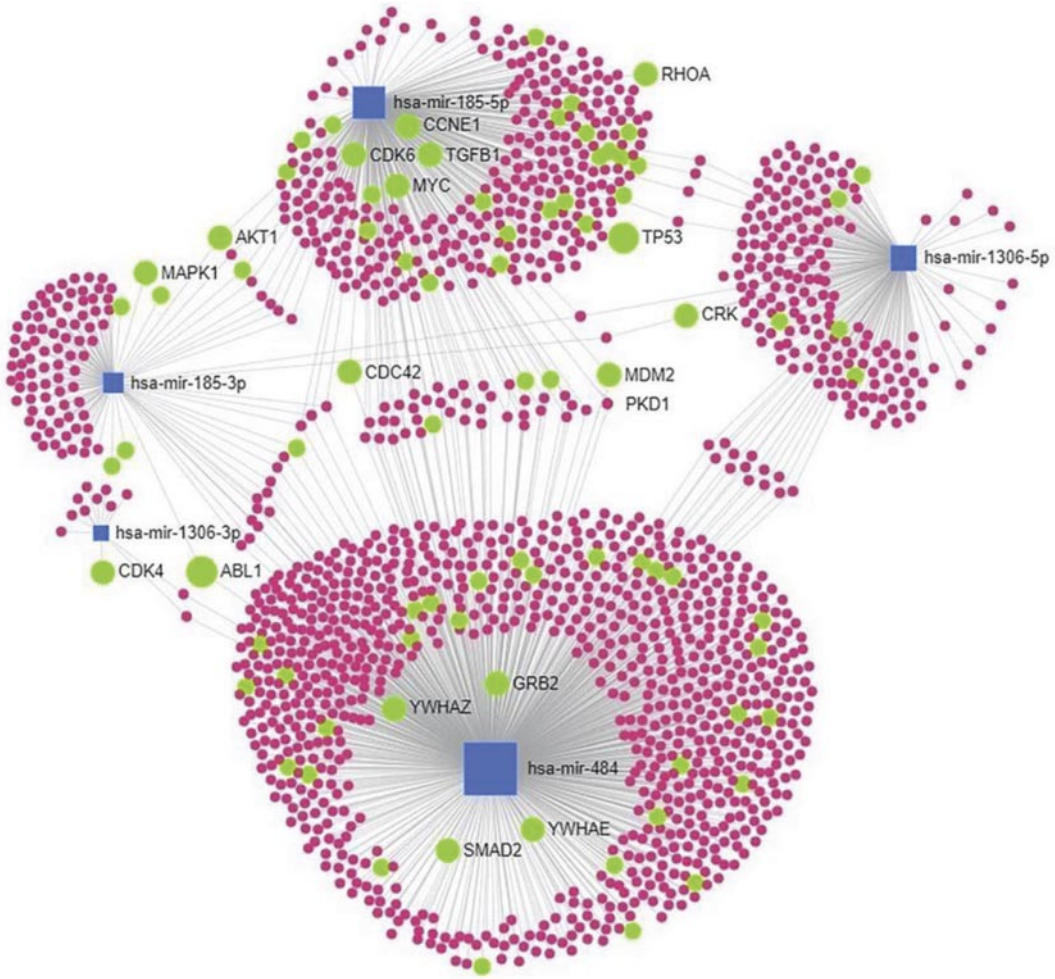

Figure 3. miRNA-gene interaction network of miRNAs most frequently affected by rare CNVs in CAKUT. Square shapes represent miRNAs while circles represent genes regulated by specific miRNAs. The size of the squares depicts the number of miRNA-gene interactions based on the miRTarBase v8.0 data. Green circles represent genes included in the significantly enriched pathways whereby the size of the circle is proportional to the number of significantly enriched pathways in which the specific gene co-localises. Published in (20).

and APAF1 upregulation and NOTCH3 downregulation, aligning with pathogenic processes relevant to CAKUT, where MDM2 overexpression contributes to cell-cycle disruption, inflammation and renal fibrosis (26, 27), APAF1 upregulation enhances apoptosis that may disturb nephrogenesis (28, 29, 30), and NOTCH3 downregulation interferes with signalling pathways essential for early kidney development and renal hemodynamic (31, 32, 33). Similarly, deletion of *MIR185* produced downregulation of miR-185 accompanied by altered expression of apoptotic and signaling mediators, where CDC42 dysregulation perturbs the Slit-Robo associated with CAKUT (34, 35) and integrin–RAC1/CDC42 signalling crucial for nephrogenesis (36) while RHOA dysregulation affects neurotrophin-mediated transmission in early kidney development (37). These findings provided direct evidence that CNV-mediated miRNA loss-of-function can perturb gene networks relevant to CAKUT pathophysiology**.**

In addition to rare variants, common CNVs, which occur in the general population yet may contribute to disease susceptibility when combined with other genetic or environmental factors, have been investigated in CAKUT. Using public CNV datasets and careful bioinformatic filtering, we identified miRNA loci recurrently affected by common CNVs and bioinformatically associated with molecular and regulatory processes implicated in CAKUT (21). Copy number distributions of *MIR9-3* and *MIR1299* in CAKUT patients

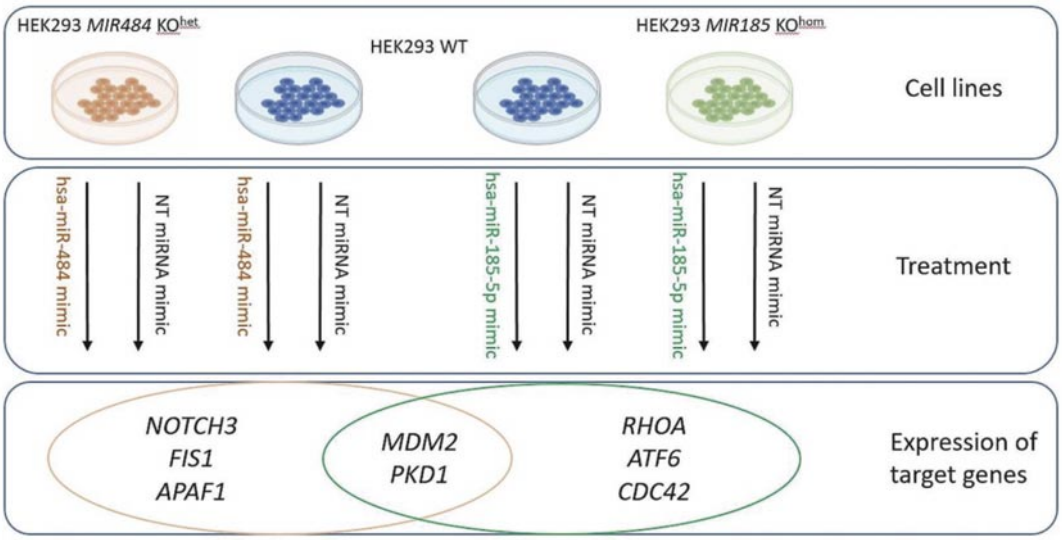

Figure 4. Heterozygous MIR484 knockout HEK293 cell line (HEK293 MIR484 KOhet) and homozygous MIR185 knockout HEK293 cell line (HEK293 MIR185 KOhom) were used as models of CNV-affected miRNAs in CAKUT. Cell lines were treated simultaneously with the corresponding miRNA mimics and NT miRNA mimic. Two of the investigated genes (MDMD2 and PKD1) have been selected as targets of both hsa-miR-484 and hsa-miR-185-5p mimics. Additional three candidate target genes with a potential role in CAKUT have been selected for each miRNA. As candidate target genes of hsa-miR-185-5p RHOA, ATF6 and CDC42 have been selected, while NOTCH3, FIS1 and APAF1 have been chosen as candidate target genes of hsa-miR-484. Both miRNA mimic treatments have been performed on HEK293 WT cell lines as well. Expression of target genes for miRNA mimic treatments have been compared to the corresponding NT miRNA treatments to explore the responsiveness of the expression of target genes to treatment with hsa-miR-484 and hsa-miR-185-5p mimics. Published in (22).

compared to controls were significantly different. Notably, both deletions and duplications of these loci were more frequent in patients than in controls, supporting the idea that deviation from normal diploid dosage, either reduced or increased copy number, can disrupt developmental homeostasis. This effect suggests why CNVs can produce variable expressivity: the same locus, when altered in different ways, may contribute to divergent phenotypic outcomes.

To further test the translational potential, restitution experiments were performed by transfecting synthetic miRNA mimics into the CRISPR-edited cell models (Figure 4) (22). While partial normalization of dysregulated targets was observed, responses were often complex and context-dependent. In some cases, restitution even produced unanticipated expression shifts, consistent with the notion of competing endogenous RNA (ceRNA) networks (23,24). Because miRNAs act within multi-layered interaction webs, the restoration of a single miRNA can redistribute regulatory pressure across the transcriptome, leading to nonlinear outcomes. This complexity stems from the fact that miRNAs exert their function through microRNA response elements (MREs), which are present in multiple transcripts, often within long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), and protein-coding genes. These molecules compete for binding to the same pool of miRNAs, thereby influencing each other's stability and translation in a dosage-dependent manner. In the context of CAKUT, where dosage sensitivity of regulatory elements is already a central pathogenic theme, ceRNA interactions may amplify or buffer the phenotypic impact of specific miRNA alterations. Restitution experiments in CRISPR/Cas9 models highlight that re-introducing a miRNA mimic of previously deleted miRNA gene does not simply reverse the deletion phenotype, but rather probably reconfigures the ceRNA landscape, occasionally resulting in paradoxical or context-dependent changes in gene expression. These findings reinforce the idea that therapeutic manipulation of miRNAs cannot be considered in isolation, but must account for the broader MRE-based interaction networks that shape developmental trajectories. These findings underscored the biological reality that miRNAs rarely operate in isolation, and that therapeutic strategies targeting miRNAs must account for ceRNA effects and network redundancy.

Taken together, these studies moved the CAKUT field beyond descriptive transcriptomics into functional validation of structural–regulatory interactions. By demonstrating that CNVs frequently intersect with miRNA loci, and that deletion of these loci can dysregulate relevant developmental pathways, the research implied to a novel potential mechanistic explanation for unresolved heritability. Importantly, it also offered a conceptual advance: miRNA genes themselves can represent primary drivers of congenital malformations, not merely modulators of downstream signalling. This insight bridged cytogenetics, transcriptomics, and functional genomics, placing CAKUT at the forefront of integrative non-coding RNA biology in human disease.

Advances, Broader Implications, and Future Perspectives

The trajectory from transcriptome profiling to CNV mapping and functional validation transformed CAKUT research into a paradigm for studying complex congenital disorders. Each methodological step addressed a central limitation of traditional genetics: the inability to fully explain variable expressivity and incomplete penetrance with coding mutations alone. By demonstrating how regulatory RNAs and structural variants converge on developmental pathways, this work provided both conceptual and methodological advances with relevance far beyond CAKUT.

Perhaps the most significant legacy of this body of work lies in its methodological framework**.** The sequential pipeline, beginning with transcriptome-wide differential expression, progressing through integrative bioinformatic prioritization, expanding into CNV–miRNA mapping, and culminating in functional CRISPR/Cas9 validation with restitution experiments provides a reproducible template (22). This multi-layered approach demonstrated how diverse omics technologies can be unified to interrogate unresolved heritability. The lessons learned are directly transferrable: transcriptome integration with non-coding RNA prediction is broadly applicable to other congenital malformations, cancer predisposition syndromes, and immune dysregulation disorders where monogenic explanations fall short.

Beyond CAKUT, the studies contributed to a conceptual reframing of miRNAs as dosage-sensitive genomic elements. Unlike protein-coding genes, which typically follow a loss-of-function versus gain-of-function paradigm, miRNAs exert subtle, network-wide regulatory effects. Alterations in copy number or expression may not abolish developmental programs outright but instead bias morphogenetic trajectories, producing variable phenotypes (11). This property explains why identical point mutations and CNVs can lead to divergent clinical presentations in different patients, a phenomenon long observed in CAKUT and other congenital diseases (12). Furthermore, restitution experiments revealed the importance of competing endogenous RNA (ceRNA) networks**,** underscoring that therapeutic strategies aimed at correcting miRNA deficits must consider indirect interactions and non-linear responses (38,39).

From a translational perspective, the research underscores the potential of miRNA profiling as a biomarker strategy in congenital disorders. Tissue-derived signatures such as miR-144 upregulation could inform diagnosis or prognosis, while CNV–miRNA mapping might improve genetic counselling by highlighting non-coding drivers of disease. Functional assays with CRISPR-engineered cell models provide a preclinical platform to test targeted interventions, including miRNA mimics or inhibitors. Yet, caution remains warranted: the restitution experiments revealed complexity and unpredictability inherent to miRNA therapeutics. Future translation will require sophisticated modeling of precise miRNA delivery mechanisms and context-specific effects.

Looking ahead, several avenues are poised to extend this work. First, RNA sequencing at single-cell resolution could unravel cell-type specific expression dynamics within CAKUT tissue, distinguishing primary developmental alterations from secondary injury responses (40). Second, integration of long non-coding RNAs

(lncRNAs) and circular RNAs into the regulatory framework will capture additional layers of ceRNA interaction (41). Third, multi-omics approaches, combining transcriptomics, epigenomics, and proteomics may provide systems-level signatures capable of predicting phenotypic outcomes with higher accuracy (42). Finally, the development of machine learning models trained on multi-layered omics and clinical data holds promise for generating predictive models that could aid clinical decision-making, stratifying CAKUT patients according to risk, likely progression, and optimal monitoring strategies (43).

Taken together, the journey through CAKUT miRNA research illustrates how a clinically challenging disorder can be leveraged as a model system to pioneer integrative molecular methodologies**.** The identification of miRNAs as both targets and drivers of disease reshaped the understanding of CAKUT inheritance and highlighted the critical role of regulatory RNAs in developmental biology. By unifying transcriptome profiling, bioinformatic prioritization, CNV mapping, and functional validation, this work offers a transferable blueprint for untangling the molecular basis of other congenital anomalies. The broader message is clear: to resolve the complexity of human developmental disorders, one must look beyond coding genes to the regulatory networks that orchestrate them.

Acknowledgements

This work was funded by the the Ministry of Science, Technological Development and Innovation of the Republic of Serbia, Grant No. 451-03-136/2025-03/200017 and the Science Fund of the Republic of Serbia, PROMIS, #6066923, miFaDriCa.

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Noncoding RNAs as biomarkers of gestational diabetes mellitus

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Abstract

Non-coding RNAs (ncRNAs), including microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs), show potential to serve as biomarkers of metabolic disorders, oxidative stress and inflammatory status, and thus may have diagnostic and prognostic significance in gestational diabetes mellitus (GDM). Aberrant expression of specific ncRNAs has been identified in placental tissue, plasma, serum, extracellular vesicles, peripheral blood mononuclear cells (PBMCs), as well as in other maternal and fetal sources. These ncRNAs exhibit high stability in circulation and can reflect underlying molecular alterations, making them promising non-invasive biomarkers for diagnosis, prognosis, and disease monitoring. Therefore, this article will review the major findings of GDM-related ncRNAs and highlight their potential role in improving clinical outcomes through biomarker-based approaches.

Keywords: gestational diabetes mellitus, miRNA, lncRNA, circRNA, liquid biopsy

Nekodirajuće RNK kao biomarkeri gestacijskog dijabetesa

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Apstrakt

Nekodirajuće RNK (nkRNK), uključujući mikroRNK, duge nekodirajuće RNK i kružne RNK, pokazuju potencijal da služe kao biomarkeri metaboličkih poremećaja, oksidativnog stresa i inflamatornog statusa, i stoga mogu imati dijagnostički i prognostički značaj kod gestacijskog dijabetesa melitusa (GDM). Aberantna ekspresija specifičnih nkRNK je identifikovana u tkivu placente, plazmi, serumu, ekstracelularnim vezikulama, mononuklearnim ćelijama periferne krvi, kao i u drugim izvorima poreklom od majke ili fetusa. Ove nkRNK pokazuju visoku stabilnost u cirkulaciji i mogu odražavati osnovne molekularne promene, što ih čini obećavajućim neinvazivnim biomarkerima za dijagnozu, prognozu i praćenje bolesti. Stoga će ovaj članak pregledati glavna saznanja o nkRNK povezanih sa GDM i istaći njihovu potencijalnu ulogu u poboljšanju kliničkih ishoda kroz pristupe zasnovane na biomarkerima.

Ključne reči: gestacijski dijabetes melitus, mikroRNK, duge nekodirajuće RNK, cirkularne RNK, tečna biopsija

Introduction

Gestational diabetes mellitus (GDM) is recognized as one of the three most common types of diabetes by the International Diabetes Federation. It is defined as a pathological condition of glucose intolerance, first recognized during pregnancy, and represents one of the most frequent metabolic complications affecting pregnant women worldwide, with an average prevalence of approximately 14% of pregnancies [1].

If left undiagnosed or poorly managed, GDM can lead to serious health risks for both the mother and the offspring. It is associated with a wide range of both short- and long-term complications, including preeclampsia, fetal macrosomia, neonatal hypoglycemia, shoulder dystocia, preterm labor, cesarean delivery, and fetal mortality. Children of mothers with GDM are at significantly increased risk of developing type 2 diabetes mellitus (T2DM), obesity, and cardiovascular diseases later in life. Furthermore, women diagnosed with GDM have a significantly elevated risk of developing T2DM postpartum, and female infants are also at increased risk of developing GDM during their own pregnancies [2].

Despite the well-established consequences of GDM, its pathogenesis remains incompletely understood. It is thought to arise from a combination of genetic, epigenetic, environmental, and neurohormonal factors that contribute to insulin resistance, hyperglycemia, and β-cell dysfunction during pregnancy. There is also evidence that (glyco)oxidative stress, low-level chronic inflammation, adipose tissue expandability, and placental factors contribute to the pathology of GDM [3].

Currently, GDM is most commonly diagnosed using the oral glucose tolerance test (OGTT), performed between 24 and 28 weeks of gestation, although there is a lack of consensus on the use of diagnostic criteria or protocols. Moreover, the OGTT is time-consuming and may cause significant discomfort, vomiting, or even more serious consequences in women with pre-existing glucose intolerance or undiagnosed diabetes [4, 5]. Since this test has limited predictive value and currently there are no GDM biomarkers that are in clinical use, there is a growing need for novel biomarkers which could aid in diagnosing, predicting, or monitoring GDM, resulting in a reduced risk of maternal and fetal complications.

In recent years, the focus of biomarker research has moved towards molecules that possess these three types of clinically relevant properties and are also accessible through non-invasive sampling. Circulating biomarkers, detectable in many biological fluids, including plasma, serum, urine, extracellular vesicles, or in blood cells, have gained increasing attention due to their ability to be monitored throughout pregnancy. Among them, non-coding RNAs (ncRNAs), particularly microRNAs (miRNAs), long noncoding RNAs (lncRNAs), and circular RNAs (circRNAs), have emerged as promising candidates [6].

Non-coding RNAs represent a diverse class of functional RNA molecules that, generally, do not encode proteins but instead regulate gene expression at epigenetic, transcriptional and post-transcriptional levels. These ncRNA species have been implicated in key physiological and pathological processes such as glucose metabolism, insulin signalling, inflammation, oxidative stress, and placental development, all of which are closely linked to the pathophysiology of GDM [6, 7].

NcRNAs possess several features that make them attractive as potential biomarkers, including a high level of stability in many body fluids, within extracellular vesicles, associated with RNA-binding proteins, or, in the case of circRNAs, due to circular structure, which protects them from RNase degradation [8]. Additionally, they have tissue- and disease-specific expression patterns and act through direct involvement in regulatory pathways. Furthermore, the desirable features of ncRNAs as biomarker candidates are non-invasive sample acquisition through liquid biopsy, detectability early in the disease, which is extremely im-

portant for timely diagnosis, and the availability of sensitive molecular techniques, such as next generation sequencing (NGS), microarray analysis, digital droplet PCR (ddPCR) and quantitative real-time PCR (qRT-PCR), which allows accurate quantification [9].

In the context of GDM, numerous studies have reported altered expression of ncRNAs in maternal plasma, serum, placental tissue, peripheral blood mononuclear cells (PBMCs), as well as in other maternal and fetal biological sources [7]. NcRNAs, especially in combination with clinical, metabolic, and redox-status parameters, may serve as valuable components of novel advanced algorithms constructed for risk prediction, early diagnosis, disease monitoring or prediction of adverse outcomes and treatment strategy guidance. Furthermore, understanding the regulatory networks mediated by ncRNAs may reveal novel therapeutic targets and provide insight into mechanisms through which dysregulated ncRNAs participate in the development of metabolic diseases [7].

In this review, we summarize the current evidence regarding the role of circulating ncRNAs in the pathophysiology of GDM and evaluate their potential utility as biomarkers for detection, prediction, and disease monitoring. By highlighting recent findings, we aim to contribute to the growing body of knowledge that supports the integration of ncRNA profiling into clinical practice.

MiRNAs

MiRNAs are highly conserved non-coding RNA sequences that are expressed in plants, animals, and some viruses [10]. They play an essential role in the post-transcriptional regulation of gene expression, which is achieved through endonucleolytic degradation of target RNAs, destabilization of target mRNAs, translational repression, or, in some cases, translational activation [11]. This regulation is mediated by a phenomenon known as RNA interference (RNAi), a natural mechanism that also protects cells from the harmful propagation of viruses and transposons [12].

The interaction between miRNA and the target mRNA is achieved by base pairing. The most miRNA-binding sites (MREs) are located in the 3 -untranslated region (3'-UTR) of mRNA. Target recognition is generally determined by the complementarity between the bases 2 to 8 of the miRNA, called the seed sequence, and the 3'-UTR of mRNA. One mRNA contains MREs for a large number of the same and different miRNAs, and one miRNA can regulate hundreds of target mRNAs, which indicates a complex post-transcriptional regulation by miRNAs that occur within regulatory networks [13].

MiRNAs play a pivotal role in normal development and are involved in a variety of biological processes. Apart from their role in cells in which their biogenesis occurs, they also serve as signaling molecules, mediating cell-to-cell communication [14]. Aberrant expression of miRNAs has been associated with numerous human diseases, including cancer, immunological disorders, and developmental abnormalities. Since miRNAs are secreted into extracellular fluids, they are also recognized as potential biomarkers for a wide range of diseases [15].

One of the most remarkable features of miRNAs is their stability in body fluids, such as blood, urine, saliva, and amniotic fluid. Unlike most RNA molecules, which are rapidly degraded by RNases, circulating miRNAs are resistant to enzymatic degradation due to their association with RNA-binding proteins (miRISC/AGO2 complexes) and incorporation in lipoprotein complexes, including high-density lipoproteins (HDL) or low-density lipoproteins (LDL), or extracellular vesicles (EVs) [16]. In the context of biomarker research and mechanistic studies on between-cell communication, EVs are particularly interesting biological entity, since they carry bioactive molecules such as proteins, lipids, and RNAs, including miRNAs, and me-

diate intercellular communication by carrying their cargo to recipient cells. Additionally, their cargo reflects the biological processes active in the cells which produce them [17]. Therefore, miRNAs enveloped in EVs, which are selectively incorporated and are among the most extensively analyzed RNA species enriched in EVs, may represent promising biomarker candidates. In GDM, EVs miRNA content reflects the physiological state of the affected maternal tissues and the placenta, while EV-associated miRNAs also modulate gene expression in distant cells, influencing insulin sensitivity, immune responses, and endothelial function [18, 19]. Importantly, placenta-derived EVs are released in increasing amounts throughout pregnancy and can be detected in maternal circulation, making their miRNA content a promising source of non-invasive biomarkers for early detection and monitoring of GDM [20].

Multiple studies have reported tissue-specific dysregulation of miRNAs in GDM, including placenta-specific changes [21, 22]. Dysregulation of a variety of miRNAs is linked to altered trophoblast proliferation, migration, invasion, and mitochondrial function [23, 24]. Specific miRNAs in pancreatic islets are involved in insulin gene expression and β-cell mass regulation [25]. These changes contribute to inadequate insulin secretion in response to increased insulin demand during pregnancy. Furthermore, altered levels of various miRNAs in adipose tissue are affecting adipocyte differentiation, lipid metabolism, and inflammatory cytokine production [26]. Taken together, these findings supported the concept that miRNAs are not just byproducts but active regulators in the pathophysiology of GDM and that miRNAs released by GDM-relevant tissues may change circulating miRNA profiles which would reflect the changes in the cells of origin.

Despite their promise as biomarkers, results from circulating miRNA studies in GDM are heterogeneous and difficult to compare across different investigations. Several factors contribute to this variability. First, miRNA expression is influenced by maternal age, BMI, ethnicity, lifestyle, diet, and pre-existing metabolic conditions [27], which can confound the results if not properly matched or adjusted. Additionally, miRNA profiles change dynamically throughout pregnancy, reflecting ongoing physiological adaptations in both maternal and placental tissues [28]. Namely, pregnancy is a highly dynamic physiological state, and miRNA expression changes across trimesters to accommodate maternal-fetal adaptation. Studies have shown that some miRNAs, especially placenta-specific miRNAs, like those from the C19MC cluster, are progressively upregulated during pregnancy and hyperexpressed in the third trimester [16]. Therefore, the timing of sample collection is critical when evaluating miRNAs as biomarkers, and results obtained at different gestational stages may not be directly comparable. Differences in sample preference (serum, plasma, whole blood, or extracellular vesicle-enriched fractions) can significantly affect miRNA content and stability. Furthermore, variability in sample collection procedure, processing time, and storage temperature can alter miRNA integrity [29]. Finally, a lack of universal endogenous controls for circulating miRNA quantification leads to inconsistent normalization methods, further contributing to inter-study variation [30].

Considering these obstructions, interpreting and comparing findings across studies remains difficult. Nevertheless, several investigations have identified matching candidate circulating miRNAs associated with GDM, or their main findings referred to members of the same miRNA families. Table 1 provides an overview of miRNA-oriented studies on GDM biomarkers [31-75], illustrating both their findings and methodological differences.

One of the commonly reported dysregulated miRNA in plasma or serum samples of GDM patients was miR-16-5p. However, these results need to be taken with caution, since miR-16-5p is highly expressed in erythrocytes and the quantity in blood fractions is severely affected by hemolysis, for which reason it is generally avoided as a potentially usable biomarker [76]. Other miRNAs were rarely reported as upregulated or downregulated in more than a single study. For instance, miR-146a-5p was reported as upregulated in two

studies on GDM in similar pregnancy stage, but with different sample types: plasma, serum-derived EVs and PBMCs [55, 67]. Similarly, miR-423-5p was among the upregulated miRNAs in plasma and plasma-derived EVs from GDM patients [55, 65], while an upregulation was also detected for miR-210-3p, miR-195-5p, miR-520h, miR-23a-3p and miR-342-3p in serum and plasma [39, 46, 49, 53, 55, 57, 63]. When it comes to miR-17-5p, higher levels of expression in plasma samples of GDM patients were reported in two studies [50, 51], while for other two members of the same family, miR-19a-3p and miR-19b-3p, two studies supported an upregulation in GDM [35, 50]. As for miR-330-3p and miR-223-3p, there were three or more reports on their upregulation in serum or plasma samples [34, 37, 40, 52, 53, 57, 77].

Besides inconsistencies regarding the panels of dysregulated miRNA hits, the data presented in the table indicate that certain miRNAs exhibited increased expression in pregnant women with GDM compared to normoglycemic controls in some studies, while in others they show decreased expression. For instance, miR-132-3p, miR-20a-5p, miR-122-5p, miR-29a-3p and miR-222-3p were reported as both downregulated and upregulated miRNA in GDM [31, 33, 34, 36, 45, 55, 50, 51, 63, 65]. In the case of miR-20a-5p, which belong to the same miRNA family as miR-17-5p and originate from a miRNA gene cluster miR-17~92, the observed differences may be caused by sample selection, since matching results were found for two studies that utilized plasma as a source of analyzed miRNAs [50, 51]. The direction of the change in the expression of specific miRNAs varied even between subgroups of samples within the same study, such was the case with miR-125b-5p and miR-183-5p in different pregnancy trimesters [32]. Several of the reported dysregulated miRNAs belong to the same family, which may be explained by similarities in their target mRNA pools and the common regulation of gene expression for those originating from clustered genes. Most prominent are the reports on the dysregulation of the members of miR-17~92 family: miR-17-5p, miR-18a-5p, miR-19a-3p, miR-19b-3p, miR-20a-5p, miR-92a-3p, which are mostly reported as upregulated in GDM (Table 1).

The interpretation of circulating miRNA profiles in GDM is highly dependent on the type of biological sample analyzed, since miRNA content and stability vary between these sources. Despite the increasing number of studies, blood cells are still rarely exploited as a source of circulating miRNAs. However, there have been studies GDM biomarkers focusing on miRNAs from whole blood or PBMCs, which demonstrated the potential diagnostic and/or prognostic significance of several candidate miRNAs, as well as their role as indicators of metabolic and redox status in hyperglycemic pregnancy [67-75, 78]. Although the selection of the most adequate type of biological sample for biomarker analysis is an important issue, studies that compare different sources of potentially relevant miRNAs in the same cohorts are rare [67, 79]. One such study is ours, in which we analyzed matched samples of EVs and PBMCs obtained from the same individuals [67]. This approach allowed us to directly compare miRNA expression patterns across two biological sources within the same physiological context. Notably, we observed consistent trends in the expression of selected miRNAs, including miR-146a-5p and miR-21-5p, in both PBMC- and EV-derived fractions [67]. The agreement in direction of change between these paired sources further reinforces the potential of these miRNAs as biomarkers of GDM.

Beside the necessity of validating the diagnostic utility of potential GDM biomarker miRNAs in large prospective studies, another important issue is determining the major factors which may affect the expression of candidate miRNAs, such as age of study participants, their life habits and previously mentioned pregnancy and sample characteristics. For certain miRNAs reported as dysregulated in GDM, previous studies have reported a substantial influence of common genetic variants on their biogenesis, or even on the sequence of mature miRNA molecules [80-83], which may interfere with quantification and results interpretation. Therefore, integration of miRNA quantification results with genetic data, clinical characteristics and other potentially relevant sources of bias should be considered during GDM biomarker evaluation.

Table 1. Summary of dysregulated miRNAs in GDM

Authors	Year	No. GDM/ Controls	Gestation week	Method	qPCR normalization	Up-regulated in GDM	Down-regulated in GDM	Reference
Serum								
Zhao et al.	2011	24/24	16–19	TLDA, qRT-PCR	cel-miR-39-3p		hsa-miR-132-3p, hsa-miR-29a-3p, hsa-miR-222-3p	[31]
Lamadrid-Romero et al.	2017	14/27	first trimester	qRT-PCR	cel-miR-39-3p	hsa-miR-125b-5p		[32]
Lamadrid-Romero et al.	2017	26/26	second trimester	qRT-PCR	cel-miR-39-3p		hsa-miR-125b-5p	[32]
Lamadrid-Romero et al.	2017	27/21	third trimester	qRT-PCR	cel-miR-39-3p		hsa-miR-125b-5p	[32]
Lamadrid-Romero et al.	2017	13/12	first trimester	qRT-PCR	cel-miR-39-3p	hsa-miR-183-5p, hsa-miR-200b-3p, hsa-miR-1290		[32]
Lamadrid-Romero et al.	2017	24/24	second trimester	qRT-PCR	cel-miR-39-3p	hsa-miR-183-5p,	hsa-miR-128-5p	[32]
Lamadrid-Romero et al.	2017	20/16	third trimester	qRT-PCR	cel-miR-39-3p		hsa-miR-183-5p, hsa-miR-200b-3p	[32]
Pheiffer et al.	2018	28/53	13-31	qRT-PCR	cel-miR-39-3p		hsa-miR-20a-5p, hsa-miR-222-3p	[33]
Martínez-Ibarra et al.	2019	18/22	second trimester	qRT-PCR	hsa-miR-454	hsa-miR-9-5p, hsa-miR-29a-3p, hsa-miR-330-3p		[34]
Wang et al.	2019	100/100	24-28	qRT-PCR	RNU6	hsa-miR-19a-3p, hsa-miR-19b-3p		[35]
Zhou et al.	2019	108/50	24-28	qRT-PCR	RNU6	hsa-miR-132-3p		[36]
Abdeltawab et al.	2020	109/103	third trimester	qRT-PCR	cel-miR-39-3p	hsa-miR-223-3p		[37]
Feng et al.	2020	12/12	NA	qRT-PCR	RNU6	hsa-miR-33a-5p		[38]
Wang et al.	2020	102/102	25	qRT-PCR	RNU6	hsa-miR-195-5p		[39]
Xiao et al.	2020	30/10	16-28	qRT-PCR	RNU6	hsa-miR-330-3p		[40]
Hua et al.	2021	30/38	third trimester	qRT-PCR	RNU6	hsa-miR-377-3p		[41]
Li et al.	2021	93/93	24–28	qRT-PCR	RNU6		hsa-miR-497-5p	[42]
Liu et al.	2021	110/78	24-28	qRT-PCR	RNU6	hsa-miR-1323-5p		[43]
Shen et al.	2021	25/30	NA	qRT-PCR	RNU6	hsa-miR-181d-5p		[44]
Sørensen et al.	2021	82/41	<20	qRT-PCR	RNU6, ath-miR-159, cel-miR-39-3p	hsa-miR-16-5p, hsa-miR-29a-3p, hsa-miR-134-5p		[45]
Wen et al.	2021	32/48	second/third trimester	qRT-PCR	RNU6	hsa-miR-520h		[46]
Juchnicka et al.	2022	24/24	9-12	qRT-PCR	miR-103a-3p	hsa-miR-16-5p, hsa-miR-142-3p, hsa-miR-144-3p		[47]
Li et al.	2022	118/65	23-28	qRT-PCR	RNU6	hsa-miR-518		[48]
Jamalpour et al.	2023	24/24	first trimester	PCR array	SNORD61, SNORD68, SNORD72, SNORD95, and SNORD96A	hsa-miR-193a, hsa-miR-21-5p, hsa-miR-23a-3p, hsa-miR-361	hsa-miR-130a-3p	[49]
Jamalpour et al.	2023	24/24	second trimester	PCR array	SNORD61, SNORD68, SNORD72, SNORD95, and SNORD96A	hsa-let-7i-5p, hsa-miR-126, hsa-miR-129	hsa-miR-125, hsa-miR-129–2, hsa-miR-130a, hsa-miR-34, hsa-miR-375	[49]
Jamalpour et al.	2023	24/24	third trimester	PCR array	SNORD61, SNORD68, SNORD72, SNORD95, and SNORD96A	hsa-let-7e, hsa-miR-107, hsa-miR-361, hsa-miR-370	hsa-miR-125, hsa-miR-129, hsa-miR-130a	[49]
Plasma								
Zhu et al.	2015	10/10	16–19	NGS, qRT-PCR	miR-221	hsa-miR-16-5p, hsa-miR-17-5p, hsa-miR-19a-3p, hsa-miR-19b-3p, hsa-miR-20a-5p		[50]
Cao et al.	2017	85/72	24–28	qRT-PCR	cel-miR-39, cel-miR-54, cel-miR-238	hsa-miR-16-5p, hsa-miR-17-5p, hsa-miR-20a-5p		[51]
Sebastiani et al.	2017	21/10	24-33	TAC, qRT-PCR	miR-320, miR-374a	hsa-miR-330-3p, hsa-miR-483-5p	hsa-miR-548c-3p , hsa-miR-532-3p	[52]
Wander et al.	2017	36/80	7-23	qRT-PCR	cel-miR-39-3p	hsa-miR-155-5p, hsa-miR-21-3p, hsa-miR-146b-5p, hsa-miR-210-3p, hsa-miR-223-3p, hsa-miR-517-5p		[53]
Peng et al.	2018	11/12	24-28	qRT-PCR	RNU6	hsa-miR-137-3p		[54]
Tagoma et al.	2018	13/9	23-31	qRT-PCR, qRT-PCR arrays	cel-miR-39-3p	Let-7e-5p, let-7g-5p, hsa-miR-100-5p, hsa-miR-101-3p, hsa-miR-146a-5p, hsa-miR-18a-5p, hsa-miR-195-5p, hsa-miR-222-3p, hsa-miR-23b-3p, hsa-miR-30b-5p, hsa-miR-30c-5p, hsa-miR-30d-5p, hsa-miR-342-3p, hsa-miR-423-5p, hsa-miR-92a-3p		[55]

Balci et al.	2020	30/30	24–28	qRT-PCR using 96.96 Dynamic Array IFCs	global mean	hsa-miR-7-5p		[56]
Yoffe et al.	2020	23/20	9-12	qRT-PCR		hsa-miR-23a-3p, hsa-miR-223-3p		[57]
Wang et al.	2021	53/46	37-40	microarray, qRT-PCR	cel-miR-39-3p		hsa-miR-574-5p, hsa-miR-3135b	[58]
Yu et al.	2021	123/123	24-28	qRT-PCR	RNU6		hsa-miR-96-5p	[59]
Filardi et al.	2022	12/12	third trimester	qRT-PCR	RNU6, ath-miR-159a	hsa-miR-222-3p, hsa-miR-409-3p		[6]
Liu et al.	2022	30/30	39	qRT-PCR	RNU6		hsa-miR-143-3p	[60]
Leng et al.	2023	60/60	NA	qRT-PCR	RNU6		hsa-miR-138	[61]
EVs								
Nair et al.	2018	12/12	>37	miRNA sequencing, qRT-PCR	RNU6-2	hsa-miR-125a-3p, hsa-miR-224-5p, hsa-miR-584-5p, hsa-miR-186-5p, hsa-miR-22-3p, hsa-miR-99b-5p, hsa-miR-433-3p, hsa-miR-197-3p, hsa-miR423-3p	hsa-miR-208a-3p, hsa-miR-335-5p, hsa-miR-451a, hsa-miR-145-3p, hsa-miR-369-3p, hsa-miR-483-3p, hsa-miR-203a-3b, hsa-miR-574-3p, hsa-miR-144-3p, hsa-miR-6795-5p, hsa-miR-550a-3-3p, hsa-miR-411-5p, hsa-miR-140-3p	[62]
Gillet et al.	2019	23/46	6-15	qRT-PCR	cel-miR-39-3p	hsa-miR–122-5p, hsa-miR–132-3p, hsa-miR–1323-5p, hsa-miR–136-5p, hsa-miR–182-3p, hsa-miR–210-3p, hsa-miR–29a-3p, hsa-miR–29b-3p, hsa-miR–342-3p, hsa-miR-520h		[63]
Zhang et al.	2021	57/61	26-40	qRT-PCR	RNU6	hsa-miR-144-3p	hsa-miR-125b-5p	[64]
Ye et al.	2022	102/101	24-28	qRT-PCR	cel-miR-39–3p	hsa-miR-423–5p	hsa-miR-122–5p, hsa-miR-148a-3p, hsa-miR-192–5p, hsa-miR-99a-5p	[65]
Zhang et al.	2023	30/30	24-28	qRT-PCR	RNU6	hsa-miR-135a-5p		[66]
Stevanović et al.	2025	50/50	24-28	qRT-PCR	miR-191-5p	hsa-miR-146a-5p, hsa-miR-21-5p		[67]
Blood/PBMC								
He et al.	2017	20/20	NA	qRT-PCR	RNU6	hsa-miR-494-3p		[68]
Xu et al.	2017	25/25	NA	qRT-PCR	NA	hsa-miR-503		[69]
Stirm et al.	2018	15/15	24-32	qRT-PCR	RNU6	hsa-miRNA-340		[70]
Bian et al.	2022	30/30	23-27	qRT-PCR	RNU6	hsa-miR-296-3p		[71]
Hocaoglu et al.	2019	19/28	third trimester	qRT-PCR	RNU6		hsa-miR-21-3p	[72]
Hu et al.	2020	35/35	24-28	qRT-PCR	RNU6		hsa-miR-4646-5p hsa-miR-5196-5p	[73]
Zhang et al.	2020	30/30	NA	qRT-PCR	RNU6	hsa-miR-770-5p		[74]
Radojičić et al.	2022	42/34	24-30	qRT-PCR	RNU6-1	hsa-miR-27a-3p		[75]
Stevanović et al.	2025	50/50	24-28	qRT-PCR	miR-191-5p	hsa-miR-146a-5p, hsa-miR-21-5p		[67]

Abbreviations: TLDA - TaqMan Low-Density Array; qRT-PCR - quantitative Reverse Transcription Polymerase Chain Reaction; NGS - Next-Generation Sequencing; TAC - TaqMan Array Card; miRNA seq - miRNA sequencing; NA – not avalilable

LncRNAs

Long non-coding RNAs (lncRNAs) are a diverse class of transcripts longer than 200 nucleotides, that generally lack the protein-coding potential, but have critical regulatory roles in gene expression at multiple levels. They can act as molecular scaffolds, signals, guides, decoys, or sponges for miRNAs, thereby modulating various signaling pathways and cellular processes [84]. LncRNAs exhibit highly specific spatial and temporal expression patterns, often characteristic of certain cell types or developmental stages, suggesting their involvement in finely tuned physiological and pathological processes, due to their exceptionally regulated expression [85].

Table 2. Summary of dysregulated lncRNAs in GDM

Authors	Year	No. GDM/ Controls	Gestation week	Method	qPCR normalization	Up-regulated in GDM	Down-regulated in GDM	References
Serum								
Zhang et al.	2017	50/47	24-28	qRT-PCR	β-actin	*MALAT1*		[94]
Li et al.	2021	93/93	24-28	qRT-PCR	*GAPDH*	*XIST*		[42]
Su et al.	2021	99/98	25-29	qRT-PCR	*GAPDH*	*HOTAIR*		[95]
Huang	2024	70/50	24-28	qRT-PCR	*GAPDH*	*DLX6-AS1*		[96]
Chen et al.	2025	118/112	24-28	qRT-PCR	*GAPDH*	*HCG18*		[97]
Ma et al.	2025	128/125	24-28	qRT-PCR	*GAPDH*	*SNHG14*		[98]
Plasma								
Li et al.	2021	60/60	24–28	qRT-PCR	*GAPDH*		*SNHG17*	[99]
Li et al.	2021	2/3	24–40	qRT-PCR		*ERMP1, TSPAN32, MRPL38, RPL13P5*		[90]
Ran et al.	2021	52/164	0.9-2.8 months	qRT-PCR	18S rRNA	*SOX2OT*		[100]
Jiang et al.	2023	56/58	24–28	LncRNA microarray, qRT-PCR	*GAPDH*	*NONHSAT054669.2, ENST00000525337*		[101]
Jiang et al.	2023	27/45	12–14	LncRNA microarray, qRT-PCR	*GAPDH*	*NONHSAT054669.2, ENST00000525337*		[101]
Leng et al.	2023	60/60	NA	qRT-PCR	*GAPDH*	*UCA1*		[61]
Zhao et al.	2023	34/186	resampling every month (from 1M)	qRT-PCR	18S rRNA	*HCP5*		[102]
Blood/PBMC								
Zhang	2019		28	qRT-PCR	*GAPDH*	*MEG3*		[103]
Li et al.	2021	25/19	24-28	qRT-PCR		*RPL13P5*		[104]
Bian et al.	2022	30/30	23-27	qRT-PCR	*GAPDH*	*MEG8*		[105]
Stevanović et al.	2024	50/50	24-30	qRT-PCR	*GAPDH*		*MALAT1, H19*	[106]

Abbreviations: TLDA - TaqMan Low-Density Array; qRT-PCR - quantitative Reverse Transcription Polymerase Chain Reaction; NGS -Next-Generation Sequencing; TAC - TaqMan Array Card; miRNA seq-miRNA sequencing; NA – not available.

In the field of metabolic disorders such as GDM, lncRNAs are of particular interest due to their regulatory roles in glucose and lipid metabolism, insulin signaling, and inflammatory pathways [6]. Their dysregulation has been reported in various tissues relevant to diabetes pathophysiology, including pancreatic islets, adipose tissue, muscles, liver, and placenta, emphasizing their involvement in both maternal and fetal physiology [86-89]. In addition, lncRNAs are important regulators of inflammation and oxidative stress, both of which are hallmarks of GDM pathophysiology. Aberrant lncRNA expression can increase proinflammatory cytokine production and disrupt redox balance, thereby exacerbating metabolic dysfunction [7, 90].

The placenta, a key organ in pregnancy, is a critical site where lncRNAs exert their effects. Placenta-derived lncRNAs regulate trophoblast proliferation, invasion, and apoptosis, processes essential for proper placental development and nutrient transfer [3]. Dysregulated placental lncRNAs have been linked to abnormal angiogenesis and impaired fetal growth, reflecting their dual impact on maternal and fetal outcomes [91]. Furthermore, lncRNAs have been implicated in β-cell dysfunction, a contributing factor to glucose intolerance in GDM. Certain lncRNAs negatively affect insulin biosynthesis and secretion, while others interfere with β-cell survival under conditions of glycotoxicity and lipotoxicity [6]. Collectively, the dysregulation of lncRNAs in GDM points to their involvement in a network of interrelated processes: insulin resistance, inflammation, oxidative stress, placental dysfunction, and β-cell function impairment [3]. Due to a fact that changes in the expression of specific lncRNAs in placental tissue were followed by their up- or downregulation in body fluids, lncRNAs are not only recognized as mechanistic players in disease pathogenesis, but also as potential biomarkers for early diagnosis and prognosis, as well as possible therapeutic targets.

In contrast to miRNAs, for which numerous studies have reported aberrant expression in GDM [31-75], research on lncRNAs remains relatively rare or non-existing, especially for studies investigating the expression of lncRNAs derived from EVs. One of the main reasons for this scarcity is technical complexity associated with the isolation and analysis of lncRNAs from EVs, serum and plasma. These RNAs are typically expressed at low levels, may be fragmented, and require highly sensitive and standardized methods for reliable detection and quantification [92, 93]. Even when such studies are conducted, there are inconsistencies in the reported expression patterns across different investigations [42, 61, 90, 94-106]. For instance, *RPL13P5* is a rarely reported upregulated lncRNA within more than a one study on this topic. A notable example is also our study on the expression of *MALAT1*, for which we showed a downregulation in PBMCs obtained from GDM patients [106], while a previous study reported upregulation [94]. However, these two studies used different biological sources of lncRNAs for the analysis, while the sampling period during pregnancy also differed [94, 106]. Despite the contradictory results obtained in these two studies, both demonstrate a correlation between *MALAT1* and *H19*, which may indicate the presence of common stimuli inhibiting the expression of these lncRNAs. Another example of inconsistencies is our result which did not replicate previously observed upregulation of *MEG3* in blood samples of GDM patients [103]. However, this previous study [103] included merely 20 participants and analyzed whole blood instead of PBMCs, which were used in our recent study [106].

Apart from their diagnostic role, many of the reported dysregulated lncRNAs displayed a correlation between the level of expression and the values of glycemic or lipid status parameters [42, 61, 95-98, 101, 104, 106]. Additionally, a correlation was detected with the parameters of redox status and zinc concentration, all reported as altered in GDM and indicative of pregnancy outcome [106, 107]. Furthermore, several lncRNA hits from GDM studies demonstrated association with the adverse pregnancy outcomes [61, 98, 100].

CircRNA

CircRNAs are a class of endogenous non-coding RNA molecules characterized by a covalently closed loop structure without 5'-3' polarity and a poly-A tail. Typically, circRNAs originate from pre-mRNA transcripts through a "back-splicing" process and contain circularized exonic sequences [108]. Their high chemical stability, caused by specific structure, as well as the tissue specificity, represent desirable features of potentially reliable disease biomarkers. However, these ncRNAs are often expressed at low level, which associate with low abundance in biological samples, while their accurate and sensitive quantification has proved challenging due to their circular structure, lack of poly-A tail, homology with parental mRNA and technical limitations of quantification methods [109].

The most extensively investigated mode of action of circRNAs is their sponging activity toward miRNAs. However, regarding biomarker significance, this type of non-coding RNA molecules is much less analyzed, compared to circulatory miRNAs. Therefore, there is a limited data on the functional significance and biomarker properties of circRNA molecules in GDM [110]. In a relatively recent study, which was multicentric and included a relatively large group of pregnant women in different stages of pregnancy, hsa_circ_0031560 and hsa_circ_0000793 were identified and validated as potentially reliable early biomarkers for GDM. Serum-derived circRNAs from this study also proved predictive for the adverse pregnancy outcome [111]. In an earlier study, plasma samples of GDM patients and matched controls were used for circRNA profiling by NGS, which demonstrated an upregulation of hsa_circ_0008285 and downregulation of hsa_circ_0001173 in

GDM [112]. Furthermore, the expression of these two circRNAs correlated with the values of various lipid and glycemic status parameters, while the knockout of hsa_circ_0008285 counteracted the increase in proliferation, migration and invasion induced by hyperglycemia in trophoblast cell line HTR-8/SVneo [112]. Previously, this circRNA (also known as circCDYL) was reported as dysregulated in different malignant diseases and cardiovascular disorders [113]. Another study, focused on plasma EV-derived circRNAs, identified hsa_circRNA_0039480 and hsa_circRNA_0026497 as potential GDM biomarkers through microarray analysis [114]. Hsa_circRNA_0039480 demonstrated significant diagnostic significance even in the first trimester of pregnancy, which was enhanced by combining with hsa_circRNA_0026497 in a novel potentially valuable early diagnostic panel [114]. A microarray profiling also identified hsa_circRNA_102893 as a downregulated circRNA in plasma samples of GDM patients, with a good diagnostic significance in early pregnancy [115].

Patients of non-Asian origin were rarely included in circRNA-oriented GDM biomarker studies. A relatively recent study in Polish population identified over 50 transcripts (both linear and circular) corresponding to a large panel of circRNA-producing genes which are dysregulated in GDM plasma samples. The relative expression of hsa_circ_0002268 (circPHACTR1) was elevated in GDM patients, compared to controls [116].

Apart from studies that relied on circRNA profiling by high-throughput methods, several candidate circRNA-based GDM biomarker studies were conducted to date. CircVEGFC-focused analysis revealed an upregulation of this circRNA in GDM, with higher values in plasma corresponding to increased incidence rates of fetal malformation and hypertension [117]. Similarly, an upregulation of circACTR2 in GDM plasma samples was determined in a candidate-based study, associated with higher rates of GDM-related adverse events [118]. One of the promising candidates for GDM biomarkers among circRNAs, hsa_circRNA_0054633, was initially determined as dysregulated in type 2 diabetes (T2DM). The supposed upregulation of this circRNA in serum samples was confirmed in a GDM study, while the expression level correlated with the values of glycemic status parameters [119] An increased expression in GDM vs. controls was determined for second and third trimester samples, as well as for placental tissue [119]. Diagnostic significance as early biomarker of GDM was not evaluated for this circRNA. However, potential significance for diabetic disorders is supported by the findings suggesting that hsa_circRNA_0054633 may serve as an indicator of the clinical characteristics and the effect of insulin therapy in T2DM [120, 121].

Conclusion

Although a multitude of evidence has suggested a role of different species of circulatory ncRNAs as novel potentially valuable biomarkers of GDM, results are still inconsistent and require carefully planned validation. The main objective is to identify clinically useful early biomarkers of GDM, enabling more precise risk stratification. The key strengths of ncRNA as potential biomarkers for GDM are their chemical stability in body fluids, minimal invasiveness of sampling procedure and the reliability of quantification procedures.

Acknowledgement

Previous original articles cited within this publication and authored by the team of researchers from the University of Belgrade – Institute for the Application of Nuclear Energy originated from studies supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Agreement nos. 451-03-68/2022-14/200019, 451-03-47/2023-01/200019, 451-03-66/2024-03/200019 and 451-03-136/2025-03/200019).

We owe our gratitude to the members of our research department who participated in research on the topic of GDM biomarkers, as well as to our collaborators from the Clinic for Gynecology and Obstetrics "Narodni Front" and University of Belgrade – Faculty of Medicine.

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MicroRNAs in Oral Cancer: Oncogenic Drivers, Tumor Suppressors, and Clinical Implications

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Abstract

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity, characterized by aggressive local invasion and high metastatic potential. Due to its often late diagnosis and poor prognosis, there is a critical need to refine early detection methods and develop novel therapeutic strategies to improve patient outcomes.

MicroRNAs (miRNAs) have emerged as pivotal regulators of gene expression, exerting profound effects on tumor initiation, progression, and therapeutic response in oral squamous cell carcinoma (OSCC). Their dual role as oncogenic miRNAs (oncomiRs), and tumor-suppressor miRNAs, highlights the complex regulatory networks driving oral carcinogenesis. OncomiRs promote proliferation, invasion, and immune evasion by modulating cancer pathways, while tumor-suppressive miRNAs counteract these effects by restoring apoptosis, inhibiting epithelial–mesenchymal transition, and reducing metastatic potential.

Recent studies underscore their potential as non-invasive diagnostic and prognostic biomarkers, detectable in saliva, serum, and tissue, and as therapeutic targets through anti-miR or miRNA mimic strategies.

This review synthesizes current evidence on the molecular mechanisms, clinical implications, and therapeutic opportunities of miRNA dysregulation in OSCC, providing a framework for integrating miRNA-based strategies into personalized oral cancer management.

Key words: oral squamous cell carcinoma, microRNA, exosomes, tumor microenvironment

MikroRNK u oralnom karcinomu: onkogeni, tumor supresori i kliničke implikacije

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Apstrakt

Oralni planocelularni karcinom (OPK) predstavlja najčešći malignitet usne duplje, poznat po izraženoj lokalnoj invazivnosti i visokom metastatskom potencijalu. Usled česte kasne dijagnoze i nepovoljne prognoze, postoji imperativ za unapređenje metoda njegovog ranog otkrivanja i za razvoj novih terapijskih pristupa kako bi se poboljšali ishodi lečenja kod obolelih.

U tom kontekstu, mikroRNK (miRNK) su se izdvojile kao ključni regulatori ekspresije gena, sa značajnim uticajem na inicijaciju tumora, njegovu progresiju i odgovor na terapiju kod pacijenata sa OPK. Njihova dvostruka funkcija, kao onkogene mikroRNK (onkomiR) i tumorsupresorske mikroRNK, ukazuje na složene regulatorne mreže koje doprinose patogenezi oralnog karcinoma. OnkomiR promovišu proliferaciju, invaziju i izbegavanje imunskog nadzora, preko modulacije različitih onkogenih signalizacionih kaskada, dok tumorsupresorske mikroRNK deluju suprotno: podstiču apoptozu, inhibiraju epitelno-mezenhimalnu tranziciju (EMT) i smanjuju sposobnost tumora za metastaziranje.

Najnovija istraživanja ističu mikroRNK kao neinvazivne dijagnostičke i prognostičke biomarkere, koji se mogu detektovati u pljuvački, serumu i tumorskom tkivu. Pored toga, sve je veće interesovanje za njihovu upotrebu kao terapijskih meta, putem strategija koje uključuju anti-miR molekule ili miRNK mimike, sa ciljem modulacije njihove patološke ekspresije.

Ovaj pregledni rad sumira novija saznanja o molekularnim mehanizmima, kliničkim implikacijama i terapijskom potencijalu deregulacije mikroRNK u OPK, nudeći osnovu za integraciju miRNK-zasnovanih pristupa u personalizovano lečenje oralnog karcinoma.

Ključne reči: oralni planocelularni karcinom, mikroRNK, egzozomi, tumorska mikrosredina

1. Introduction

Oral cancer, predominantly oral squamous cell carcinoma (OSCC), accounts for over 90% of malignancies affecting the oral cavity (1). Globally, an estimated 377,000 new cases of oral cancer and 177,000 related deaths were reported in 2020, according to GLOBOCAN data, positioning oral cancer among the leading causes of mortality from malignant diseases (2). The highest incidence rates are observed in South and Southeast Asia, parts of Latin America, and Eastern Europe, with Serbia occupying a high position in European morbidity and mortality ranking (3,4). Projections further suggest a nearly 30% increase in the incidence of head and neck cancers by 2030, highlighting the growing global burden (5).

OSCC arises from multifactorial etiologies, including tobacco use, excessive alcohol consumption, betel quid (BQ) chewing, human papillomavirus (HPV) infection, and nutritional deficiencies (6). Among the most significant risk factors for oral cancer are tobacco use and alcohol consumption, which exert a well-established synergistic effect on carcinogenesis (7). Human papillomavirus (HPV)—particularly HPV-16—is also linked to oropharyngeal cancers; additional risk factors include poor oral hygiene, chronic irritation, dietary deficiencies, etc. Chronic exposure to these factors may promote carcinogenesis, progression, and metastasis by inducing genetic mutations and dysregulation of the tumor microenvironment (8). Oral cancer predominantly affects individuals aged 50 to 70 years, although incidence in younger patients—often without classical risk factors—is increasing (9). Men are typically affected two to three times more frequently than women, but this ratio is decreasing (10). In women, daily alcohol intake markedly elevates the risk of oral cancer, primarily due to reduced gastric alcohol metabolism, which results in higher blood alcohol levels and greater accumulation of acetaldehyde in oral tissues (11). Recently, oral dysbiosis, i.e. a disbalance between pathogenic and commensal bacterial communities within the oral cavity, has also emerged as a contributing factor to oral cancer (12) (Fig. 1).

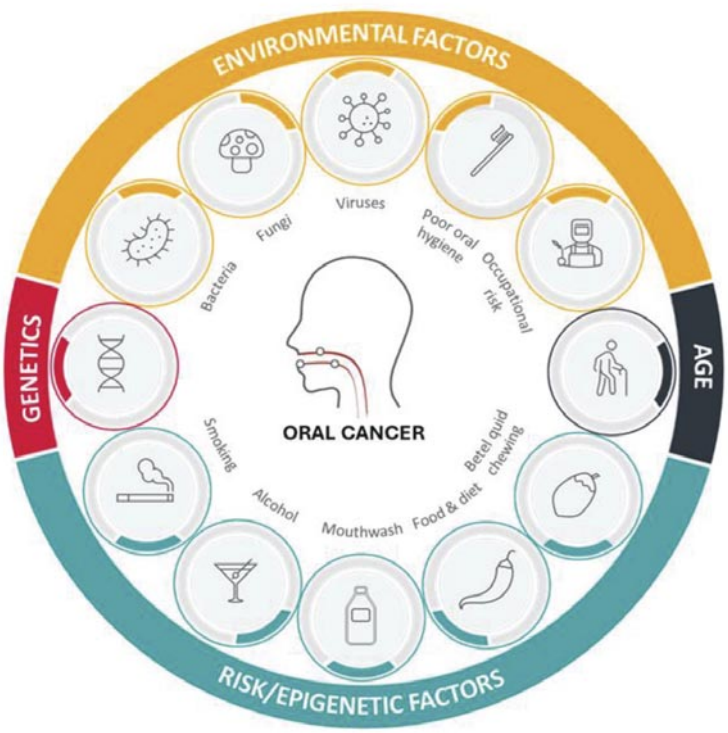

Figure 1. Schematic representation of etiological factors involved in oral cancer pathogenesis. Published in (20).

Despite significant advances in treatment modalities, oral cancer remains an aggressive malignancy characterized by frequent local recurrences and regional or distant metastases. The five-year survival rate has stagnated at approximately 50% over the past two decades, reflecting only minimal improvement in patient outcomes (13). In addition to poor survival statistics, patients often face substantial physical and psychological sequelae as a result of surgical resection and/or radiotherapy. The unfavorable prognosis is largely attributable to late-stage diagnosis, with more than 60% of oral carcinomas identified at advanced stages, while achieving earlier and more reliable detection remains one of the greatest challenges in head and neck oncology (14).

Substantial research efforts have been directed toward unraveling the fundamental mechanisms driving the onset and progression of oral cancer, reflecting the complexity of its pathogenesis. The development of carcinoma is a complex, multifactorial process that begins with the transformation of a subset of normal keratinocytes, which, under the influence of epigenetic processes, biological factors, or cytogenetic alterations, undergo changes in the cell cycle, DNA repair mechanisms, cell differentiation, and apoptosis, ultimately leading to malignant neoplastic transformation of keratinocytes (15). A large body of work has focused on genetic and epigenetic alterations, including also mutations in tumor suppressor genes and oncogenes, chromosomal instability, and aberrant DNA methylation patterns, all of which contribute to the stepwise transformation from oral potentially malignant disorders (OPMDs) to invasive carcinoma (16). The molecular assessment of surgical margins has also emerged as a critical area, as histologically negative but molecularly altered margins are strongly associated with local recurrence and poor clinical outcomes (17).

Another important line of investigation has examined the influence of oncogenic microorganisms, particularly periodontal pathogens and oncogenic viruses such as HPV and EBV, which promote chronic inflammation, immune evasion, and direct modulation of oncogenic signaling pathways. In parallel, the concept of cancer stem cells (CSCs) has gained increasing recognition, as these subpopulations within tumors exhibit self-renewal capacity, resistance to therapy, and are believed to act as key initiators of tumorigenesis and recurrence. Together, these converging fields highlight the multifactorial nature of oral cancer development and underscore the importance of integrated approaches in both basic and translational research.

Within this framework, microRNAs (miRNAs) have emerged as crucial regulators linking genetic, epigenetic, microbial, and stem cell–related mechanisms, positioning them as both promising biomarkers and potential therapeutic targets in oral carcinogenesis.

2. MicroRNAs

MicroRNAs (miRNAs) are a class of small, non-coding RNAs, typically 18–25 nucleotides in length, that regulate gene expression at the post-transcriptional level by binding to complementary sequences within target mRNAs, leading to translational repression or degradation (18) . Through this fine-tuning of gene expression, miRNAs play pivotal roles in virtually all cellular processes, including proliferation, differentiation, apoptosis, and stress responses (19).

In cancer, dysregulation of miRNA expression has been widely documented, with certain miRNAs acting as oncogenic drivers (oncomiRs) by silencing tumor suppressor genes, while others function as tumor suppressor miRNAs by inhibiting oncogenes or pathways promoting malignant transformation (20). Abnormal miRNA expression can drive cancer progression, as miRNAs may act either as oncomiRs or as tumor suppressors. Such dysregulation may result from gene amplification or deletion, epigenetic modifications,

altered transcription factor regulation, or disturbances in miRNA biogenesis and processing, ultimately contributing to malignant transformation and therapy resistance (Fig.2) (21).

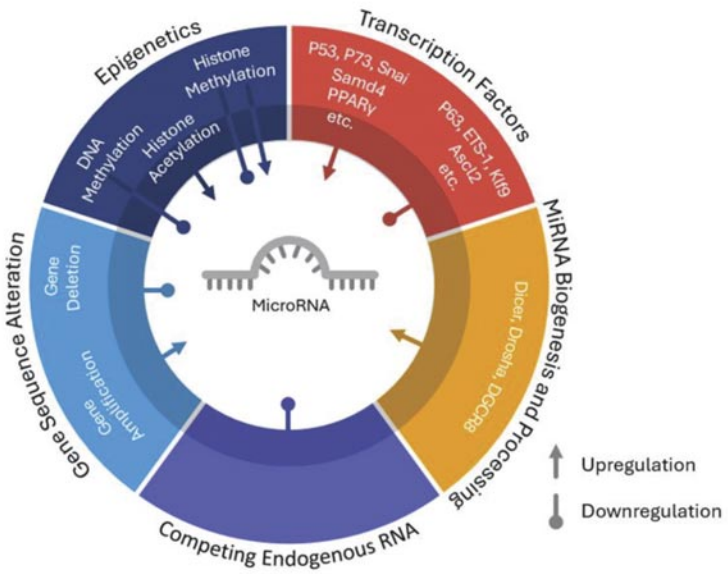

Figure 2. Schematic reprsentation of mechanisms of microRNA regulation

Their unique stability in body fluids, combined with their context-dependent roles in tumor initiation and progression, has made them highly attractive both as diagnostic and prognostic biomarkers and as therapeutic targets (22). In the context of oral cancer, miRNAs represent a converging point for multiple pathogenic mechanisms, bridging genetic alterations, epigenetic dysregulation, microbial influences, and cancer stem cell biology.

Aberrant expression of miRNAs has been well documented in OSCC and plays a pivotal role in tumor initiation, progression, and therapeutic resistance (Fig. 3).

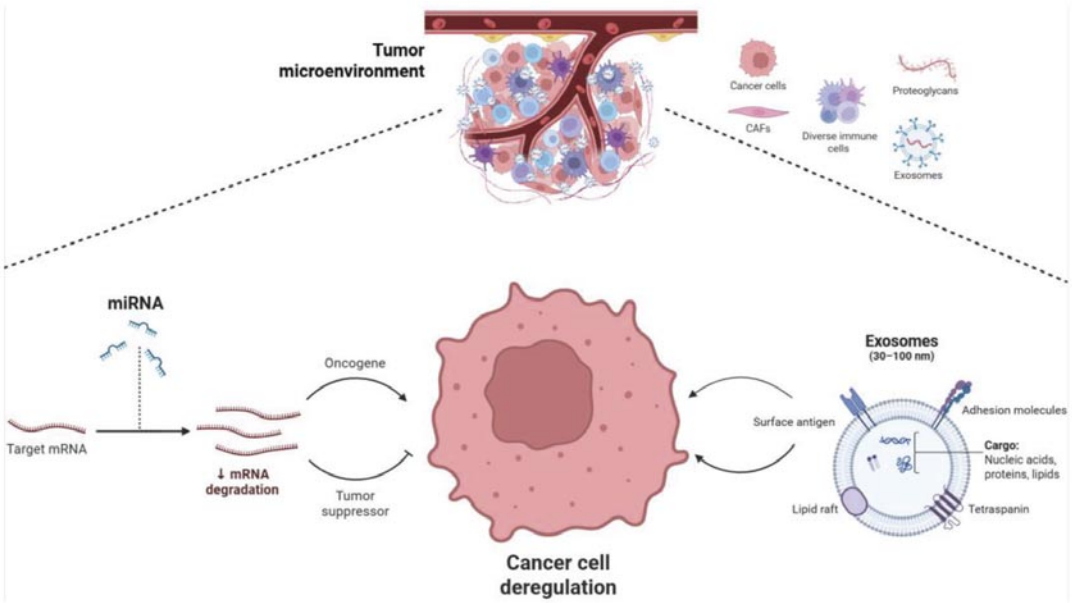

Figure 3. Schematic representation of cancer cell deregulation via different miRNAs

2.1. Oncogenic miRNAs (oncomiRs)

OncomiRs are a class of microRNAs (miRNAs) that contribute to tumor initiation and progression by silencing tumor suppressor genes (TSG) and activating oncogenic signaling pathways. In oral cancer, several oncomiRs have been identified as critical molecular drivers (Table 1). Among them, miR-21 is one of the most consistently upregulated across various malignancies, including OSCC (23). It directly targets tumor suppressors such as PTEN and PDCD4, thereby activating the PI3K/AKT signaling pathway and promoting cell proliferation, survival, and resistance to apoptosis (24). MiR-21 is also a key regulator of cancer stem cell (CSC) properties. Its silencing in $CD44^+$ CSC populations significantly downregulates stemness-associated markers like OCT4, SOX2, and NANOG (25). Moreover, co-treatment of miR-21-silenced $CD44^+$ cells with BET protein inhibitors (iBETs) has been shown to suppress Cyclin D1 and induce Caspase-3, resulting in G1 cell cycle arrest and apoptosis activation (26). Additionally, miRNA-21 has been proposed as a potential biomarker for malignancies, detectable in body fluids such as blood, sputum, cerebrospinal fluid, and feces, thus supporting its use in non-invasive cancer diagnostics (27).

Table 1. miRNA–target gene Interactions and their mechanistic roles in cancer-related pathways

miRNA	TARGET GENE	REGULATION	MECHANISM	REFERENCE
miR-21	CASP3	Inhibition	Apoptosis	(51,52)
miR-21	BAX	Inhibition	Pro-apoptotic	(52,53)
miR-21	CCND1	Activation	PI3K/Akt signaling pathway	(54)
miR-21	BCL-2	Activation	Cell survival	(51)
miR-21	CTNNB1	Activation	Wnt signaling pathway	(55)
miR-21	OCT4, SOX2, NANOG	Activation	Pluripotency	(56,57)
miR-31	CCND1	Inhibition	Cell cycle	(58)
miR-31	CTNNB1	Activation	Wnt signaling pathway	(59)
miR-133	CASP3	Activation	Apoptosis	(60)
miR-133	CCND1	Inhibition	Cell cycle	(61)
miR-133	BCL-2	Inhibition	Apoptosis	(62)
miR-155	CDC73	Activation	Proliferation	(63)
miR-155	FOXO3a	Activation	Invasion	(64)
miR-221/222	PTEN	Inhibition	Apoptosis	(65)
miR-221	PIK3R1	Activation	Angiogenesis	(66)
miR-99	mTOR	Inhibition	Proliferation	(67)

Another prominent oncomiR in OSCC is miR-31, which is frequently upregulated and orchestrates a complex regulatory network involving multiple target genes—RhoA, FIH, ACOX1, VEGF, SIRT3, etc.—and signaling pathways such as the EGF-AKT axis, ERK-MMP9 cascade, and Wnt signaling (28). Epithelial-to-mesenchymal transition (EMT), is considered a hallmark of oral cancer where epithelial cells lose their characteristics and gain mesenchymal ones (29). MiR-31 facilitates EMT and invasion, particularly through modulation of FIH and RhoA (30,31).

MiR-155 plays a dual role in immune evasion and chronic inflammation. It has been proposed as a predictive biomarker for neoadjuvant chemotherapy efficacy in OSCC (32,33). By downregulating SOCS1, miR-155 enhances STAT3 signaling, thereby promoting angiogenesis and metastasis (34,35).

The miR-221/222 cluster is frequently overexpressed in OSCC tissues relative to normal oral mucosa and is associated with enhanced tumor cell proliferation, migration, and invasion (36). Specifically, miR-222 correlates with advanced clinical stage, poorer overall survival, and more aggressive tumor phenotypes. This effect is mediated through direct targeting of CDKN1B, whose overexpression inhibits OSCC cell proliferation and enhances apoptosis (37). Additional targets of this cluster include PTEN and PUMA; suppression of miR-221/222 restores their function, leading to increased apoptosis and decreased invasiveness (38,39).

Several other oncomiRs are emerging as important regulators of oral epithelial cell fate. MiR-27a promotes OSCC cell growth and metastasis by targeting Prohibitin; miR-93 enhances angiogenesis and proliferation via modulation of the PTEN/PI3K/AKT pathway; and miR-196 contributes to metastasis and proliferation through repression of tumor suppressors such as Annexin A1. These findings highlight the diverse and critical roles of oncomiRs in OSCC pathogenesis and highlight their potential as therapeutic targets and prognostic biomarkers.

2.2. Tumor suppressor microRNAs

Tumor suppressor microRNAs (ts-miRNAs) act as negative regulators of oncogenesis by targeting oncogenes and inhibiting signaling pathways involved in malignant transformation (Table 1). Functionally, they oppose the actions of oncomiRs. In oral cancer, the downregulation of tumor suppressor-miRNAs is strongly associated with tumor initiation, progression, metastasis, and resistance to conventional therapies. Among the most well-studied tumor suppressor miRNAs OSCC are miR-34a**,** miR-99a**,** miR-125b**,** miR-133**,** miR-195, and members of the miR-200 family. These miRNAs play essential roles in regulating cell cycle progression, cancer stemness, EMT, and invasive behavior.

MiR-34a, a direct transcriptional target of *p53*, exerts its tumor-suppressive effects by inhibiting key oncogenes such as *BCL2*, *CDK6*, and *MET*, resulting in apoptosis induction, cell cycle arrest, and reduced metastatic potential. Loss of miR-34a expression in OSCC is frequently associated with disease progression and poor prognosis. Mechanistically, miR-34a suppresses the Axl/Akt/GSK-3β signaling pathway (40). It also inhibits tumor progression by repressing the interleukin-6 receptor (*IL6R*) (41).

MiR-99a and miR-125b both inhibit the *mTOR* signaling pathway, which is central to cell growth, metabolism, and survival. Reduced expression of miR-99a correlates with enhanced proliferation and decreased radiosensitivity in OSCC. A number of functional studies suggested that the inhibition of AKT/mTOR signalling pathways by members of miR-99 family is exerted through insulin-like growth factor 1 receptor (IGF1R) targeting (38). Loss of miR-125b facilitates tumor progression by failing to suppress ERBB2 and components of the MAPK pathway (42).

MiR-133 downregulation in OSCC, specifically in CSCs, is also a common characteristic of oral cancer and various cancer cell lines, while its overexpression has been shown to inhibit the proliferation, viability, and invasion of cancer cells (23,43,44). Functionally, miR-133 inhibits key oncogenic pathways by targeting genes involved in proliferation (eg. *EGFR)* (45), invasion and migration (*eg. FSCN1*) (46). MiR-133 also regulates EMT through targeting PDE1C (47), enhances chemoresistance—particularly to cisplatin—and reduces apoptosis, via CXCR4 (48). Restoration of miR-133 has been shown to suppress tumor growth, increase radiosensitivity, and reverse invasive phenotypes.

The miR-200 family (e.g., miR-200a/b/c, miR-141, miR-429) plays a critical role in maintaining the epithelial phenotype by directly targeting *ZEB1* and *ZEB2*, two key transcription factors driving EMT. Downregulation of these miRNAs leads to increased invasiveness and metastasis in oral cancer (49,50).

Taken together, the downregulation of tumor suppressor miRNAs in oral cancer contributes to uncontrolled proliferation, increased metastatic potential, EMT induction, and resistance to therapy. Their well-characterized roles in tumor suppression make them promising candidates for use as early diagnostic biomarkers and as targets for therapeutic restoration strategies.

3. MicroRNAs in CSCs

Fang and colleagues (2012) were among the first to investigate the relationship between microRNAs and colorectal cancer stem cells. Using surface markers, they isolated CD133+/CD44+ (CSCs) and CD133–/CD44– (non-stem) cell populations from the human adenocarcinoma cell line CW1116 and identified significant differences in microRNA expression. A total of 62 microRNAs were differentially expressed, with half being upregulated and the other half downregulated in CSCs compared to non-stem cells (51). In colorectal cancer stem cells, dysregulation of miR-21 (upregulated) and miR-145 (downregulated) was observed, influencing the expression of CD44, β-catenin, and SOX2 (52). Elevated expression of miR-19a/19b in lung cancer stem cells plays a critical role in maintaining stemness characteristics by regulating the Wnt/β-catenin signaling pathway (53). In glioblastoma CSCs, increased miR-21 expression has been shown to directly affect apoptosis and proliferation by reducing the expression of the FASLG protein (54).

In oral cancer stem cells, little is known about microRNA expression. The association of miR-21 with stemness markers CD44+ and Nanog–Stat-3 has been documented, and this interaction is considered a potential cause of resistance to existing therapies (55). Conversely, miR-145 acts as a tumor suppressor in oral cancer stem cells by directly targeting SOX9, thereby reducing sphere-forming ability and invasiveness (56). Moreover, stemness markers OCT4, SOX2, and NANOG in CSCs may be regulated by miR-200c, which functions as a tumor suppressor by repressing stemness in both head and neck cancers and breast cancer (57).

4. MicroRNAs and exosomes

In oral cancer, exosomes have emerged as crucial vehicles for the horizontal transfer of functional microRNAs (miRNAs) that modulate gene expression in recipient cells. These nanoscale extracellular vesicles (30–150 nm) are actively secreted by various cell types, including tumor cells, and serve as key mediators of intercellular communication within the tumor microenvironment. They shuttle proteins, lipids, and nucleic acids—including miRNAs. Tumor-derived exosomal miRNAs participate in multiple cancer-related processes such as proliferation, migration, invasion, angiogenesis, EMT, immune modulation, and resistance to chemotherapy and radiotherapy. They are also perceived as important players in metastasis (58). Notably, the miRNA cargo within exosomes is often enriched compared to that of their parent cells, highlighting selective packaging mechanisms (59).

The regulation of EMT by exosomal miRNAs involves repression of epithelial markers and induction of mesenchymal markers, promoting metastatic potential (58). Hypoxia, a hallmark of OSCC tumors, upregulates miR-21 expression in both cells and their exosomes, linking microenvironmental stress to aggressive tumor phenotypes via cell-to-cell communication. For instance, exosomal miR-21 derived from hypoxic OSCC cells increases mesenchymal markers Snail and Vimentin, while decreasing the epithelial marker E-cadherin in recipient normoxic cells, thereby driving a pro-metastatic phenotype (49). Similarly, exosomal miR-200 family members modulate EMT dynamics through direct targeting of the EMT-inducers ZEB1 and ZEB2 (60).

Moreover, exosomes from cisplatin-resistant OSCC cell lines can transfer miR-21 to cisplatin-sensitive parental cells, leading to downregulation of tumor suppressors PTEN and PDCD4, impaired DNA damage response, and enhanced drug resistance both *in vitro* and *in vivo* (61). Exosomal miRNAs further alter signaling pathways in recipient cells by targeting tumor suppressors. In particular, exosomes enriched with oncomiRs like miR-155 and miR-21 promote OSCC cell proliferation and invasion through suppression of TSG PTEN and Bcl-6, while treatment with miR-126-rich exosomes inhibits oncogenic behaviors and oncogene EGFL7 expression in OSCC cells (59). In addition, functional studies revealed that treatment of normal fibroblasts with OSCC-derived exosomes containing high levels of miR-21 and miR-31 led to significant upregulation of oncogenic signaling components including PIK3CA, AKT, NOTCH, and HES1 (24).

5. Impact of exosomal miRNAs on Tumor Microenvironment (TME)

The tumor microenvironment (TME) in OSCC is a complex ecosystem composed of cancer cells, stromal fibroblasts, endothelial cells, immune cells, and extracellular matrix components. Crosstalk within this microenvironment is increasingly recognized as a critical driver of tumor progression, therapeutic resistance, and metastasis. A key mediator of this intercellular communication are the exosomes with their cargo, including miRNAs, that are shuttled between cells. These exosomal miRNAs act as potent regulators of gene expression in recipient cells, thereby reprogramming the TME to favor tumor growth and immune evasion. For instance, hypoxic tumor-derived exosomal miR-21 induces cancer-associated fibroblast activation (CAF) to promote OSCC metastasis, i.e. normal fibroblast are transformed into CAFs via miR-21 targeting YOD1 (62). Enhanced activity of CSC derived extracellular vesicles is also positively correlated with upregulated β-catenin, PI3K, STAT3, mTOR and TGF-β1 (63). Similarly, exosomal miR-221/222 can modulate endothelial cells by suppressing PIK3R1, thereby stimulating angiogenesis (64). Tumor-derived exosomal miR-31 has been implicated in remodeling the extracellular matrix and enhancing invasive potential.

Conversely, stromal and immune cells within the TME also release exosomes that influence tumor cells. For instance, exosomal miRNAs (miRNA-23a) derived from tumor associated macrophage (TAM) induced tumor cell migration and invasion, as well as EMT in OSCC, with increased mesenchymal (MMP-2, MMP-9) and decreased epithelial markers (E-calmodulin) in tumor cells. It was also determined that PTEN was the miRNA-23a target (65). CSC-derived exosomes enriched in miR-21, STAT3, β-catenin transform normal gingival fibroblasts into CAFs, and enhance migration and drug resistance (63).

6. MicroRNAs from a clinical perspective

MicroRNAs (miRNAs) hold significant clinical promise in oral cancer as non-invasive biomarkers for diagnosis, prognosis, and as potential therapeutic targets. For diagnosis, multiple studies have demonstrated that circulating or salivary miRNA signatures can effectively distinguish oral cancer patients from healthy or premalignant individuals with high sensitivity and specificity. For instance, an 8-miRNA salivary panel (miR-7-5p, miR-10b-5p, miR-182-5p, miR-215-5p, miR-431-5p, miR-486-3p, miR-3614-5p, and miR-4707-3p) achieved an AUC of \~0.95 for oral cancer vs. controls and \~0.91 for oral cancer vs. OPMD (66).

Prognostically, elevated miR-21 levels in tissue or circulation correlate with larger tumor size, local invasion, lymph node metastasis, and poor survival, with meta-analyses confirming its strong predictive value (67). Moreover, salivary profiles of OSCC patients responding well to neoadjuvant chemotherapy (NACT) showed lower miR-21 and miR-155 and higher miR-375 levels compared to resistant cases, supporting their utility as prognostic markers (33). Meta-analyses further highlight miR-21, miR-155, and miR-375 as clini-

cally relevant biomarkers across HNSCC, including OSCC (68). Other circulating markers, such as miR-483-5p (poor prognosis, late-stage disease, and nodal metastasis (69)) and miR-9 (low levels linked to poor overall and disease-free survival (70)), have also been reported. Similarly, miR-200b-3p emerged as an independent prognostic predictor in multivariate analyses. Exosomal miRNAs add another layer of prognostic insight: in OSCC, exosomal miR-21 and miR-155 were upregulated, while miR-126 was downregulated in both cells and patient serum. Notably, reduced exosomal miR-126 was associated with late-stage disease and poorer survival (59).

Therapeutically, miRNA-based interventions are still at an experimental stage, but both replacement strategies (miRNA mimics) and silencing approaches (anti-miRs) are being explored. Anti-miR-21 oligonucleotides and restoration of tumor-suppressive miRNAs such as miR-375 have demonstrated efficacy in preclinical OSCC models by reducing proliferation, inducing apoptosis, and enhancing radiosensitivity (71). Differentiation-based approaches in CSCs offer another innovative strategy: induction of CSC differentiation was shown to downregulate oncogenic miR-21 while restoring tumor-suppressive miRNAs such as miR-133 and miR-491, suggesting a promising therapeutic avenue (43). Natural compounds may also influence miRNA biology; for example, Ovatodiolide suppressed OSCC progression by reducing miR-21 cargo in extracellular vesicles (EVs) secreted by CSCs, thereby impairing the transformation of gingival fibroblasts into cancer-associated fibroblasts and disrupting EV–tumor microenvironment (TME) interactions (63).

7. Conclusion

Altogether, the body of evidence strongly supports the central role of miRNAs in the pathogenesis and clinical course of OSCC. Their dysregulation drives key oncogenic processes such as proliferation, invasion, metastasis, therapeutic resistance, and tumor–microenvironment crosstalk, while their presence in easily accessible biofluids makes them attractive non-invasive biomarkers. Salivary and circulating miRNA panels have already shown high diagnostic accuracy and hold promise as prognostic indicators of survival and treatment response.

Beyond their role as biomarkers, functional studies highlight miRNAs as potential therapeutic targets, with anti-miR and miRNA mimic strategies demonstrating efficacy in preclinical OSCC models. Approaches such as cancer stem cell differentiation and modulation of extracellular vesicle cargo further underscore the versatility of miRNA-based interventions in reshaping the tumor microenvironment. However, despite rapid advances, the translation of miRNA research into routine clinical practice remains hampered by challenges such as delivery systems, stability and off-target effects.

In this context, miRNAs can be envisioned as the next generation of tools in precision oncology for oral cancer, integrating diagnostic, prognostic, and therapeutic applications. Continued interdisciplinary research is essential to bridge experimental findings with clinical implementation, ultimately improving outcomes for patients with this aggressive malignancy.

Acknowledgments

This research was funded by the Science Fund of the Republic of Serbia, #GRANT NO 7750038, ORAL CANCER—NEW APPROACHES IN PREVENTION, CONTROL, AND POST-OPERATIVE REGENERATION—AN IN VITRO STUDY—ORCA–PCR.

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PERSONALIZOVANA MEDICINA

PERSONALIZED MEDICINE

Personalized medicine: looking back and forward

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Abstract

Although medicine always aimed to be personalized, true implementation of personalized medicine in health care practice has started recently. The term "personalized medicine" has been first mentioned in 1999. It was in the era of evidence-based medicine which promoted the concept of medicine dedicated to an average patient. Fascinating development of molecular biology and bioinformatics shifted medicine to more personalized approach, which tailors medical diagnosis and treatments to an individual's unique biological characteristics. Nowadays, modern medicine, precision medicine, is based on availability of great amount of biological data, and it is in line with the biomedical model of health. There are four cornerstones of precision medicine: genome-based diagnostics, pharmacogenomics, specific molecular-targeted, gene and cellular therapy and predictive genomics. Enormous progress in the field of each of these cornerstones has strongly contributed to the great achievements of modern medicine. The door for precision medicine is wide-open in the everyday clinical practice and there is no doubt that it has already brought great benefits to patients. The future of medicine lies in personalized medicine, a more comprehensive approach to health. It considers not only biological, but also environmental, socio-economic and psychological factors, and represents the biopsychosocial model of health. Personalized medicine is still a great challenge for medical researchers and practitioners as well as for healthcare systems, but it is expected that it will be fully implemented in medical practice worldwide in 21st century.

Keywords: personalized medicine, precision medicine, genome-based diagnostics, pharmacogenomics, molecular targeted therapy, gene therapy, cellular therapy, predictive genomics

Personalizovana medicina: prošlost, sadašnjost i budućnost

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Apstrakt

Iako je medicina uvek težila personalizaciji, prava primena personalizovane medicine u zdravstvenoj praksi počela je nedavno. Termin „personalizovana medicina" prvi put je pomenut 1999. godine. Bilo je to u eri medicine zasnovane na dokazima koja je promovisala koncept medicine posvećene prosečnom pacijentu. Fascinantan razvoj molekularne biologije i bioinformatike pomerio je medicinu ka personalizovanijem pristupu, koji prilagođava medicinsku dijagnozu i tretmane jedinstvenim biološkim karakteristikama pojedinca. Danas se savremena medicina, precizna medicina, zasniva na dostupnosti velike količine bioloških podataka i predstavlja biomedicinski model zdravlja. Postoje četiri stuba precizne medicine: dijagnostika zasnovana na genomu, farmakogenomika, specifična molekularna, genska i ćelijska terapija i prediktivna genomika. Ogroman napredak u svakoj od ovih oblasti značajno je doprineo velikim dostignućima moderne medicine. Precizna medicina je prisutna u svakodnevnoj kliničkoj praksi i nema sumnje da je već donela velike koristi pacijentima. Budućnost medicine leži u personalizovanoj medicini, sveobuhvatnijem pristupu zdravlju. Ona uzima u obzir ne samo biološke, već i ekološke, socio-ekonomske i psihološke faktore i predstavlja biopsihosocijalni model zdravlja. Personalizovana medicina je i dalje veliki izazov za istraživače i lekare, kao i za zdravstvene sisteme, ali se očekuje da će biti u potpunosti implementirana u medicinskoj praksi celog sveta u 21. veku.

Ključne reči: personalizovana medicina, precizna medicina, dijagnostika zasnovana na genomu, farmakogenomika, molekularna terapija, genska terapija, ćelijska terapija, prediktivna genomika

Past

"It's far more important to know what person the disease has than what disease the person has"-Hippocrates

The concept of personalized medicine dates back for centuries. In ancient times, in the 8th century BCE, the first evidence about medicine adapted to individual's health appeared in the *Odyssey* written by Homer, introducing a Greek word "pharmakon", which could be ambiguously either a remedy or a poison to different persons (1). It is also well-known that Hippocrates, recognized as the father of modern medicine (4th century BCE), communicated to future generations of doctors with a message: "It's far more important to know what person the disease has than what disease the person has" (1). He practiced to individualize diagnosis and treatment, for example, by giving cold food to a "phlegmatic" person (2). Hippocrates was ahead of his time, as he suggested that every human is distinct, affecting both the disease prediction and the treatment (3). During the twenty-five centuries, despite the ancient visions, medical approach was not focused on each patient, but physicians used experience acquired from earlier therapeutic procedures applied for heterogeneous populations of patients.

Development of science brought substantial changes in medicine at the end of the 19th century. Chemistry, histochemistry and microscopy enabled physicians to identify the cause of the disease leading to more specific therapy in some patients. In the early 1950s, "scientific medicine" led to the concept of "evidence-based medicine (EBM)", a systematic approach to medical decision-making, based on large-scale population data and randomized controlled trials, to guide care for the average patient (4). EBM is guided by evidence from clinical trials, systematic reviews and meta-analyses, which provided recommendations for treatment of specific disease. It is assumed that the statistical homogeneity of the patients with the same diagnosis is present, and the outliers are ignored. EBM improved the quality of health care worldwide, but the individuality of each patient was not significantly considered. At the same time experienced medical experts practiced more personalized approach, considered as "an art of medicine" (5).

Personalized medicine had limited achievements in the 20th century. One of the first examples of application of the personalized medicine in clinical practice was in 1907, when Reuben Ottenberg recognized Mendelian inheritance patterns in human blood groups and suggested that patient and donor blood should be grouped and cross matched before a blood transfusion procedure. Fascinating development of molecular biology and especially molecular genetics, which started with the discovery of the secondary structure of DNA molecule (6), transformed the personalized medicine from an idea and an "art" to a practice. The first pharmacogenetic markers were discovered in 1956, when the genetic basis of fava beans toxicity ("favism") and the antimalarial drug primaquine is discovered as glucose-6-phosphate dehydrogenase (G6PD) deficiency (7). Cytochrome P450 2D6 polymorphism was first reported in 1977 with information that approximately 10% of Europeans could not metabolize an antihypertensive drug debrisoquine (8).

The term "personalized medicine" was introduced for the first time in 1999, when a short article entitled "New Era of Personalized Medicine: Targeting Drugs for Each Unique Genetic Profile", was published in *The Wall Street Journal*. A few months later, it was reprinted in *The Oncologist* (9). In the article, poor efficacy of the current pharmacotherapy, disease heterogeneity, and genetic variability were underlined. It was also emphasized that "one-size-fits-all" approach is not acceptable in modern medicine, what paved the path of pharmacogenomics, the main cornerstone of personalized medicine.

Human genome mapping (10) was a breakthrough providing almost complete information on human DNA and better understanding of human genetic profile. Personalized medicine got the scientific background through genetics and genomics and it was even named a "genome-based medicine". The golden age of personalized medicine has begun.

Present

"We must learn to treat the person, not the disease; the system, not just the symptoms. This is personalized medicine " - Mark Hyman

Nowadays medicine tends to be personalized, and to use the knowledge of molecular basis of the disease in order to individualize prevention, diagnosis and treatment of each patient.

There are several definitions of personalized medicine. For instance, the Personalized Medicine Coalition defines it as "the use of new methods of molecular analysis to better manage a patient's disease or predisposition to disease" (11). "Providing the right treatment to the right patient, at the right dose at the right time" is a definition of personalized healthcare used by the EU (12), while according to the National Cancer Institute, NIH, personalized medicine is "form of medicine that uses information about a person's genes, proteins, and environment to prevent, diagnose, and treat disease" (13).

At the present time, there are four cornerstones of personalized medicine: genome-based diagnostics, pharmacogenomics, specific molecular-targeted, gene and cellular therapy and predictive genomics.

1. Genome-based diagnostics

Huge advancements in technology and collection of big data (databases) related to genomics enabled discovery of numerous molecular-genetic tests leading to precise diagnostics in patients.

There are several approaches for the comprehensive analysis of genetic profiles of many people, which have provided sufficient data on molecular genetic markers that can be used in the diagnosis and prognosis of certain diseases. The most well-known are platforms for DNA analysis, such as DNA microarrays and next-generation sequencing (NGS). A special type of study that contributed to the application of personalized medicine in clinical practice is the analysis of a large number of genetic markers in different patients suffering from the same type of disease and the determination of the association of these markers with the pathological phenotype (genome-wide association study, GWAS) (14). However, medical science and practice have strived for a more complete understanding of the personal genetic profile of each person. Sequencing of the whole genome (WGS) using NGS and long read sequencing is therefore imposed as the ultimate genetic test (15).

Genomic testing became essential for the diagnosis of rare diseases (prenatal and postnatal) (16), as well as for more precise classification of cancer patients in distinct prognostic groups, leading to follow up of the disease and specific, more successful treatment protocols (17). Nowadays, standard clinical protocols for the treatment of oncological patients cannot be applied without the detection of specific genetic markers, representing either important diagnostic and prognostic indictors, or targets for the implementation of specific therapeutics (18).

2. Pharmacogenetics/Pharmacogenomics (PGx)

Pharmacogenetics/Pharmacogenomics (PGx) studies the human response to drugs, determined by individual genetic profile responsible for differences in drug metabolism (pharmacokinetics) and physiological drug response (pharmacodynamics), identifying responders and non-responders to a drug, and predicting the efficacy and/or toxicity of a drug. PGx is the basis for the application of personalized medicine. The goal of PGx is to identify genes and allelic variants of genes that could influence the response to drugs already used in therapy, with a goal to predict individual response to therapy.

PGx completely changes the old therapeutic paradigm of "one dose fits all patients" and "trail-and-error" prescription, to a novel, personalized concept of "matching the right therapeutic and the right dose to the specific genetic signature of the patient" (19).

PGx tests have become available and are routinely used in clinical practice. Before the administration of certain drugs, it is mandatory to perform an analysis of the relevant PGx markers. The application of pharmacogenetic tests enables patients to receive adequate therapy (the right drug and the right dose) in accordance with their genotype. This reduces the possibility of developing complications and the occurrence of side effects (adverse drug reactions) due to which the therapy should be stopped. This approach also prevents administration of inadequate drugs, shortens the treatment time and saves on hospital days. FDA's Center for Drug Evaluation and Research (CDER) has supported PGx for more than a decade by providing regulatory advice, reviewing applications, and developing policies and processes centered on genomics and individualized therapeutics (20). Nowadays, PGx markers are determined for more than 170 drugs, and there are recommendations for pre-emptive PGx testing for 64 drugs.

3. Molecular-targeted therapy

Molecular genetics has also found its place in the design of therapeutics for certain diseases whose cause is defined at the molecular level. Molecular therapeutics are a true example of causal therapy.

Trastuzumab is a key example of a personalized medicine, and it is often seen as the "poster child" for personalized medicine. It only improves overall survival and slows disease progression for patients whose cancer overexpress the HER2 protein. Trastuzumab is not beneficial, and may cause harm to patients with cancers that do not overexpress HER2. Today, HER2 testing is a routine part of clinical diagnosis for breast cancer patients. It was the first biomarker-driven targeted therapeutic (21).

Imatinib-mesylate is the first successful story of molecular-targeted therapy (22). It targets a genetic defect, a reciprocal translocation between chromosomes 9 and 22 (Philadelphia chromosome), which creates the *BCR/ABL* gene fusion, that is prevalently found among chronic myeloid leukemia (CML) patients. The consequence of this gene fusion is a constitutively active tyrosine kinase enzyme, which further initiates a signaling cascade for cancer development. Imatinib-mesylate works by inhibiting this fusion enzyme to constantly activate proteins in a consecutive sequence and thus prevent the growth of cancer cells, leading to their apoptosis. The BCR-ABL tyrosine kinase enzyme exists only in cancer cells. This is why this drug only works on these cells.

Based on the success of application of molecular-targeted therapy in oncology, there is an optimistic scenario that molecular-targeted therapy will contribute to the transformation of cancer into a chronic condition.

Table 1. Molecular-targeted therapy in cancer

Genetic aberration-target	Drug	Disease
BCR/ABL fusion gene	Imatinib Mesylate	Chronic Myeloid leukemia
BRAF V600E	Vemurafenib	Metastatic melanoma
PML-RARa fusion gene	ATRA (all-trans retinoic acid) ATO (Arsenic trioxide)	Acute promyelocytic leukemia
ALK mutations	Crizotinib	Non-small-cell lung cancers
EGFR mutations	Erlotinib	Non-small-cell lung cancers

The molecular targeted therapy made a great contribution to monogenic diseases, such as cystic fibrosis (CF), which is the most common rare disease in Caucasians and the most common lethal inherited disease in this population. It is the consequence of pathogenic variants in *CFTR* gene. Recently, a molecular-targeted therapy for CF, the *CFTR* modulator consisting of the triple combination, elexacaftor–tezacaftor–ivacaftor, has brought benefits to a large percentage of CF patients with the F508del mutation, the most common disease-causing variant in CF, realizing the "dream of molecularly targeted therapies" for CF (23).

4.Gene therapy

Gene therapy is a medical technology aiming to use therapeutic delivery of genetic material into patient's cells, as a drug to treat disease. The ultimate goal of gene therapy is to restore normal function of the gene whose defect is causing a disease. The genetic basis of the disease is targeted using several approaches. If an endogenous gene is missing or mutated it can be replaced with a healthy copy. Also, new genetic material can be delivered into cells to overpass the effect of genetic defects and restore gene's normal function. DNA can be edited, and a pathogenic variant can be corrected (like with CRISPR-Cas9 methodology). Finally, a disease-causing gene can be turned off. Various therapeutic genetic material is usually delivered to a cell using viral vectors or nanoparticles.

Today, somatic gene therapy is applied, the introduction of a therapeutic gene into the cells of a certain tissue, usually the one in which the mutation manifests itself most dramatically. This approach can be performed *in situ*, *ex vivo* and *in vivo*. *In situ* gene therapy introduces a therapeutic gene into affected patient's tissue, while the *ex vivo* approach involves taking a certain tissue (usually blood cells), treating it with a therapeutic gene *in vitro* and returning the modified cells to the body. The *in vivo* approach implies the application of a therapeutic gene intramuscularly, intravenously, etc.

The first FDA approved gene therapy trial was for severe combined immunodeficiency (SCID), caused by variants in the *ADA* (adenine deaminase) gene (24). It was an example of *ex vivo* gene therapy. SCID was the first disease cured using genetic engineering of human DNA. However, due to the inefficiently controlled gene transfer, the incorporation of a therapeutic gene into the patient's genome probably led to the activation of genes that caused hematological malignancies in treated patient (25). Consequently, gene therapy protocols were postponed for decades.

The concept of gene therapy for tumors is different from that for genetic diseases. In malignant tumors, the breakthrough in gene therapeutic strategy involved designing suicide gene therapy, which was first applied for malignant glioma in 1992 (26, 27).

After many years the optimization of various types of safe vectors for genetic transfer and the introduction of new genome editing tools, first gene therapy-based drug (Glybera, alipogene tiparvovec) was approved by EMA for lipoprotein lipase (LPL) deficiency in 2012. It was in clinical practice from May 2013. Thirty patients were successfully treated. The drug was withdrawn in 2017, due to lack of demand (28).

There are different gene therapy approaches which were developed simultaneously with new discoveries in molecular biology. siRNA therapy uses short, double-stranded small interfering RNA (siRNA) molecules to silence specific genes that cause disease, by suppressing gene expression. The US Food and Drug Administration (FDA) has approved three therapeutic siRNA drugs since 2022, ONPATTRO® (patisiran) for hereditary transthyretin amyloidosis, GIVLAARI® (givosiran) for acute hepatic porphyria and OXLUMO® (lumasiran) for primary hyperoxaluria type 1 (29). In addition, several other drugs are in the late stages of clinical trials (30).

Gene therapy has experienced rapid flourishing in recent years. It has been fully introduced in clinical practice and numerous regulatory approvals have been obtained, mostly for rare diseases and cancers. Some of the most important are: Luxturna®, for inherited retinal diseases, the first FDA approval for inherited disease in 2018, ZOLGENSMA® for spinal muscular atrophy, Vyjuvek® for dystrophic epidermolysis bullosa, Casgevy®**,** for beta/thalassemia and sickle cell disease, the first approved medical treatment based on CRISPR gene-editing technology (31)**.**

The FDA expects to see a doubling of new gene therapy applications every year. It is predicted that by the year 2025, the US will be approving between 10 and 20 different gene therapies every year (32).

5. Cellular therapy

Cellular therapy is a medical approach which involves the transfer of a specific cell type, or types, into a person to treat or prevent a disease. A life of cells in human body includes processes of growth, division and differentiation. When cells grow old or become damaged, they usually die, and new cells get created to divide and to take their place. However, sometimes damaged cells continue to replicate, as a consequence of genetic and epigenetic changes accumulated in the cell. Many human diseases are caused by cells not functioning properly.

The role of cellular therapy is to replace damaged cells or to correct cellular dysfunction. New approaches based on immune system are also used in the treatment of diseases, particularly cancer.

Medicine has decades of experience with bone-marrow or blood stem cell transplantations, a procedure for the replacement of defective or cancerous bone marrow with new, healthy bone marrow cells. It is used for treatment of hematological malignancies, bone marrow disorders (aplastic anemia) and genetic blood disease (thalassemia, sickle cell anemia, autoimmune diseases).

Regenerative medicine is also a sort of cellular therapy. Regenerative medicine deploys a body's own cells and growth factors to repair tissues by restoring their lost functions (33). Several cell therapies in regenerative medicine have been introduced in clinical practice. Some of them are commercially available with FDA approval, such as keratinocyte- and/or fibroblast-derived skin substitutes for treatment of diabetic foot ulcers (34) or burns (35). Although commercial cell therapies are beneficial in repairing tissues, they are unable yet to regenerate them (36).

Mesenchymal stem cell (MSC) treatment has been proposed as a novel approach for tissue engineering and regenerative medicine. To date, there are numerous clinical studies involving MSCs as an intervention in various conditions, such as chondral and bone defects (37). In 2016, the first MSC-based therapy has received regulatory approval for the treatment of Buerger's disease in India. In 2017, orphan designation was granted by the European Commission for autologous adipose tissue-derived MSC for the treatment of Buerger's disease (38).

Nowadays, cellular therapy is in the midst of a boom in oncology, in a form of immunotherapy that includes collecting a patient's immune cells, genetically modifying them *in vitro* to better target and destroy cancer cells, and then reinfusing them into the patient to create a "living drug" that fights the disease (39).

Dendritic cell (DC)-based vaccines is a new cancer immunotherapy regimen. DCs are the most potent group of antigen-presenting cells. The DC-based vaccine is created from cancer patient's monocytes obtained by leukapheresis. Monocytes are differentiated *in vitro (ex vivo)* into immature DCs which are maturated in a cocktail of cytokines, while being simultaneously exposed to autologous tumor cell lysates or cancer-specific antigens (such as peptides or RNAs). The mature, antigen-loaded DCs are reinfused to the patient, initiating and modulating antigen-specific immunity and tolerance, and improving antitumor immune

response obtained by therapeutic vaccines (Figure 1). Sipuleucel-T (the brand name Provenge) is the first therapeutic cancer vaccine approved by the FDA for treating metastatic castration-resistant prostate cancer (40). The Nobel Prize in Physiology or Medicine 2011 was awarded to Ralph M. Steinman, Jules A. Hoffman and Bruce A. Beutler for the discovery of essential elements of innate immunity, in particular dendritic cells (DCs) and toll-like receptors (TLRs). This was the most important step on a way to development of DC-based vaccines to immunize patients infected with HIV or affected by cancer.

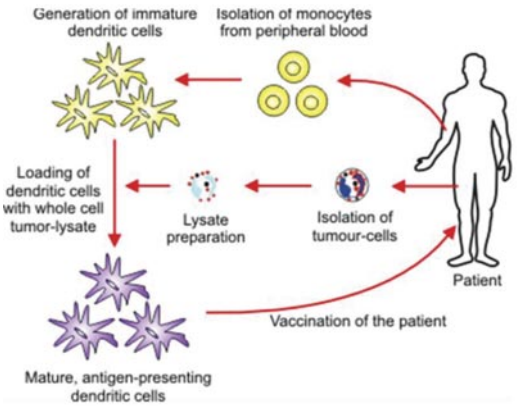

Figure 1. Schematic diagram of DC-based antitumor vaccine

Another powerful immune therapy, **CAR (Chimeric Antigen Receptor) T-cell therapy**, represents an example of *ex vivo* gene therapy. In this medical procedure, patient's own T-cells are genetically modified *in vitro*, in a way that they start to express CAR that targets cancer. These CAR T-cells are reinfused back into the patient, to recognize and destroy cancer cells. CAR T-cell therapy provides a long-lasting immune attack against cancer. It can induce long-term remission for many patients but also can have serious side effects. CARs are engineered receptors consisting of a combination of three components: a variable region of an antibody capable of recognizing and binding to cancer-specific antigens (ectodomain), an anchoring transmembrane domain, and an intracellular region consisting mainly of the TCR-signaling domain, which is capable of activating T-cells (endodomain).

FDA has approved six CAR-T cell therapies for the treatment of various hematologic malignancies. Kymriah®, CAR-T cell therapy targeting the B cell antigen CD19, was the first CAR-T cell therapy to receive approval by the FDA and the EMA for the treatment of children and young adults with ALL (41). The FDA and EMA have subsequently approved three additional CAR-T cell therapies targeting the CD19 antigen: Yescarta®,Tecartus®, and lisocabtagene maraleucel (Breyanzi®). In addition, two B cell maturation antigen (BCMA) CAR-T cell products have been approved for the treatment of relapsed/refractory B-cell malignancies and multiple myeloma, idecabtagene vicleucel (Abecma®) in 2021 and Carvykti® in 2022 (42). Currently, several clinical trials are ongoing testing these six CAR-T cells for additional indications. CAR T-cell therapy has achieved great success in the treatment of B-cell leukemia. In some trials, over 90% complete remission rates in B-cell malignancies, including pediatric and young adult B-ALL, have been reported. Also, long-term remission in patients has been demonstrated, with some remaining cancer-free for several years. CAR T-cell therapy has been particularly effective in patients with relapsed or refractory aggressive B-cell cancers who have failed other treatments. Despite that, a major limitation of CAR T-cell therapy is the lack of an optimal target antigen in solid tumors and acute myeloid leukemia.

Tumor-infiltrating lymphocytes (TILs) are also used as cellular immunotherapy. As cancers grow, T and B lymphocytes of a patient recognize cancer cells as abnormal and penetrate the tumor. These TILs begin

working to kill cancer cells. Sometimes, they're prevented from doing that by brakes in the immune system or signals from the tumor that weaken the immune response.

TILs already recognize many targets on the cancer cells, and they do not need to be genetically engineered to recognize cancer, as in CAR T-cell therapy is the case. So, TILs themselves can be used as a form of cell therapy, only by expanding the TILs *ex vivo*. However, this approach was not particularly successful, until 2024 when FDA has approved lifileucel (Amtagvi), the first treatment for cancer that uses TILs. Lifileucel is the first cellular therapy to be approved for a solid tumor, the skin cancer melanoma (43).

In the meantime, immunotherapy using TILs has been significantly improved by engineering them with genetic elements of the checkpoint blockade (a blockade of immune checkpoint inhibitory pathways activated by cancer cells). **Checkpoint inhibitors therapy** represents a paradigm-shifting approach for cancer therapy.

Patient's immune system recognizes that cancer cells express foreign antigens, since somatic gene mutations and epigenetic changes produce altered proteins on the membrane of cancer cells. Immune response to cancer is observed in cancer patients, but it is not efficient. Actually, tumors induce tolerance among tumor-specific T cells and hijack the immune checkpoint system by expressing ligands that bind receptors on T cells (44).

Immune checkpoint inhibitors were developed to escape some of those cancer mechanisms to block patient's immune system in order to unleash the immune cells to attack cancer and to enhance anti-tumor immune response. Several checkpoint inhibitors have been FDA approved drugs for cancer treatment. James P. Allison and Tasuku Honjo were awarded the 2018 Nobel Prize in Physiology or Medicine for their pioneering work in this area.

The first target for checkpoint inhibitors is CTLA4, an immunoglobulin superfamily member, an immune checkpoint receptor expressed by T cells, which inhibits T cell activation and proliferation (45). Ipilimumab is an antibody drug against CTL4, used for melanoma treatment from 2011 (46).

The second target for checkpoint inhibitors is an interaction between PD-L1, a ligand expressed on the surface of tumor cells and PD-1, a receptor expressed by activated effector T cells. The interaction between tumor PD-L1 and PD-1 on patient's T cells results in blockage of production and secretion of cytotoxic mediators required for tumor killing (45). Several checkpoint inhibitors targeting PD-1 (nivolumab, pembrolizumab, cemiplimab) are FDA approved for treatment of melanoma, non-small-cell lung cancer, Hodgkin lymphoma, head and neck squamous cell carcinoma, urothelial carcinoma, hepatocellular carcinoma, gastric and gastroesophageal carcinoma and cutaneous squamous-cell carcinoma (44). Checkpoint inhibitors, antibody drugs against PD-L1 (atezolizumab, durvalumab, avelumab), are used in treatment of urothelial carcinoma, non-small-cell lung cancer, triple-negative breast cancer and small-cell lung cancer (44).

It is evident that cancer is a great challenge for modern medicine and that personalized medicine approach has led to the cutting-edge cancer therapies.

6. Predictive genomics

"An ounce of prevention is worth a pound of cure." Benjamin Franklin

Predictive genomics is a field that aims to direct modern medicine and healthcare to prevention and personalization. Using individual's genetic data for pre-symptomatic risk assessment can lead to avoiding illness and maintaining health and well-being.

There are several predictive genomic tests which can help healthy individuals predict the development of a certain disease and undertake some actions, such as additional regular control testing or change of lifestyle. One of the most used is a BRCA1 and BRCA2 test. Detection of pathogenic variants in these genes significantly increases the risk of breast, ovarian, prostate, and pancreatic cancers (47).

Also, some genetic markers can predict development of cardiovascular diseases in young population. The identification of individuals with at-risk genotypes at their early age can be utilized throughout their life to follow and allow adjustments to the preemptive treatment strategy (48).

Nutrigenetics and nutrigenomics are unavoidable disciplines of predictive genomics. They pave a path toward personalized nutrition.

Personalized nutrition is emerging as a transformative approach to health, rooted in the understanding that individual genetic variability influences how dietary regimes affect the human body. Nutrigenetics explores how a person's genetic profile impacts nutrient metabolism, while nutrigenomics investigates how diet influences gene expression and disease development (49).

Certain genetic alterations, such as those in the PAH gene, can lead to metabolic disorders like phenylketonuria, which requires strict dietary management from birth. Predictive neonatal genetic testing is obligatory to prevent severe neurological damage.

Similarly, celiac disease and lactose intolerance are linked to specific gene variants. Genetic screening can help identify individuals at risk and guide preventive dietary strategies.

Advancements in genome-wide association studies (GWAS) have identified predictive nutrigenetic markers. Variants in genes like *FTO*, *PPARG*, and *VDR* influence susceptibility to obesity, vitamin D metabolism, and bone health and must be considered in prevention and management of chronic diseases (50).

Ultimately, personalized nutrition aims to tailor dietary habits to genetic profiles, promoting disease prevention and optimal health. With growing access to DNA-based dietary recommendations, this field holds promise for revolutionizing nutritional science and public health.

Predictive genomics leads to the goal of modern medicine - preventive medicine. It moves focus from treatment of the disease to its prevention. It enables early interventions, lifestyle adjustments, and screenings to prevent or delay the onset of disease. It also contributes to cost-effective healthcare.

Future

"Personalized medicine is an art that advocates for the patient, not the pocket or convenience of the medical system" - Melissa Cady

In future, it is expected that personalized medicine will be fully implemented in medical practice worldwide.

Nowadays, the idea of true personalized medicine has not been realized yet. Actually, we are in the era of precision medicine, which uses the knowledge of molecular basis of the disease in order to individualize prevention, diagnosis and treatment. Precision medicine is based on the availability of great amount of biological data, and it is in line with the biomedical model of health.

A more comprehensive, precise, and even "personal" approach to health, namely, personalized medicine, would require taking into account not only biological, but also environmental, socio-economic and psychological determinants, an approach more in line with the biopsychosocial model of health (51).

But, initially, even precision medicine has to make significant progress. We are still at the level of genome-based medicine (52-54). Only genomics, and partially epigenomics and transcriptomics are considered in modern medicine. Data related to multi-omics, a broader, integrated view of molecular changes and interactions, should be included in precision medicine and applied in diagnosis, prognosis, treatment and prevention of the disease. Novel and more accurate markers will be discovered by analyzing multiple data layers (genomics, epigenomics, transcriptomics, proteomics, metabolomics, microbiomics). Therefore, the four cornerstones of future precision medicine will be: omics-based diagnostics, pharmaco-omics, specific molecular-targeted and cellular therapy and predictive multi-omics.

Moving forward to the future, personalized medicine will be defined by the "4P" framework: Predictive, Preventive, Personalized, and Participatory. This model will shift healthcare from a reactive approach to a proactive, individual-centered model that uses omics data, lifestyle factors, and patient involvement (55).

A great hope is given to the innovations driving change. Technological leaps are necessary for progress of personalized medicine. Also, the intersection of big data analytics and omics science will become the backbone of personalized medicine. Bioinformatics and artificial intelligence (AI) tools have already revolutionized precision medicine. Even now, deep learning algorithms can predict drug responses based on a patient's genetic profile with accuracy rates exceeding 85% in some applications (56). Advancements in AI and machine learning will be able to identify patterns in vast datasets that would be impossible for human analysts to detect. We need algorithms to make better computational predictions of experiments we have never performed in the lab or in clinical trials. Since there is confidence in science and innovation, these are reachable goals.

But the prospect is not all rosy. There are ethical concerns among which the most important one is if personalized medicine will be available worldwide. We need a paradigm shift such that medicines are no longer lucrative market commodities but are global public health goods available to all those who need them.

BOHEMIA is a foresight study designed specifically to identify priority directions for EU research and innovation in Horizon Europe programme**.** An innovative methodology is utilized, such as a comprehensive foresight process, including scenario building, a Delphi survey with experts, and online consultations with stakeholders. *BOHEMIA* explicitly referenced the UN Sustainable Development Goals (SDGs) as a fundamental framework for its objectives. One of the proposed priority directions for EU R&I policy is precision medicine. An accepted scenario is:

"It is 2040. Individualized precision medicine combining mass data analyses, genetic engineering, epigenetics, and knowledge about the personal microbiome and the biotic environments helps anticipate and cure illnesses."

We believe that we will collectively transform healthcare from a population-based to an individual-focused paradigm and that personalized medicine will be fully implemented in medical practice worldwide.

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Induced pluripotent stem cells as a model to study drug induced pancreatitis: past, present and future

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Abstract

Drug-induced pancreatitis (DIP) represents a significant clinical challenge, accounting for approximately 0.1-2% of all acute pancreatitis cases. Among immunosuppressive therapies, thiopurines are particularly notorious for causing pancreatitis, with an incidence of 3-5% in inflammatory bowel disease (IBD) patients. The idiosyncratic and dose-independent nature of thiopurine-induced pancreatitis (TIP) has hindered the development of predictive biomarkers and mechanistic understanding. Traditional *in vitro* models using immortalized cell lines and animal models have proven insufficient to recapitulate the complexity of human pancreatic responses to drugs. The advent of induced pluripotent stem cell (iPSC) technology has opened new avenues for personalized disease modeling and drug toxicity assessment. This review explores the evolution of iPSC-based models for studying drug-induced pancreatitis, from early bidimensional cultures to sophisticated three-dimensional organoids, discussing their applications in elucidating pharmacokinetic and pharmacodynamic mechanisms, identifying genetic risk factors, and developing precision medicine approaches. Recent breakthrough studies demonstrate that iPSC-derived pancreatic models from patients who developed TIP show enhanced sensitivity to thiopurine cytotoxicity, with distinct mechanisms involving TPMT expression in stem cells and Rac1 protein levels in differentiated pancreatic cells. These findings highlight the potential of iPSC technology for predictive toxicology and therapy personalization, particularly in vulnerable pediatric populations where clinical trials are limited by ethical constraints.

Keywords: Drug-induced pancreatitis, thiopurines, induced pluripotent stem cells, inflammatory bowel disease, personalized medicine, pediatric gastroenterology

Indukovane pluripotentne matične ćelije kao model za proučavanje pankreatitisa izazvanog lekovima: prošlost, sadašnjost i budućnost

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Apstrakt

Pankreatitis izazvan lekovima (DIP) predstavlja značajan klinički izazov, čineći približno 0,1-2% svih slučajeva akutnog pankreatitisa. Među imunosupresivnim terapijama, tiopurini su posebno poznati po izazivanju pankreatitisa, sa incidencom od 3-5% kod pacijenata sa inflamatornom bolešću creva (IBC). Idiosinkratična i od doze nezavisna priroda pankreatitisa izazvanog tiopurinom (TIP) ometala je razvoj prediktivnih biomarkera i mehanističko tumačenje. Tradicionalni *in vitro* modeli koji koriste immortalizovane ćelijske linije i životinjske modele pokazali su se nedovoljnim da rekapituliraju složenost odgovora pankreasa na lekove kod ljudi. Pojava tehnologije indukovanih pluripotentnih matičnih ćelija (iPSC) otvorila je nove mogućnosti za personalizovano modeliranje bolesti i procenu toksičnosti lekova. Ovaj pregledni rad istražuje evoluciju modela zasnovanih na iPSC za proučavanje pankreatitisa izazvanog lekovima, od ranih dvodimenzionalnih kultura do sofisticiranih trodimenzionalnih organoida, razmatrajući njihovu primenu u razjašnjavanju farmakokinetičkih i farmakodinamičkih mehanizama, identifikovanju genetskih faktora rizika i razvoju pristupa precizne medicine. Nedavne revolucionarne studije pokazuju da modeli pankreasa izvedeni iz iPSC pacijenata koji su razvili TIP pokazuju povećanu osetljivost na citotoksičnost izazvanu tiopurinima, uzrokovanu različitim mehanizmima koji uključuju ekspresiju TPMT u matičnim ćelijama i nivoe Rac1 proteina u diferenciranim ćelijama pankreasa. Ovi nalazi ističu potencijal iPSC tehnologije za prediktivnu toksikologiju i personalizaciju terapije, posebno kod ranjive pedijatrijske populacije gde su klinička ispitivanja ograničena etičkim pravilima.

Ključne reči: pankreatitis izazvan lekovima, tiopurini, indukovane pluripotentne matične ćelije, inflamatorna bolest creva, personalizovana medicina, pedijatrijska gastroenterologija

1. Drug-induced pancreatitis: clinical significance and unmet needs

Drug-induced pancreatitis (DIP) represents an underrecognized yet clinically important cause of acute pancreatitis, characterized by pancreatic inflammation triggered by pharmacological agents (1). While the overall incidence of DIP appears relatively low (0.1-2% of all acute pancreatitis cases), certain medications carry substantially higher risk. The clinical presentation of DIP is indistinguishable from other forms of acute pancreatitis, manifesting with severe abdominal pain, elevated serum amylase and lipase levels (typically >3 times the upper limit of normal), and potentially life-threatening complications including pancreatic necrosis, systemic inflammatory response syndrome, and multi-organ failure (2).

More than 500 drugs have been implicated in causing pancreatitis, with varying levels of evidence supporting causality (3). Among immunosuppressive agents, thiopurines—including azathioprine (AZA), mercaptopurine (MP), and thioguanine (TG)—are well-established culprits. These drugs are widely used in the management of inflammatory bowel disease (IBD), acute lymphoblastic leukemia, and as immunesuppressants following organ transplantation (4,5). The incidence of thiopurine-induced pancreatitis (TIP) in IBD patients ranges from 3-5%, with Crohn's disease (CD) patients showing higher susceptibility (3-5%) compared to those with ulcerative colitis (UC) (1-2%) (6,7) .

The idiosyncratic nature of TIP presents significant challenges for clinical management. Unlike many dose-dependent adverse drug reactions, TIP typically occurs within the first month of treatment and shows no clear relationship with drug dosage or metabolite levels (8). This unpredictability, combined with the absence of reliable biomarkers, leaves clinicians unable to identify at-risk patients before therapy initiation. Once TIP occurs, permanent discontinuation of thiopurine therapy is typically required, necessitating alternative treatment strategies that may be less effective or associated with different toxicity profiles (9).

2. Thiopurine pharmacology and mechanisms of pancreatotoxicity

Thiopurines are pro-drugs requiring extensive hepatic metabolism to generate their active metabolites, thioguanine nucleotides (TGNs), which exert cytotoxic and immunosuppressive effects (10). Azathioprine is rapidly converted to mercaptopurine through both enzymatic (glutathione-S-transferase-mediated) and non-enzymatic pathways involving reduced glutathione (11,12) . Mercaptopurine subsequently undergoes metabolism via multiple enzymatic routes, including hypoxanthine-guanine phosphoribosyltransferase (HPRT), thiopurine methyltransferase (TPMT), aldehyde oxidase (AO), and xanthine oxidase (XO) (13). The balance between activation and inactivation pathways determines the intracellular accumulation of cytotoxic TGNs.

The mechanisms underlying thiopurine cytotoxicity are multifaceted and include: (1) incorporation of thioguanine nucleotides into DNA and RNA, disrupting replication and transcription; (2) inhibition of *de novo* purine synthesis through methylthioinosine monophosphate (meTIMP)-mediated blockade of phosphoribosyl pyrophosphate amidotransferase (PPAT); (3) inhibition of Rac1, an anti-apoptotic GTPase, leading to T lymphocyte apoptosis; and (4), for azathioprine specifically, depletion of intracellular glutathione levels, resulting in increased oxidative stress and reactive oxygen species (ROS) generation (14–16) .

Despite comprehensive understanding of general thiopurine pharmacology, the specific mechanisms responsible for TIP remain poorly defined. Several hypotheses have been proposed:

Immunological mechanisms: The temporal pattern of TIP onset (typically within 30 days of treatment initiation) suggests possible immune-mediated pathology. The strong genetic association between HLA-

DQA1-HLA-DRB1 variants and TIP risk (with heterozygous individuals showing 9% incidence and homozygous individuals 17% incidence) supports an immunological component (17).

Direct cytotoxicity: Thiopurines may exert direct toxic effects on pancreatic acinar cells through metabolite accumulation or oxidative stress. Azathioprine shows higher TIP incidence compared to mercaptopurine in CD patients, potentially related to azathioprine's capacity to generate ROS through glutathione depletion (18).

Pharmacodynamic alterations: Recent evidence suggests thiopurines inhibit Rac1 activity in pancreatic ductal cells, impairing cystic fibrosis transmembrane conductance regulator (CFTR) localization to the plasma membrane, which could contribute to TIP pathogenesis (19).

Genetic predisposition: Beyond HLA associations, variations in thiopurine-metabolizing enzymes (TPMT, NUDT15, ITPA, PACSIN2) influence drug response and toxicity, though their specific role in TIP remains unclear (20–22).

3. Limitations of conventional models for studying drug-induced pancreatitis

The investigation of drug-induced pancreatitis has historically relied on animal models and immortalized cell lines, each with significant limitations that have impeded progress in understanding mechanisms and developing preventive strategies.

Animal models: Rodent models of acute pancreatitis have been valuable for studying general pancreatic pathophysiology but show poor predictivity for drug-induced toxicity (23). Species differences in drug metabolism, pancreatic anatomy, immune responses, and genetic background create substantial translational gaps. For example, the expression and activity of thiopurine-metabolizing enzymes differ significantly between rodents and humans (24). Additionally, ethical concerns and the inability to capture individual patient variability limit the utility of animal models for personalized medicine approaches.

Immortalized cell lines: Pancreatic cell lines, including AR42J (rat pancreatic acinar cells), HPAF-II (human pancreatic adenocarcinoma), and various ductal epithelial lines, have been used to study pancreatic cell biology and drug responses (25). However, immortalization processes involving viral oncogenes, telomerase overexpression, or chemical/radiation treatments fundamentally alter cellular phenotypes, metabolic profiles, and stress responses (26). These cells often lack key functional characteristics of primary pancreatic cells and fail to recapitulate the complex multicellular architecture of the pancreas. Moreover, they cannot capture the genetic and epigenetic variability underlying individual susceptibility to drug-induced toxicity.

Primary human pancreatic cells: While primary cells would theoretically provide the most relevant model, their procurement requires invasive procedures (surgical resection or biopsy), yields limited cell quantities, cannot be expanded substantially in culture, and is essentially impossible to obtain from healthy individuals or prospectively from at-risk patients. These practical constraints have severely limited the use of primary pancreatic cells for mechanistic studies or predictive toxicology.

These limitations underscore the critical need for innovative model systems that combine human relevance, genetic fidelity, experimental tractability, and the capacity for personalized investigation—requirements that induced pluripotent stem cell technology is uniquely positioned to address.

4. The iPSC revolution: from discovery to disease modeling

The discovery of induced pluripotent stem cells (iPSCs) by Shinya Yamanaka and colleagues in 2006 revolutionized regenerative medicine and disease modeling (27). By introducing four transcription factors

(OCT3/4, SOX2, c-MYC, and KLF4) into differentiated somatic cells, Yamanaka demonstrated that cellular identity could be reset to an embryonic-like pluripotent state, capable of differentiating into any cell type of the human body.

Evolution of reprogramming technologies: The original retroviral reprogramming method raised concerns about genomic integration and mutagenesis. Subsequent developments introduced non-integrative approaches including Sendai virus (a single-stranded RNA virus that replicates cytoplasmically without nuclear entry), episomal plasmids, modified mRNA, microRNA, and small molecule-based reprogramming (28–30). Among these, Sendai virus has emerged as a particularly attractive method, combining high efficiency with safety by avoiding genomic integration while allowing complete viral clearance after several passages (31).

Advantages of iPSC technology: iPSCs offer several transformative advantages for biomedical research: (1) they can be generated from easily accessible somatic cells (skin fibroblasts, peripheral blood mononuclear cells, urinary cells); (2) they retain the donor's genetic and epigenetic information, enabling personalized disease modeling; (3) they provide virtually unlimited cell quantities through self-renewal capacity; (4) they can be differentiated into any cell type, including those difficult or impossible to obtain from patients; and (5) they enable longitudinal studies and high-throughput screening applications (32,33).

Particular relevance for pediatric populations: iPSC technology holds special promise for pediatric medicine, where clinical trials are limited by ethical concerns, small patient populations, and developmental differences in pharmacokinetics and pharmacodynamics (34). Children with IBD represent a population where thiopurine therapy is common but TIP can be particularly severe, with more extensive anatomical involvement and greater impact on growth and development (35,36). iPSC-derived models bypass ethical constraints while capturing the patient-specific factors contributing to adverse drug reactions in this vulnerable population.

5. iPSC differentiation toward pancreatic lineages

Recapitulating pancreatic development *in vitro* requires understanding the stepwise specification events that occur during embryogenesis. The pancreas develops from the definitive endoderm through a series of well-characterized stages involving specific transcription factors and signaling pathways (37).

Definitive endoderm formation: The first critical step involves differentiating iPSCs toward definitive endoderm, marked by expression of SOX17 and FOXA2. This is typically achieved through combined activin A and Wnt signaling (via CHIR99021, a GSK3β inhibitor), mimicking the embryonic signals that specify endodermal fate (38).

Pancreatic specification: Definitive endoderm cells are then patterned toward posterior foregut and pancreatic progenitor identity through FGF, retinoic acid, and BMP/TGF-β pathway modulation. Key markers of this stage include PDX1 (pancreatic and duodenal homeobox 1), the master regulator of pancreatic fate (39).

Exocrine pancreatic differentiation protocols: Efficient exocrine differentiation requires specific signals, particularly FGF7 (also known as keratinocyte growth factor, KGF), which plays a pivotal role in acinar cell specification (40). The protocol developed by Takizawa-Shirasawa and colleagues represents one of the most efficient approaches, involving sequential treatment with activin A/CHIR99021 for definitive endoderm, FGF7 for primitive gut tube, cyclopamine/noggin/retinoic acid for pancreatic progenitors, and FGF7/GLP-1/nicotinamide for final exocrine differentiation (41). Additional factors including nicotinamide

(acting as a ROCK kinase inhibitor) and GLP-1 (stimulating enzyme secretion) promote maturation of acinar cells expressing digestive enzymes such as amylase (AMY2A and AMY2B isoforms).

Despite significant progress, current iPSC-derived exocrine pancreatic cells often retain some immature or fetal characteristics, such as lower digestive enzyme expression compared to adult pancreas. This limitation may actually be advantageous for studying developmental toxicology but necessitates careful interpretation when extrapolating to adult pancreatic function.

6. From 2D cultures to 3D organoids: advancing model complexity

While two-dimensional (2D) monolayer cultures of iPSC-derived pancreatic cells represent a significant advance over immortalized cell lines, they lack the three-dimensional architecture, cell-cell interactions, and tissue-level organization that characterize native organs and influence drug responses (42).

Organoid technology: Organoids are self-organizing, three-dimensional multicellular structures that recapitulate key aspects of organ architecture, cellular heterogeneity, and functionality (43). iPSC-derived organoids combine the advantages of patient specificity and genetic tractability with the structural complexity of organoid systems (44).

Pancreatic organoid generation: iPSC-derived pancreatic organoids can be generated through several approaches. One strategy involves differentiating iPSCs into pancreatic progenitors in 2D culture, then embedding these cells in extracellular matrix (typically Matrigel®) where they self-organize into three-dimensional structures (45). Alternative protocols generate anterior spheroids from definitive endoderm, which are then embedded and differentiated toward pancreatic fate (46).

Advantages of organoid models: Compared to 2D cultures, organoids offer: (1) more physiological cell-cell and cell-matrix interactions; (2) establishment of apical-basal polarity and formation of luminal structures mimicking pancreatic ducts; (3) appropriate localization of secretory enzymes and transporters; (4) enhanced cellular maturation and functionality; and (5) better recapitulation of tissue-level responses to injury, inflammation, and drug exposure (47).

Current limitations: Organoid technology faces several challenges including: variability in differentiation efficiency and organoid characteristics; difficulty achieving full maturation; absence of vascular networks limiting size and nutrient delivery; lack of immune cells and other non-parenchymal cells present in native organs; and limited standardization of protocols across laboratories (48,49).

7. Recent breakthrough: Patient-derived iPSC models reveal distinct mechanisms of thiopurine-induced pancreatitis

A landmark 2025 study by Rispoli and colleagues represents the first comprehensive investigation of thiopurine-induced pancreatitis using patient-specific cellular models (50). This case-control study enrolled 10 pediatric inflammatory bowel disease patients from multiple Italian centers: 5 cases who developed pancreatitis during azathioprine therapy (mean age 15.3 ± 3.4 years, all male, including 3 UC and 2 CD patients) and 5 matched controls who tolerated treatment without pancreatitis. All TIP cases presented with mild pancreatitis, developing symptoms after an average of 78.8 ± 94.6 days of therapy, compared to controls who remained pancreatitis-free for 1105.4 ± 819.8 days.

Patient-specific iPSCs were generated via Sendai virus-based reprogramming of peripheral blood mononuclear cells, ensuring genomic integrity through non-integrative methodology (51). These iPSCs

were then differentiated into functional pancreatic exocrine cells using a stepwise 13-day protocol involving sequential treatment with activin A/CHIR99021 (definitive endoderm), FGF7 (primitive gut tube), cyclopamine/noggin/retinoic acid (pancreatic progenitors), and FGF7/GLP-1/nicotinamide (exocrine maturation).

The central finding revealed that both iPSCs and iPSC-derived pancreatic cells from TIP patients exhibited significantly enhanced sensitivity to thiopurine cytotoxicity. For iPSCs, thioguanine treatment showed the most pronounced differences ($p<0.001$), with TIP cells displaying only 21.80% viability versus 43.89% for controls at 2.5×10^{-7} M. iPSC-derived pancreatic cells from TIP patients demonstrated increased sensitivity to both thioguanine ($p<0.01$) and mercaptopurine ($p<0.01$), with significant differences at 1.6×10^{-5} M thioguanine (54.56% viability for TIP vs. 72.18% for controls). Interestingly, azathioprine showed no significant differences, possibly because its additional glutathione-depleting effects masked patient-specific variations.

Mechanistic investigations revealed cell-type-specific determinants of drug sensitivity. In iPSCs, real-time PCR demonstrated that control cells expressed significantly higher levels of TPMT (thiopurine methyltransferase) compared to TIP cells ($p<0.05$), while other metabolizing enzymes (HPRT, NUDT15, ITPA, PACSIN2) showed no differences. This lower TPMT expression in TIP iPSCs likely reduces detoxification capacity, consistent with previous studies showing enhanced thioguanine sensitivity in low-TPMT cells. However, LC-MS/MS analysis revealed no differences in thioguanine metabolite concentrations (TGMP and meTGMP) between groups, and [^{3}H]-thymidine incorporation assays showed similar proliferation rates, suggesting that enhanced cytotoxicity reflects altered cellular responses to metabolites rather than their accumulation.

In differentiated pancreatic cells, the mechanism appeared distinct. TPMT expression showed no differences between TIP and control cells, but western blot analysis revealed significantly higher Rac1 protein expression in TIP pancreatic cells ($p<0.05$), while iPSCs showed no Rac1 differences. Since thiopurines inhibit Rac1 (a GTPase critical for cell survival, CFTR localization, and pancreatic secretory function), higher baseline Rac1 in TIP pancreatic cells may paradoxically increase vulnerability to thiopurine-mediated disruption of these essential cellular processes.

This study provides proof-of-concept that patient-derived iPSC models can reveal idiosyncratic drug reaction mechanisms unattainable through traditional approaches. The findings suggest that thiopurine cytotoxicity operates through pharmacokinetic mechanisms (TPMT-mediated) in stem cells and pharmacodynamic mechanisms (Rac1-mediated) in differentiated pancreatic cells (52,53). These insights open avenues for biomarker development, with TPMT expression in iPSCs and Rac1 levels in differentiated cells representing potential predictive markers for TIP risk. The approach demonstrated here could be extended to other idiosyncratic reactions and represents a significant advance toward precision medicine in gastroenterology, particularly valuable for pediatric populations where prospective clinical studies face ethical constraints. Future directions include validation in larger cohorts with healthy controls, assessment of Rac1 activity (beyond expression), and development of three-dimensional organoid models to better recapitulate the complex pancreatic microenvironment.

8. Conclusions and future perspectives

The evolution of iPSC technology from basic reprogramming to sophisticated disease modeling platforms represents a paradigm shift in our approach to studying drug-induced adverse reactions. For thiopurine-induced pancreatitis, patient-derived iPSC models have revealed previously inaccessible mechanistic

insights, demonstrating that idiosyncratic drug toxicity reflects complex interactions between individual genetic backgrounds, cell-type-specific vulnerabilities, and drug pharmacology.

The field now stands at a critical juncture. While proof-of-concept studies have demonstrated feasibility and biological plausibility, translation toward clinical utility requires: (1) validation in larger, multicenter cohorts; (2) development of standardized protocols for iPSC generation, differentiation, and toxicity assessment; (3) integration of advanced three-dimensional organoid and organ-on-chip technologies; (4) incorporation of immune cells and other non-parenchymal components that may modulate drug responses; (5) prospective validation studies comparing *in vitro* predictions with clinical outcomes; and (6) cost-effectiveness analyses to determine practical implementation strategies.

Beyond TIP, the iPSC-based approach holds promise for investigating other idiosyncratic drug reactions affecting difficult-to-access tissues, including drug-induced liver injury, cardiotoxicity, and neurotoxicity. As technologies mature and costs decrease, patient-specific toxicity testing may become feasible for high-risk medications, enabling truly personalized prescribing decisions. This vision of precision pharmacotherapy—where treatment selection is guided by individual cellular responses rather than population averages—represents the ultimate goal of personalized medicine, with iPSC technology serving as the enabling platform for its realization.

Funding

This work was supported by the Italian Ministry of Health through contributions to the Institute for Maternal and Child Health IRCCS Burlo Garofolo, Trieste, Italy (RC 44/22).

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A short introduction to pharmacoeconomics

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Abstract

Pharmacoeconomics is a branch of health economics that evaluates the costs and benefits of pharmaceutical interventions in resource-limited healthcare systems. Unlike classical economics, healthcare markets diverge from assumptions of free competition, making structured economic evaluation essential for policy and clinical decision-making. Key concepts include opportunity cost, utility, marginal utility, willingness to pay, and the incremental cost-effectiveness ratio (ICER). Pharmacoeconomic studies categorize costs into direct medical, direct non-medical, indirect, and intangible, assessed from perspectives such as patients, providers, insurers, and society at large. Evaluation methods encompass cost-effectiveness, cost-utility, cost-minimization, cost-benefit, and cost-threshold analyses, each suited to specific decision contexts. Central to these approaches is the measurement of health outcomes and utilities, often quantified in Quality-Adjusted Life Years (QALYs) through instruments like EQ-5D or SF-6D. Decision-analytic frameworks, including decision trees and Markov models, support the projection of long-term outcomes and uncertainty management in complex disease pathways. Together, these tools guide evidence-based resource allocation, aiming to balance clinical effectiveness, quality of life, and economic sustainability. This review provides a concise overview of the principles, methods, and models underpinning pharmacoeconomics, highlighting its role in optimizing therapeutic choices and informing healthcare policy.

Keywords: pharmacoeconomics, health economics, economic evaluation, cost-effectiveness, decision analysis

Kratak uvod u farmakoekonomiju

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Apstrakt

Farmakoekonomija predstavlja granu zdravstvene ekonomije koja procenjuje troškove i koristi farmaceutskih intervencija u uslovima ograničenih resursa zdravstvenog sistema. Za razliku od klasične ekonomije, tržišta zdravstvene zaštite odstupaju od pretpostavki slobodne konkurencije, što čini sistematsku ekonomsku evaluaciju neophodnom za oblikovanje politika i donošenje kliničkih odluka. Ključni pojmovi uključuju oportunitetni trošak, korisnost, graničnu korisnost, spremnost na plaćanje i inkrementalni odnos trošak-efikasnost (ICER). Farmakoekonomske studije klasifikuju troškove na direktne medicinske, direktne nemedicinske, indirektne i nematerijalne, posmatrane iz perspektive pacijenata, pružalaca usluga, fonda zdravstvenog osiguranja i društva u celini. Metode evaluacije obuhvataju analizu troškovne efektivnosti, troškovne korisnosti, minimizacije troškova, troškova i koristi, kao i pragovnu analizu, pri čemu je svaka prilagođena specifičnom kontekstu odlučivanja. Centralno mesto u ovim pristupima zauzima merenje zdravstvenih ishoda i korisnosti, najčešće izraženih kroz godine života prilagođene kvalitetu (QALY), pomoću instrumenata poput EQ-5D ili SF-6D. Analitički okviri odlučivanja, uključujući stabla odlučivanja i Markovljeve modele, omogućavaju projekciju dugoročnih ishoda i upravljanje neizvesnošću u složenim bolestima. Ovi alati usmeravaju alokaciju resursa zasnovanu na dokazima, sa ciljem uravnoteženja kliničke efikasnosti, kvaliteta života i ekonomske održivosti. Ovaj pregled daje sažet prikaz principa, metoda i modela farmakoekonomije, naglašavajući njenu ulogu u optimizaciji terapijskog izbora i kreiranju zdravstvene politike.

Ključne reči: Farmakoekonomija, zdravstvena ekonomija, ekonomska evaluacija, troškovna efektivnost, analiza odlučivanja

Introduction to core concepts and terms

Economics, as an essential part of human society, is the study of the behaviour of people in producing, exchanging, and consuming goods and services. It comprises the core of maintaining complex systems essential for human existence, like diagnostics and treatment in medicine. However, economics, like all sciences, has its own set of assumptions and constraints under which it would operate. One of the main assumptions of the field is that the amount of goods and services that can be used is limited. Another is the free market assumption, constituting three points:

1. Perfect knowledge and certainty exist on the evolution of capital and the market, consequently the prices
2. There are no external factors in the free market
3. Many producers with no market power are present

However, when it comes to health economics, none of these ideal assumptions holds. The main reason is that most health systems are state-regulated to a degree, and the goals are different compared to the standard competitive market [1]. The general health policy of most systems has a goal to guarantee equitable access, good quality and outcomes, efficiency of health technologies, and value-for-money maximization. Additionally, we can emphasize the issue of polypharmacy that increases the healthcare budget due to adverse drug reaction, where, for a treatment, multiple drugs are administered to achieve a suitable outcome. Due to the obvious problem of resource constraints and multiple goals that need to be achieved at once, a health technology assessment must be made in order to determine the best course of action.

Pharmacoeconomics deals specifically with the problem of polypharmacy in the context of health economics [2]. Pharmacoeconomics can be defined as a branch of health economics that focuses on weighing the costs and benefits of a particular intervention in comparison with an analogous alternative in the context of pharmaceutical drugs. The scientific concept that justifies the existence of pharmacoeconomics further is the presence of pharmacogenetics, a field that deals with the differences in drug response in patients based on their genetic code. Thus, such a field is essential to fulfil the objectives of most health systems.

Before delving into the methodology of pharmacoeconomics, there are several core terms that must be clarified [2].

Opportunity cost is the quantity of a good we should sacrifice in order to obtain one more unit of another good. No matter what the complexity and the robustness of a production network, choosing to produce less of one product in order to produce more of another product is a decision type which must be frequently made.

Utility is the level of satisfaction that consumers obtain through having their desires met. For example, if a person takes a drug, the level of satisfaction with the obtained outcome is a utility.

Consumer choice is the decision of a person to obtain utility by consuming goods.

Marginal utility is additional or incremental satisfaction derived from the consumption of the next unit of product. For example, certain supplements can aid in treating a disease, but, if consumed afterwards, they can aid in increasing the quality of life.

Economic evaluation is a systematic evaluation of the benefits and costs arising from the comparison of different health technologies. This is the core of pharmacoeconomics and it will be explained in detail.

Willingness to pay (λ - WTP) is the maximum amount concerning how much a decision maker is willing to pay for one extra year of perfect health.

Incremental cost-effectiveness ratio (ICER) is the ratio that calculates the additional cost required per additional unit of effectiveness.

Cost Categories and Evaluation Methods

A first step in performing pharmacoeconomic studies is data collection. Due to the nature of economics, the costs of multiple interventions and medical services are necessary to perform adequate economic evaluations [3].

It is necessary to point out that the cost of production or servicing is different from the price of the same entities declared. While the cost shows the amount of resources required to ensure a product or service exists, the price is the monetary value of exchange declared to perform the exchange of a product or a service with a consumer. In most cases, a consumer of health products or services receives a reimbursement, meaning that the consumer's spendings are refunded per a health insurance contract, depending on the health system model used.

This leads the discussion to the categorization of costs in healthcare. Most costs can be sorted into four types:

1. Direct medical costs, which deal with inputs required to perform the treatment – pharmaceuticals, diagnostic tests, and hospitalizations
2. Direct non-medical costs, which deal with costs associated with treatment, but are not medical in nature – cost of travel, food, and lodging
3. Indirect costs, which usually deal with the loss of productivity because of illness or death
4. Intangible costs, which deal with costs of pain, suffering, anxiety, or fatigue that occur because of an illness or the treatment of an illness. (These costs are addressed in cost-benefit and cost-utility analyses)

To determine what costs are important to measure, it is necessary to determine the perspective of the study. While numerous perspectives exist, the most important one is the social perspective, which includes costs to the insurance company, costs to the patient, costs to the provider/institution, and indirect costs because of loss of productivity.

Further, due to economic events, like inflation, it is necessary to adjust the costs or standardize them based on the retrospective data, viewed from a certain point in time.

Lastly, one must consider the ICER, so that any economic analysis can have a conclusion.

The resources for cost estimations are different and are referred to by category.

Medication costs are often calculated using the average wholesale price. Medical institutions or pharmacies buy medications in bulk, so this is the price to refer to.

Medical service costs are evaluated using either a list of charges or by using reimbursement rates given by the local regulating body.

Personnel costs require work measurements, meaning active monitoring of activities to determine what each action and decision requires.

Hospitalization costs have several different ways of being measured [3]. "Per diem" refers to the cost of a general hospitalization per day, but it doesn't cover any specificity. "Disease-specific per diem" refers to the

cost of hospitalization of a patient with the considered disease. "Diagnosis-Related Group" refers to the cost of hospitalization of patients with cohesive diagnoses, since they would use similar resources. Lastly, micro-costing is the most precise method, but it requires an extensive use of resources to document each cost.

Types of Economic Analyses and Key Findings

Economic evaluations are one of the most important tools of pharmacoeconomics [1,2]. However, not all economic evaluations are created equal, nor are they all useful in the same degree in different situations. The main types of economic evaluations are:

1. Cost-effectiveness analysis (CEA)
2. Cost-utility analysis (CUA)
3. Cost-minimization analysis (CMA)
4. Cost-benefit analysis (CBA)
5. Cost-threshold analysis (CTA)

Cost-effectiveness analysis (CEA)

The CEA is a method which evaluates whether a medical product improves specific clinical outcomes enough to justify the additional costs required. As it is clear from the definition, it is a quantitative approach which draws its conclusions from intermediate or final clinical endpoints (i.e. endpoints of treatment phases, control blood tests, etc.). The argumentation for CEA conclusions is based on physical units, where, for example, in Diabetes Mellitus type 2, the HbA1c percentage is measured after a certain treatment has been used.

This method considers not only the additional cost of a new health technology, but also the additional benefits for the patient. Therefore, clinical effectiveness is measured using intermediate clinical outcomes by measuring Life-Years Gained (LYG) by applying a treatment. Such prioritization is called the extra-welfarist approach, whose goal is to maximize health effects in a resource-constrained system.

We can conclude that the main objectives of CEA are:

1) Determination of the price of a new intervention
2) Investigation of the reimbursement option of the new intervention
3) Involvement in the development of clinical guidelines on medication prescription status by physicians

Cost-utility analysis (CUA)

While CEA does consider the LYG, it is questionable how acceptable and bearable the gained life years would be for a patient. For example, one could argue that chemotherapy does increase lifespan, but the quality of such a life can be lower due to several side effects, like nausea, vomiting, diarrhea, fatigue, etc. Therefore, effectiveness is not the ultimate possible value.

A life year gained can be adjusted by quality parameters, thus forming a new term, "Quality-adjusted Life Years" (QALY). This means that the main measurement isn't just an extension of life, but of a life with the utility of the patients.

Therefore, the key features of CUA are:

1) Effectiveness is measured by considering the utility of the patients (QALY)
2) QALYs are calculated by combining the remaining years of life with the quality adjustments.

Cost-minimization analysis (CMA)

Monetary units are also a possible measuring scale, especially when it comes to comparing two different medical interventions which reach a similar or even same outcome. The term for two medical interventions which similar outcomes is called clinical equipoise. Thus, CMA is used to propose how to reduce costs while keeping the outcomes of a medical intervention the same.

Cost-benefit analysis (CBA)

As mentioned in previous sections, there are direct and indirect costs of a health technology. For example, a medical intervention that increases QALYs might require additional hospitalization, which can be more expensive than providing care at home. Therefore, CBA is used to measure health outcomes in monetary terms. However, it is difficult to be used in the economic analysis of healthcare interventions.

Cost-threshold analysis (CTA)

In order to adequately classify any health technology based on either physical or monetary units, there must be a threshold on the graph accounting for both effectiveness and cost. Such a graph is demonstrated on the picture below and it is necessary to define the threshold through CTA, otherwise, any further economic evaluation would be aimless.

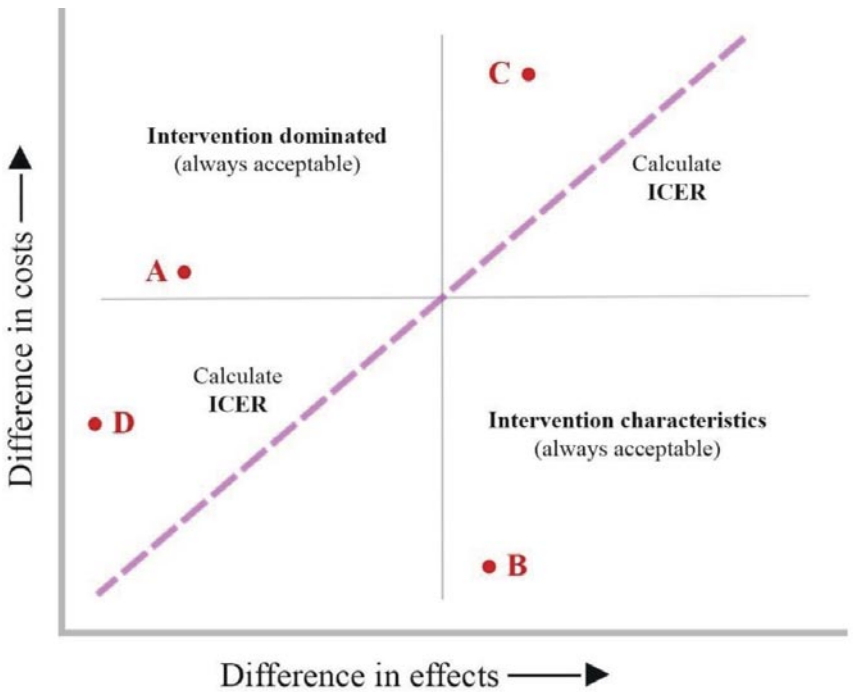

Figure 1. The figure represents a coordinate system which shows the possibilities of a health technology falling into one of the categories. Dominance refers to the property of a treatment being both less costly and has a better outcome. The dotted line is the threshold for cost and effect differences. Anything above the threshold is unacceptable and anything below it is acceptable. Points A, B, C, D represent example options of health technologies. Figure taken from "Essentials of Pharmacoeconomics" from Karen Rascati 2014.

Lastly, it is important to mention the steps of economic evaluation.

1. Define the decision problem
2. Conceptualize the model
3. Determine the relevant decision analytic model
4. Collect data to populate the decision analytic model

5. Define uncertainty
6. Calculate expected costs and consequences
7. Present results

Measuring health outcomes and utility

Measuring health outcomes and utility is a critical aspect of evaluating the effectiveness and efficiency of a healthcare intervention, a medication, a medical device, or any other sort of healthcare technology.

Health outcomes represent changes in health status resulting from healthcare interventions or treatments, encompassing multiple dimensions such as physical, mental, and social well-being [4]. These outcomes can be measured through various means, including clinical assessments (physical examination, laboratory testing, imaging), self-reported measures, or objective observations (e.g., gait or movement fluctuations noted by a healthcare provider or caregiver). Complex assessments may be necessary to establish the presence or absence of certain health outcomes, which are defined by clinical protocols to ensure scientifically valid outcome measurements. To minimize potential bias in assessing health outcomes, objective and standardized methods are applied alongside clinical assessments conducted by well-trained healthcare specialists, who may be independent and blinded to study details or subject information [4]. In contrast, health utility measures the strength of an individual's preferences for specific health outcomes or health-related quality of life. Utility measures, such as the Quality-Adjusted Life Year (QALY), quantify individual preferences for health outcomes under conditions of uncertainty. QALYs combine both quality and quantity of life into a single index and are frequently employed in cost-utility analyses to evaluate the efficiency of healthcare interventions.

To achieve the ultimate goal of estimating a patient's quality of life and facilitating pharmacoeconomic analysis, it is essential to combine both health outcome-related measurement instruments and utility measures. Examples of these include Health-Related Quality of Life (HRQoL) measures and Clinical Outcome Measures. Certain health outcome measures focus solely on a single aspect or dimension of health, such as mortality and survival. While the length of life is undeniably a significant aspect of health, the quality of life experienced is equally important. Consequently, numerous measures have been developed to describe the various dimensions or attributes of HRQoL, and the different levels achieved within each [4]. Combining health outcome measures with utility measures enables a more comprehensive understanding of the overall impact of healthcare interventions on patients' lives, ultimately informing decision-making and resource allocation in healthcare.

Several HRQoL instruments exist to collect and generate data about a patient's health state and convert them to utilities. These survey instruments are classified into different types based on their aim, approach, and preference weight. A patient's health state can be measured directly using methods such as Standard Gamble (SG), Time Trade-Off (TTO), or Visual Analog Score (VAS). In these methods, patients directly provide a score for their quality of life without considering other factors such as societal, economic, or psychological factors affecting their daily lives. Direct methods allow patients to describe their own health state rather than rely on a predefined health state description. However, they raise concerns, mainly ethical ones, as patients might be asked to choose between dying and remaining in the same health state. Additionally, it is uncertain whether all patients' health states are equally represented since patients with the poorest health states might be unwilling to provide values [5].

In contrast, preference-based instruments are more widely applied [6]. These instruments comprise a unique

descriptive health classification approach and a preference weight algorithm to generate utility scores, allowing them to cater to different needs, be more precise, and adapt to various settings on a case-by-case basis. Examples of preference-based instruments include EQ-5D (two versions: EQ-5D-3L and EQ-5D-5L), Short-Form 6-Dimension (SF-6D), Health Utilities Index (two versions: HUI2 and HUI3), Assessment of Quality of Life (several versions, e.g., AQoL 6D and 8D), and 15D. These instruments are further classified into generic-based questionnaires, condition-specific questionnaires, nationality-specific, population-specific [5,7].

According to Rowen et al., [5], a preference weight is a "numerical judgment of the desirability of a particular outcome or situation" [5]. Each health state is defined based on the chosen descriptive system. Preference weights are elicited from either the general population, patients, or healthcare experts. People are asked to rate their quality of life using a direct method (e.g., SG, TTO, VAS), and their responses serve as elicitation. Determining the preference weight at the outset is crucial since it converts health state responses into utility. Generic-based questionnaires, also known as Multi-Attribute Utility Instruments (MAUIs), assess HRQoL status across various clinical areas and interventions without focusing on a specific disease. Comprising a self-completion questionnaire, MAUIs follow the basic design of all preference-based questionnaires, enabling the acquisition of quality-adjustment weights and generating utility data for QALY calculations in economic evaluations. Their descriptive system consists of HRQoL domains for which patients provide scores to determine their health status [2].

In this case, preference weights are derived from the general population rather than patients, reflecting healthcare users' perceptions and feelings about an intervention [8,9]. Policymakers and scientists generally prefer considering taxpayers' opinions, as they represent the entire community, and many are end-users of the investigated intervention, rather than focusing solely on patients with specific conditions [8,9]. Furthermore, condition-specific questionnaires, another type of preference-based questionnaire, are dedicated to a particular disease or condition and include domains that address the signs and symptoms of that condition [7]. Being disease-sensitive, they employ preference weights derived from patient cohorts to reflect group needs. However, current pharmacoeconomic guidelines do not recommend condition-specific questionnaires as the preferred method due to the lack of comparability among different healthcare interventions and diseases. This absence of comparability hampers proper estimation of resource allocation among diseases [5]. Nationality- or population-specific questionnaires aim to provide evidence for value sets that differ across countries or populations due to language, cultural settings, ethics, social norms, and other factors [5].

Finally, it is evident it is more challenging to incorporate results from multidimensional health status measures in pharmacoeconomic ratios because measuring outcomes (i.e., effectiveness) involves multiple scores representing various aspects of the disease. Additionally, the range of possible scores varies between the numerous available health status instruments, complicating interpretation. To evaluate a patient's HRQoL, researchers can choose tools that assess general health status using generic measures or focus on particular aspects of the disease under study using disease-specific measures. Generic measures offer a broader view of the patient's overall health status, while disease-specific measures provide a more detailed understanding of the disease's impact on the patient's life.

Decision Analysis – Analytic Approaches

Undoubtedly, life sciences have made remarkable progress during the last decades. Technology and research advancements have allowed both the improvement of existing therapeutic approaches as well as the development of radical innovations to tackle major health issues. However, no matter how beneficial an

approach is, if it is not economically viable, unfortunately, it cannot stand on the market or get reimbursed by the national healthcare system. Therefore, health economists must conduct thorough investigation on the costs and benefits of different alternatives and clearly present the outcomes to the decision makers.

Through decision analysis, a suitable model is developed, aiming to construct and structure decisions. Including multiple methods and tools in order to clearly depict the crucial aspects of a decision situation, they provide quantitative support for decision makers. In particular, alternative options and their respective consequences are compared, so that decision makers can evaluate them, depending on the cost and the healthcare outcomes [10]. The most common models used in health economics for this purpose, are "Decision Trees" and "Markov Models".

Regarding decision trees, according to Gray and his colleagues, a decision tree is defined as "branching structure in which each branch represents an event that may take place in the future". The identification of different alternatives and linkage of relative events, after a certain process, allows for clarification of complex decisions.

However, what are the necessary steps to be followed in order to develop a decision model for economic evaluation?

- Defining the question – What's the decision problem to be face?
- Defining the boundaries of the model
- Structure the most appropriate model for use in economic evaluation
- Identifying and synthesizing evidence – populating the model
- Evaluating the model
- Handling uncertainty and heterogeneity
- Assessing the value of additional research

These basic stages are mandatory, independent of the model type to be used [10,11].

Before developing a decision model, two concepts of vital importance must be clearly comprehended.

Since analysts are trying to predict future outcomes in order to compare different alternatives, it's important to calculate probabilities. Probabilities as a number indicate the likelihood of an event taking place in the future. Some probability concepts frequently used in decision models are:

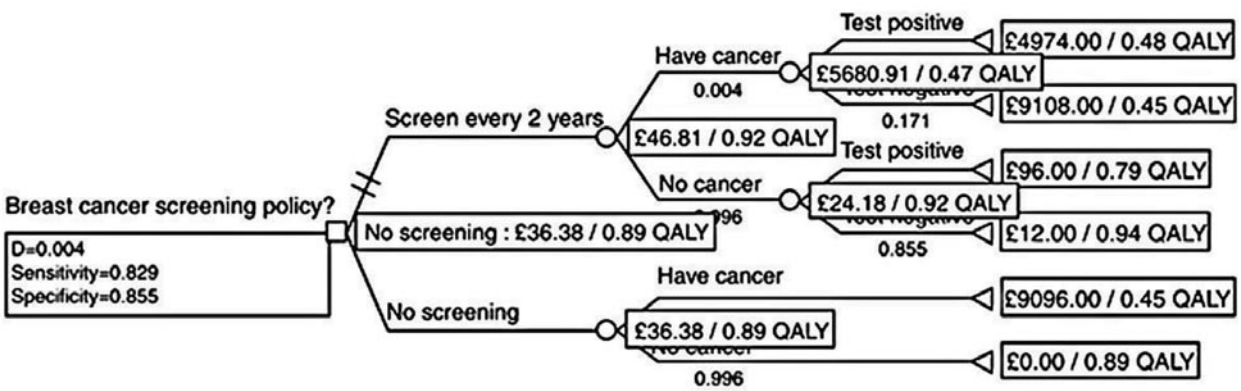

Figure 2. Decision tree mode comparing breast cancer screening policies. The costs (in £) and quality-adjusted life years (QALYs) of two alternatives are illustrated. More specifically, the option of breast cancer screening is evaluated against no screening. The model considers disease prevalence (D=0.004), test sensitivity (0.829) and specificity (0.855). The branches of the decision tree showcase the possible clinical pathways for women with and without cancer, according to the test results (true positive, false negative, false positive, true negative). The final nodes display the expected cost and QALYs for each pathway, while intermediate nodes show aggregated values. Figure taken from Gray et al. 2010.

- Joint probability: The likelihood of two events occurring together.
- Conditional Probability: If event B is known to have occurred, what's the probability of event A occurring?
- Independence: Event A does not affect the occurrence of event B and vice versa

In addition, each possible prognosis can be directed to a certain cost or outcome. These can be defined as "payoffs" [11].

In conclusion, all these stages and concepts are depicted below in an example of a decision tree, calculating expected values relating to a breast cancer screening policy [10].

Markov Models and Long-Term Projections

Even though decision trees are essential for following health consequences of decisions, multiple diseases and conditions have more complex outcomes and long follow-up periods. Moreover, patients can transition between different health state over longer periods of time, called cycles.

Markov analysis is the analytical framework that uses "disease states" to represent possible consequences of an intervention over a cycle [2]. These "disease states" are mutually exclusive and exhaustive and, thus, each individual patient represented in the model can be in one and only one of these disease states at any given time.

There are five steps in Markovian modelling:

1) Choose the health states that represent outcomes
2) Determine possible transitions between states
3) Choose the length of a cycle and how many cycles should be analysed
4) Estimate the probabilities associated with transitioning
5) Estimate the costs and outcomes associated with each option

It is essential to notice that probability plays and important role in Markov models, so uncertainty is unavoidable.

Uncertainty comes in different types:

1) Structural uncertainty (model structure)
2) Variability due to heterogeneity (sample characteristics)
3) First-order uncertainty (nature is inherently stochastic)
4) Second-order uncertainty (exact values of statistical parameters aren't always available)

Thus, whenever a Markov model is deployed, a sensitivity analysis needs to be performed to determine how much the estimations can vary. There are two main types of sensitivity analyses:

a) **Deterministic sensitivity analysis,** which is based on using confidence intervals to measure variable changes.
b) **Probabilistic sensitivity analysis,** which is based on sampling parameters from their respective distributions, and it poses that ICER has a probabilistic nature.

Furthermore, Markov analysis has two main flaws. One flaw is the less transparent nature of the analysis due to its complex nature. Another flaw is the Markovian assumption that transition between two health states is not dependent on the previous cycles. Medical history is an essential part of a patient's treatment and outcome determination, however, so Markov analysis needs to be modified with tunnel states, which involve difficult computations.

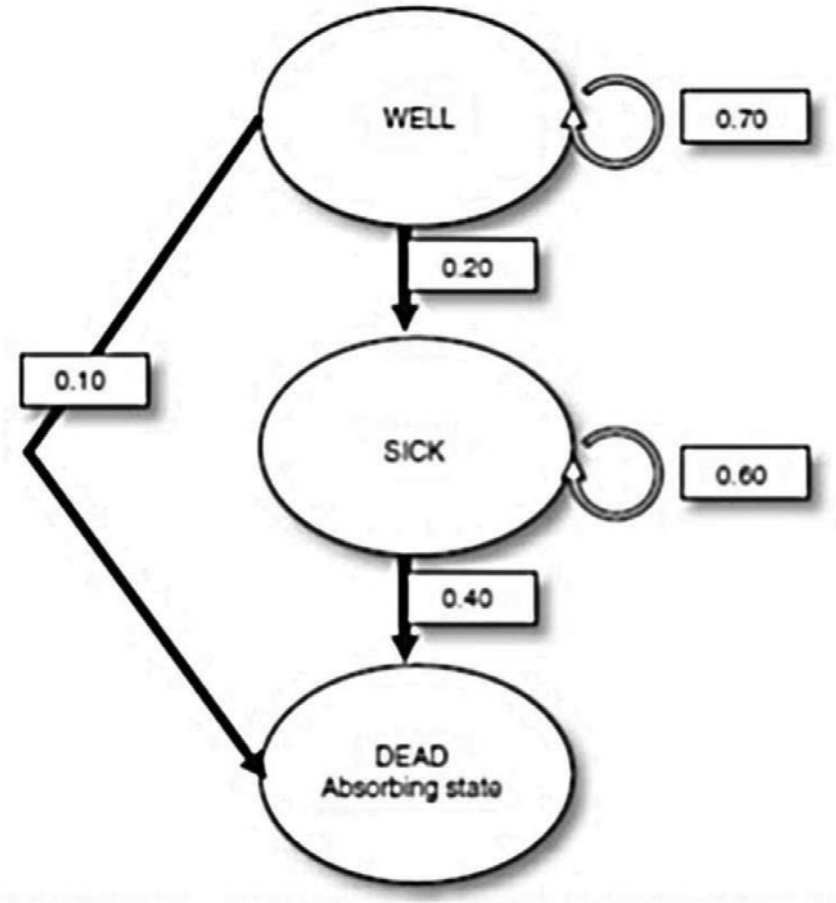

Figure 3. The figure represents a Markov model. Each ellipse represents a cycle, which can be repeated. The arrows show possible transitions and the numbers associated with them are the probabilities of transitioning to the named state. An absorbing state is a state which cannot transition to another. Figure was taken from "Essentials of Pharmacoeconomics" by Karen Rascati 2014.

Conclusion

This paper is by no means an exhaustive treatise on pharmacoeconomics, nor was it intended to be. Like any field, pharmacoeconomics deals with subjects whose details and frameworks are dealt with under scrutiny using methodology which needs to be studied, understood and practiced.

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Personalized Medicine Begins at Birth: Newborn Screening for Spinal Muscular Atrophy in Serbia as a Model of Individualized Care

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Abstract

Spinal muscular atrophy (SMA) is a leading genetic cause of infant mortality, with disease-modifying therapies achieving maximal benefit when administered presymptomatically, underscoring the medical, ethical, and public health imperative for newborn screening (NBS). In 2021, Serbia launched its first genetic NBS initiative for SMA, centralized at the Faculty of Biology, University of Belgrade, a center of SMA diagnostics and research since 1997. Over a 17-month pilot study, 12,000 newborns across two maternity hospitals were screened using dried blood spots analyzed by qPCR for *SMN1* absence, with confirmatory MLPA testing and *SMN2* copy number determination. Following pilot study success, the national SMA screening program began on September 15, 2023, now encompassing 52 public and 6 private maternity hospitals. By September 09, 2025, 119,735 newborns had been screened, identifying 19 infants with SMA; 17 received immediate therapy—6 with 2 *SMN2* copies, 7 with 3 copies, and 4 with 4 copies—while 2 infants with 5 *SMN2* copies remain under observation. Treated infants remain largely asymptomatic. A multidisciplinary Expert SMA Commission ensures individualized treatment decisions, integrating genetic, clinical, and laboratory data. The program establishes Serbia's first reliable SMA incidence estimate (1:6,302 births) and demonstrates that presymptomatic diagnosis, structured screening workflows, and personalized therapy can transform SMA from a severe, life-limiting disease into a manageable condition. Serbia's experience provides a compelling model for integrating precision medicine into national health systems through coordinated collaboration among academia, patient advocacy, industry, and government, illustrating how early genetic diagnosis and tailored interventions can fundamentally change disease trajectories.

Key words: Spinal muscular atrophy, newborn screening, *SMN2* copy number, presymptomatic treatment, personalized medicine

Personalizovana medicina na rođenju: neonatalni skrining za spinalnu mišićnu atrofiju u Srbiji kao model individualizovanog pristupa

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Apstrakt

Spinalna mišićna atrofija (SMA) predstavlja vodeći genetički uzrok smrtnosti odojčadi, pri čemu terapije koje menjaju tok bolesti imaju najveći efekat kada se primene presimptomatski. To naglašava medicinski, etički i javnozdravstveni značaj neonatalnog skrininga. Srbija je 2021. pokrenula prvi genetički neonatalni skrining za SMA, centralizovan na Biološkom fakultetu Univerziteta u Beogradu, koji od 1997. ima ekspertizu u istraživanjima i dijagnostici SMA. U 17-mesečnoj studiji izvodljivosti testirano je 12,000 novorođenčadi iz dva porodilišta. Uzorci suvih krvavih mrlja analizirani su metodom qPCR radi otkrivanja odsustva gena *SMN1*, uz potvrdno testiranje i određivanje broja kopija *SMN2* metodom MLPA. Nakon završetka studije izvodljivosti, nacionalni program skrininga za SMA počeo je 15. septembra 2023. i danas obuhvata 52 državna i 6 privatnih porodilišta. Do 9. septembra 2025. testirano je 119,735 novorođenčadi i identifikovano je 19 SMA pozitivnih beba. Od toga je 17 odmah započelo terapiju—6 sa dve kopije *SMN2*, 7 sa tri kopije i 4 sa četiri kopije—dok su dva novorođenčeta sa pet kopija pod kliničkim nadzorom. Lečene bebe su uglavnom ostali asimptomatski. Multidisciplinarna Stručna komisija za SMA donosi individualizovane odluke o terapiji, kombinujući genetičke, kliničke i laboratorijske podatke. Program je dao prvu pouzdanu procenu učestalosti SMA u Srbiji (1:6,302) i pokazao da presimptomatska dijagnoza, jasno definisani protokoli i personalizovana terapija mogu SMA pretvoriti iz teškog, životno ograničavajućeg oboljenja u (iz)lečivu bolest. Srpsko iskustvo pruža model za integraciju precizne medicine u nacionalne zdravstvene sisteme kroz saradnju akademske zajednice, pacijenata, industrije i države.

Ključne reči: spinalna mišićna atrofija, neonatalni skrining, broj kopija *SMN2*, presimptomatsko lečenje, personalizovana medicina

Spinal Muscular Atrophy: A Genetic Disorder with Devastating Consequences

Spinal muscular atrophy (SMA) is a progressive autosomal recessive neuromuscular disorder with a reported global incidence ranging from approximately 1 in 6,000 to 1 in 10,000 live births (1). Prior to the advent of disease-modifying genetically designed therapies, SMA was recognized as the leading genetic cause of infant mortality. The proximal form of the disease was first described more than a century ago—initially by Guido Werdnig in Austria in 1891, followed soon after by Johann Hoffmann in Germany—laying the foundation for the clinical recognition of SMA as a distinct entity (2,3,4,5).

The disorder is characterized by the progressive degeneration of alpha motor neurons in the spinal cord and brainstem, leading to symmetrical muscle weakness, atrophy, and—particularly in the most severe cases—early respiratory failure (6).

The clinical spectrum of SMA is broad, encompassing phenotypes ranging from the severe early-onset Type 1 form to milder, later-onset forms (Types 2–4) (7,8). Infants with **SMA Type 1 (Werdnig–Hoffmann disease, OMIM #253300)** usually present within the first six months of life with profound proximal muscle weakness, most pronounced in the legs. They often display generalized hypotonia in a characteristic "frog-leg posture," tongue fasciculations, absent reflexes, and paradoxical diaphragmatic breathing due to intercostal weakness. Feeding difficulties and risk of aspiration emerge early as bulbar weakness progresses, and despite normal cognition, these children never achieve independent sitting and typically face life-threatening respiratory complications within the first two years of life (9,10).

Children with **SMA Type 2 (Dubowitz disease, OMIM #253550)** achieve independent sitting but are never able to walk (11). Symptoms generally appear between 6 and 18 months, with some variation. Progressive weakness may lead to loss of motor abilities, scoliosis, and respiratory issues, though life expectancy is usually longer than in Type 1.

SMA Type 3 (Kugelberg–Welander disease, OMIM #253400) is milder: affected children are able to walk independently, though many lose this ability later in life (11). Onset typically occurs after 18 months, with earlier-onset cases classified as Type IIIA and later-onset as Type IIIB. Functional limitations develop gradually, but individuals often remain ambulant for years.

At the mildest end of the spectrum lies **SMA Type 4 (OMIM #271150)**, which appears in adulthood, usually after the age of 21 and most commonly in the mid-30s. Patients present with slowly progressive proximal weakness but often maintain independence for decades. Life expectancy is generally normal, and complications such as bulbar involvement or respiratory failure are rare (12).

However, this traditional classification is largely cross-sectional and does not adequately capture the dynamic evolution of the clinical picture, particularly in the era of effective treatments. The advent of novel therapeutic options and accompanying clinical trials has reshaped natural history studies and prompted an update of the international consensus. Today, overlap among SMA types is frequently observed, and the predictive value of a classification based solely on initial motor milestones is limited. As a result, the classical type-based system is becoming less practical for modern clinical studies. To ensure consistency in outcome measures and long-term follow-up, a functional reclassification has been recommended, categorizing patients as non-sitters, sitters, or walkers. This framework acknowledges the SMA phenotype as a continuum and emphasizes the patient's current functional abilities and response to therapy. Still, it is important to recognize that no single classification system fully encompasses the heterogeneity of SMA presentations and disease trajectories (8).

The underlying genetic etiology most commonly involves a homozygous absence (due to deletion or gene conversion) of the *SMN1* gene (OMIM# 600354), located on chromosome 5q13 (13). In approximately 2–5% of affected individuals, SMA results from compound heterozygosity, in which one *SMN1* allele is deleted or converted and the second carries a pathogenic intragenic variant (e.g., point mutation or small insertion/deletion) (14). In both scenarios, the result is a functional deficiency of survival motor neuron (SMN) protein, which leads to progressive motor neuron degeneration and the clinical manifestations of SMA.

The SMN protein is a 38-kDa molecule expressed in virtually all cell types, localized both in the cytoplasm and within specialized nuclear structures known as Gemini of Cajal bodies ("gems"). Its expression is ubiquitous, but its biological roles are complex and not yet fully elucidated. Together with Gemin proteins, SMN forms a multiprotein complex that acts as a molecular chaperone, facilitating the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs)- key components of the pre-mRNA splicing machinery. SMN also participates in arginine methylation of certain splicing-related proteins, in axonal mRNA transport within motor neurons, and potentially in processes at the neuromuscular junction (15). The selective vulnerability of motor neurons to reduced SMN levels may relate to their exceptionally long axons and high dependency on efficient axonal mRNA trafficking and splicing. Complete loss of SMN is embryonically lethal in model organisms, while partial deficiency leads to the motor neuron degeneration characteristic of SMA (16).

Disease severity correlates closely with *SMN2* copy number (OMIM# 601627), a nearly identical paralog of *SMN1* that partially compensates for SMN protein loss. Due to a synonymous substitution in exon 7 (c.840C>T, NM_000344.4), the majority (~90%) of *SMN2* transcripts are alternatively spliced to exclude exon 7, resulting in a truncated and non-functional protein (17). Only approximately 10% of *SMN2*-derived transcripts produce full-length, functional SMN protein. Although the number of *SMN2* copies theoretically ranges from one to eight, most individuals with SMA possess between two and four copies (18). Generally, a higher *SMN2* copy number is associated with a milder disease phenotype, although exceptions exist, and the correlation is not absolute (19,20,21). More specifically, 73% of SMA Type I patients carry two *SMN2* copies, 78% of Type II patients carry three copies, 50% of Type IIIa patients carry three copies, 61% of Type IIIb patients carry four copies, and 75% of Type IV patients carry four copies. The predictive value of three *SMN2* copies is lower than that of two or four copies, as three copies can be observed in 20% of Type I, 78% of Type II, and 51% of Type III patients (18,19,22). Despite this strong trend, *SMN2* copy number alone cannot reliably predict disease course. Additional factors are thought to contribute, including other genes influencing motor neuron survival and sequence variants within *SMN2* that alter the proportion of full-length protein produced. Intrafamilial variation further illustrates this complexity: some affected family members carry the same *SMN2* copy number, but show very different phenotypes. These findings underscore the role of splicing modulators and additional genetic modifiers beyond *SMN2* copy number in shaping disease severity (12,23).

Transformative Therapies Have Redefined the Prognosis of SMA

Until recently, there were no disease-modifying treatments for SMA, and management was limited to supportive and palliative care. For nearly a century after the first clinical descriptions, research focused primarily on characterizing the disease course and pathology across the spectrum of severity. A major turning point came in 1995 with the identification of the *SMN1* gene as the underlying cause of SMA. This discovery, combined with the creation of animal models, provided critical insights into disease pathophysiology and laid the groundwork for the development of targeted therapies.

The subsequent development and regulatory approval of three targeted therapies have revolutionized SMA treatment: nusinersen (Spinraza®, Biogen), onasemnogene abeparvovec (Zolgensma®, Novartis), and risdiplam (Evrysdi™, Roche/Genentech).

- Nusinersen is an intrathecally administered antisense oligonucleotide that promotes inclusion of exon 7 during SMN2 pre-mRNA splicing, thereby increasing functional SMN protein levels. Treatment begins with a loading phase of four doses over two months, followed by maintenance doses every four months. Due to its delivery method, nusinersen must be administered in a controlled clinical setting by trained personnel (24),
- Risdiplam is an orally administered small-molecule splicing modifier that also enhances exon 7 inclusion in SMN2 transcripts. Taken daily on a lifelong basis, it offers the advantage of home-based treatment (25),
- Onasemnogene abeparvovec is a one-time intravenous gene replacement therapy delivering a functional copy of SMN1 via a self-complementary adeno-associated viral vector (scAAV9), enabling sustained SMN protein production from a single infusion (26).

Clinical trials and real-world studies demonstrate that all three therapies significantly alter SMA's natural history. When administered in the presymptomatic stage, these interventions are associated with dramatic preservation of motor function, achievement of motor milestones previously considered unattainable, prolonged survival, and improved quality of life. These outcomes highlight the transformation of SMA from a leading genetic cause of infant mortality into a condition that can now be actively modified with targeted interventions.

In parallel with therapeutic advances, multidisciplinary care for SMA has progressed significantly, guided by international consensus guidelines and strengthened by collaboration among clinicians, researchers, families, and patient organizations (27,28,29). The success of SMA therapies has firmly established the disease as a paradigm of modern personalized medicine. The clinical paradigm has shifted from solely supportive care to active disease modification, underscoring the critical importance of early diagnosis and intervention, particularly through newborn screening (NBS).

The Imperative for Newborn Screening: Treating SMA Before Symptoms Appear

SMA often progresses rapidly and irreversibly within the first weeks or months of life. By the time clinical signs—such as hypotonia, feeding difficulties, or paradoxical breathing—become apparent, substantial motor neuron loss has already occurred. This highlights a narrow therapeutic window during which treatment must be initiated to prevent or minimize irreversible damage (30).

Early identification is therefore critical, yet diagnosis is frequently delayed due to the asymptomatic nature of SMA at birth. Median diagnostic delays vary markedly by SMA subtype. A systematic review found weighted mean delays of approximately 3.6 months for Type I, 14.3 months for Type II, and 43.6 months for Type III (31). A real-world Chinese cohort reported median delays of ~3.4 months for Type I, ~4.1 months for Type II, and ~11.4 months for Type III (32). While three months may not seem long, it can be devastating for infants with SMA Type 1, as nearly 95% of motor neurons may already have degenerated within the first weeks of life (33). Consequently, a child diagnosed two months after birth may have already permanently lost the ability to sit independently, breathe unassisted, chew, or swallow. Even with treatment initiation at that point, fatal outcomes may not be preventable.

This diagnostic delay remains the single greatest barrier to achieving the full potential of modern SMA therapies. When irreversible damage occurs before therapy begins, the clinical benefits of even costly treatments are substantially reduced.

Evidence from a recent study in Serbia demonstrates that infants identified through neonatal screening exhibit markedly elevated levels of phosphorylated neurofilament heavy chain (pNF-H) in cerebrospinal fluid and plasma, reflecting ongoing neuroaxonal injury (34). These findings confirm that even presymptomatic newborns with increased *SMN2* copy number can display early neuronal degeneration, highlighting the need for immediate intervention and validating pNF-H as a biomarker for disease activity and potential treatment response.

Newborn screening has thus emerged as the only reliable strategy to identify affected infants before symptoms appear, enabling timely intervention that can prevent irreversible neuromuscular damage. From a public health perspective, SMA fulfills multiple Wilson and Jungner criteria for screening: it is a serious, often fatal condition with a well-characterized natural history, a reliable and feasible test exists (typically PCR-based detection of *SMN1* exon 7 presence/absence), effective therapies are available, and early intervention dramatically improves outcomes.

International pilot programs and real-world data reinforce the cost-effectiveness and ethical justification of including SMA in newborn screening panels. Consequently, a growing number of countries have implemented SMA screening to ensure affected infants have equitable access to life-saving therapies (35,36,37,38). Nevertheless, disparities persist. According to the SMA Newborn Screening Alliance interactive map, only 66% of newborns in geographical Europe currently undergo SMA screening (39), highlighting the urgent need for broader implementation to ensure that all children benefit from early diagnosis and timely care.

While newborn screening is highly effective, prenatal and carrier screening programs have also been implemented in some countries. National population-based carrier screening in Israel, introduced in 2013, identified at-risk couples before pregnancy, with a carrier frequency of approximately 1 in 54 (excluding silent carriers who could not be detected with the method used for screening). Despite this, only about half of SMA cases were prenatally detected, and decisions regarding pregnancy termination were influenced by ethical, cultural, and religious considerations (40). Studies report that only a minority of pregnancies diagnosed with SMA were terminated, and social stigma or reluctance to undergo testing further limits carrier screening effectiveness.

Several genetic and technical factors reduce carrier screening accuracy:

- **Cis Configuration (2+0 Genotype):** Some heterozygous carriers of *SMN1* absence possess two copies of the *SMN1* gene on the same chromosome (cis configuration or 2/0 genotype). Standard quantitative analyses of *SMN1* copy number cannot differentiate these carriers from individuals with two *SMN1* copies on separate chromosomes (trans configuration or 1/1 genotype), leading to potential false-negative results (41,42). Studies have estimated that approximately 5.5% of SMA carriers exhibit this 2/0 genotype, which may not be detected by conventional screening methods;
- ***De Novo* Mutations:** Approximately 2% of SMA cases arise from *de novo SMN1* rearrangements, reflecting the gene cluster's high mutation rate due to repeated sequences that predispose to unequal crossover and recombination, indicating that a significant proportion of affected individuals may not have a family history of the condition (43);

- **Intragenic Mutations:** Rare intragenic mutations in the *SMN1* gene (14), when paired with the more common *SMN1* absence, may be responsible for SMA but are undetectable by standard dosage analyses. Such mutations can lead to false-negative results in carrier screening;
- **Ethnic Variability:** The residual risk of being an SMA carrier based on genotype alone varies between 1:99 and approximately 1:1, depending on ethnicity. Ethnic-specific risk values have been identified, highlighting the importance of considering ethnic background in carrier screening (44);
- **Screening Limitations:** Carrier testing does not predict SMA subtype or severity. Approximately 60% of affected infants develop Type I SMA, while 40% develop milder forms (Types II–III). This variability underscores the complexity of SMA and the limitations of carrier screening in predicting clinical outcomes;
- **Cost and Accessibility:** Widespread carrier screening is expensive, relies on public awareness, and may be hindered by stigma or reluctance to participate. These factors can limit the effectiveness and reach of carrier screening programs. A study assessing the cost-effectiveness of genetic screening for SMA found that universal screening had a high incremental cost-effectiveness ratio, indicating that it may not be cost-effective in all settings (45). Additionally, the implementation of such programs requires significant healthcare infrastructure and resources, which may not be available in all regions.

These limitations underscore why newborn screening remains the most equitable, effective, and ethically justified strategy for SMA: it identifies affected infants early, regardless of parental carrier status, cultural background, or ethical decisions, ensuring timely access to disease-modifying therapies.

Serbia's Role in SMA Diagnostics and Therapeutics

Serbia has played a proactive role in SMA research and diagnostics in the region. The Faculty of Biology at the University of Belgrade first introduced molecular genetic testing for SMA in 1997 and has since continuously advanced its diagnostic capabilities. Since 2011, multiplex ligation-dependent probe amplification (MLPA) has been routinely used for diagnostic testing, *SMN2* copy number determination, and detection of complex rearrangements such as hybrid *SMN* genes. Together with the Institute for Mother and Child Health Care of Serbia "Dr. Vukan Čupić," molecular diagnosis has been provided for hundreds of symptomatic patients, accompanied by genetic counseling and carrier or prenatal testing for at-risk families (46,47).

The therapeutic landscape in Serbia advanced significantly in 2018 with the introduction and full reimbursement of nusinersen and risdiplam by the Republic Health Insurance Fund, followed in 2023 by the inclusion of gene-replacement therapy. Initially, access to these treatments was limited to symptomatic individuals, which curtailed their full potential. This limitation underscored the urgent need for presymptomatic diagnosis through newborn screening—a decision that is clinically sound, ethically justified, and economically rational. With the national rollout of NBS for SMA, Serbia has aligned with international standards and ensured that all affected infants have equal access to timely, life-altering therapy.

Implementing National Newborn Screening for SMA in Serbia: From Pilot to Nationwide Rollout

The implementation of national newborn screening for SMA in Serbia is a story of perseverance, collaboration, and a clear public health vision. What began as a limited initiative driven by academic researchers

and patient advocates evolved into a comprehensive, government-supported national program. The transition was marked not by major technological breakthroughs, but by a concerted effort to build trust, align stakeholders, and demonstrate the life-changing value of early SMA detection (48).

Although the importance of newborn screening is widely recognised, approaches vary across European countries (49). In Serbia, prior to 2022, newborn screening was routinely performed for phenylketonuria (PKU), congenital hypothyroidism (CH), and cystic fibrosis (CF), but SMA screening had not yet been considered, despite the availability of SMA-modifying therapies.

Establishing a successful newborn screening system required detailed planning of every step—from sample collection and transport, to laboratory testing, reporting results to paediatricians, informing parents, initiating therapy, and monitoring patients. The Serbian journey began in 2021 with a feasibility study for newborn screening for SMA, prepared in collaboration with the Association SMA Serbia and the University Children's Hospital. The proposal was submitted to the Ministry of Health, but was rejected without feedback, showing that expertise and strong collaborations alone do not guarantee progress. Later that year, a round table on SMA brought together government representatives, yet newborn screening was treated more as science fiction than a pressing public health priority. Fortunately, pharmaceutical representatives present recognized the urgent need to "screen the unseen" and agreed to fully fund a one-year feasibility project. The study was then launched at the Obstetrics and Gynaecology Clinic "Narodni Front" in Belgrade, the country's largest maternity hospital, with approximately 8,000 births annually. Using an opt-in approach, only newborns whose parents consented were tested. Twelve months later, the program expanded to a second site, the University Clinical Center Kragujevac, bringing the total number of screened newborns during the feasibility study to around 12,000. This pilot program demonstrated that presymptomatic diagnosis through newborn screening is both feasible and impactful: two SMA-affected newborns identified during this phase, along with a 16-month-old sibling of one, received early treatment and remained asymptomatic. These outcomes, together with the active involvement of families and clinicians as advocates, helped shift public and institutional attitudes toward SMA screening.

Following the pilot's success, the Republic Health Insurance Fund recognized the medical and societal value of the program and took formal steps to integrate SMA screening into the national health system. A significant milestone was the establishment of a national Expert Commission for SMA. This body ensures that every diagnosed child receives a personalized treatment recommendation, based not only on genetic findings such as *SMN2* copy number, but also on clinical context and biochemical data. In this way, the Serbian approach has embedded personalized medicine directly into the public health infrastructure.

Since the national rollout in September 2023, 119,735 newborns have been screened. Nineteen infants with SMA have already been identified and seventeen received timely therapeutic intervention. Among these, six children had 2 copies of *SMN2*, seven had 3 copies, four had 4 copies, and two had 5 copies. From these data, the current SMA incidence in Serbia is estimated at 1 in 6,302 live births—the first reliable national estimate ever established. This incidence rate not only reflects the power of systematic newborn screening but also provides a critical epidemiological foundation for future planning of healthcare resources and therapy allocation. Therapy was initiated according to individual needs: children with two *SMN2* copies received onasemnogene abeparvovec (n = 5) or nusinersen (n = 1); those with three copies received risdiplam (n = 5) or nusinersen (n = 2); children with four copies were treated with Risdiplam, while the two children with five copies were not immediate candidates for therapy (27,28). These outcomes are summarized in Figure 1. One child received therapy late at 676 days (patient P12), after SMA was identified in a sibling through newborn screening, and the child had already developed symptoms. Patient P13 also received

therapy late because initial medical follow-up was neglected by the mother; the child developed herpes simplex virus–associated encephalitis, and only upon hospital admission could a definitive SMA diagnosis be established. The dashed line in the figure marks 42 days of life, the internationally recognized benchmark for initiating presymptomatic therapy. This threshold is based on pivotal clinical trials, most notably SPR1NT**,** which showed that infants treated before 6 weeks of age (<42 days) achieved age-appropriate motor milestones (50). In the cohort with two *SMN2* copies, all 14 children sat independently for ≥30 seconds, with 11 reaching this within the WHO-defined developmental window for normal development (48). Comparable outcomes were reported in presymptomatic risdiplam studies, such as RAINBOWFISH, where earlier treatment was strongly associated with improved functional achievements, including independent sitting and survival without permanent ventilation (51).

The Serbian experience thus confirms what global evidence has established: the first six weeks of life represent a critical therapeutic window in SMA, where early intervention can transform prognosis from severe disability or early death to near-normal development.

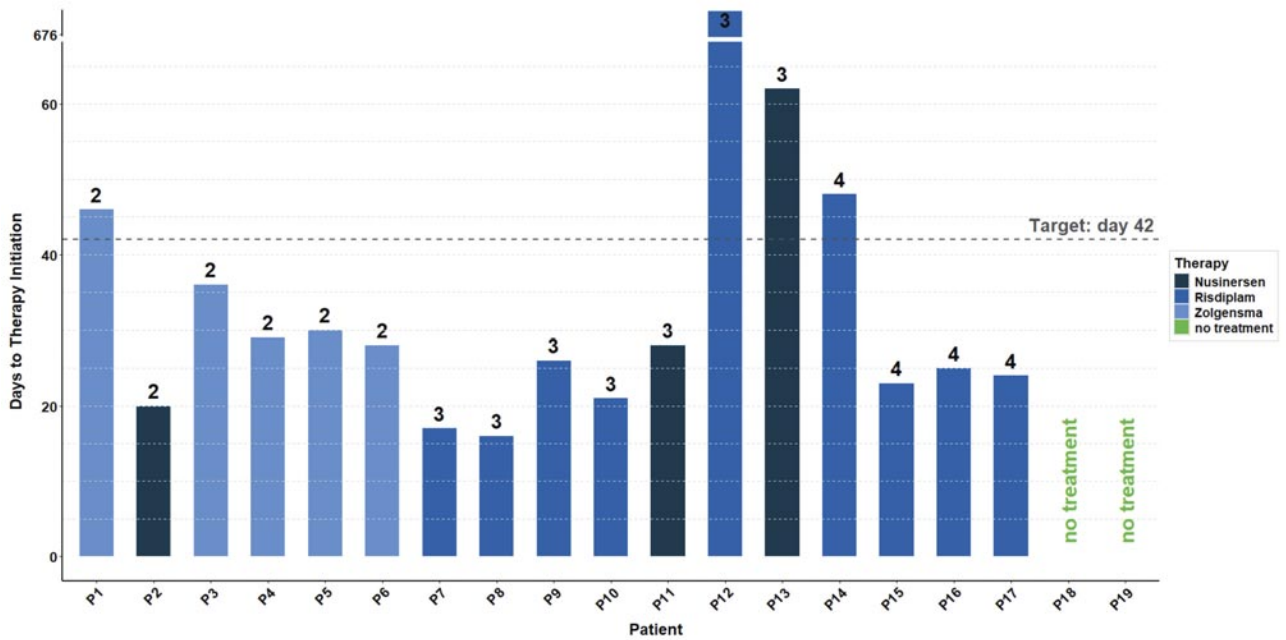

Figure 1. Age at therapy initiation, type of therapy, and *SMN2* copy number in children with spinal muscular atrophy identified through newborn screening. Bars represent the time from birth to therapy start for each patient (P1–P19). Bar color indicates the type of therapy: *Nusinersen* (dark blue), *Risdiplam* (blue), *Zolgensma* (light blue), and *no treatment*. Patients P18 and P19 had 5 *SMN2* copies and were not candidates for any approved SMA therapies. Numbers above each bar indicate the number of *SMN2* gene copies. Patient P12 received therapy late (at 676 days), after SMA was identified in a sibling through newborn screening, and already presented with symptoms. The dashed horizontal line indicates the target age for therapy initiation (Target: day 42). This cut-off reflects the inclusion criterion from pivotal clinical trials, in which treatment was initiated before 6 weeks of life (<42 days) to maximize therapeutic benefit (50,51). Light gray dashed horizontal lines mark 5-day intervals for easier visual reference.

The above-mentioned numbers reflect more than statistics—they represent lives fundamentally altered by early diagnosis and personalized treatment. The Serbian experience illustrates that introducing newborn screening for SMA does not necessarily require vast resources or advanced technology. Rather, it depends on strong intersectoral cooperation, the willingness to start small, and a commitment to scale based on evidence and trust. It also affirms that even smaller countries can become leaders in public health innovation when patient needs, scientific insight, and institutional support are aligned.

Establishing a Reliable Screening and Diagnostic Algorithm: The Backbone of Effective SMA Detection

The national newborn screening program for SMA in Serbia follows a well-defined and efficient algorithm that ensures rapid identification and confirmation of affected newborns (Figure 2). Screening begins with the collection of dried blood spot (DBS) samples from newborns within the first few days after birth, as part of the routine newborn screening panel. These samples are analyzed using a real-time quantitative PCR (qPCR) assay specifically designed to detect the homozygous absence of exon 7 in the *SMN1* gene, the most common genetic cause of SMA. This initial test differentiates screen-negative from screen-positive samples.

Crucially, during the pilot phase, a clear diagnostic workflow was developed to ensure accuracy and robustness. Newborns who screen positive are immediately subjected to a second-tier confirmatory MLPA test, performed twice: once from the DNA extract used for initial screening and once from an additional punch from the same Guthrie card. This second-tier assay serves two essential purposes: confirming the absence of *SMN1* (reduces false positives, minimizing unnecessary anxiety) and quantifying *SMN2* copy number that helps predict disease severity and guide therapeutic decisions. Once three screen-positive results are obtained, the finding is immediately communicated to the maternity hospital and the designated pediatric neurologist. The neurologist contacts the family without delay and schedules confirmatory blood sampling and a clinical check-up within 24 hours. The fresh blood sample is used not only for final confirmation of the screening result using MLPA, but also for final determination of *SMN2* copy number and potential baseline biomarker testing.

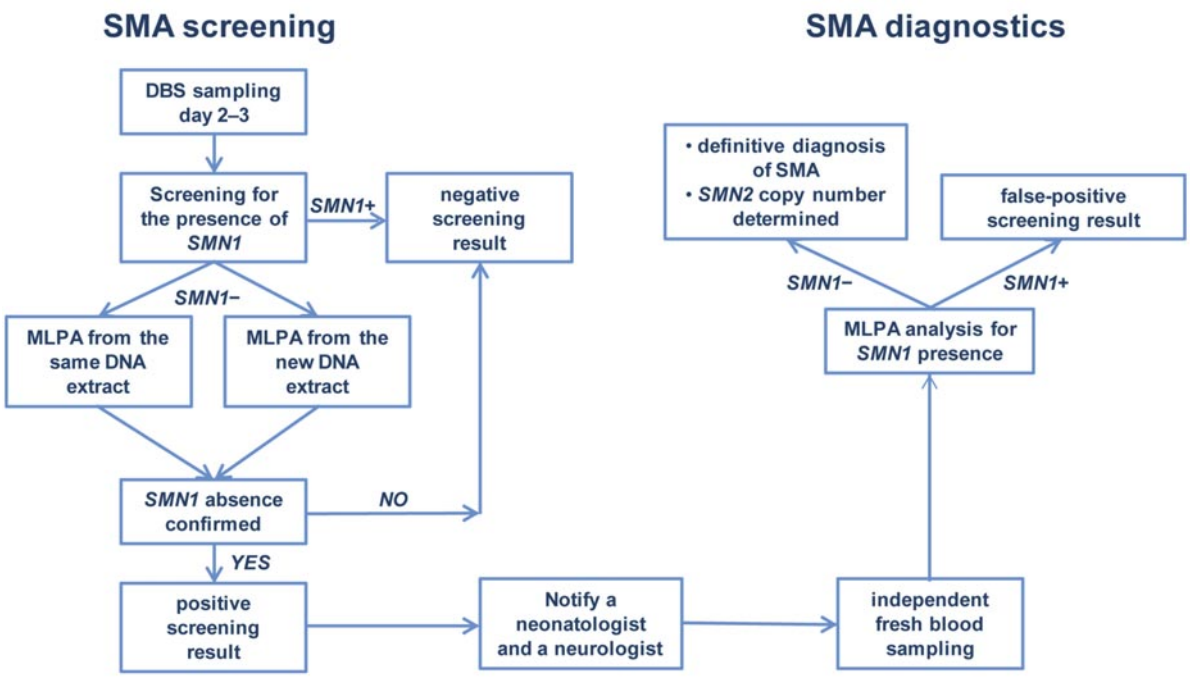

Figure 2. Schematic overview of the spinal muscular atrophy (SMA) newborn screening and diagnostic algorithm in Serbia. Abbreviations: SMA, spinal muscular atrophy; DBS, dried blood spot; *SMN1*+: *SMN1* gene present; *SMN1*–: *SMN1* gene absent; MLPA: multiplex ligation-dependent probe amplification. All MLPA analyses are performed using the SALSA MLPA Probemix P021 SMA kit (MRC Holland, Amsterdam, The Netherlands).

Notably, since the launch of the program, no false positive results have been observed, underscoring the reliability of the applied workflow. Importantly, *SMN2* copy number information is available early, al-

lowing clinicians to assess the urgency of treatment initiation and anticipate the most suitable therapeutic option for each patient.

By combining accurate molecular techniques with a streamlined workflow, Serbia's screening and diagnostic algorithm facilitates early detection and rapid initiation of care, improving long-term outcomes and aligning with international best practices.

One Child, One Plan: The National Expert SMA Committee's Role in SMA Treatment Decisions

SMA serves as a prime example of the paradigm shift brought about by the advent of personalized medicine, which has revolutionized the management of rare genetic disorders by enabling the tailoring of therapeutic strategies to an individual's unique genetic profile. Early diagnosis through newborn screening not only facilitates timely intervention but also provides critical genetic information essential for personalized care from birth. The clinical presentation and therapeutic response in SMA are significantly influenced by the number of copies of the *SMN2* gene, a key modifier of disease severity and treatment efficacy (19,20,21).

In Serbia, the integration of SMA into the national newborn screening program has been complemented by the establishment of a multidisciplinary National Expert SMA Committee operating under the Republic Healthcare Insurance Fund. This committee, comprising pediatricians, neurologists, a patient representative, and a molecular biologist, oversees treatment decisions for newly diagnosed children. Each case is meticulously reviewed, incorporating clinical and molecular data, with particular emphasis on *SMN2* copy number. While this genetic parameter plays a central role in therapeutic decision-making, it is never considered in isolation; the committee ensures a holistic approach to patient care.

Infants with more than four *SMN2* copies are currently not candidates for approved disease-modifying therapies and are instead carefully monitored through regular clinical check-ups (27,28). Children with two *SMN2* copies are typically recommended by the Committee for onasemnogene abeparvovec due to its unique ability to deliver a functional *SMN1* gene. In contrast, therapies such as nusinersen and risdiplam work by enhancing *SMN2* splicing to increase SMN protein production, which may be less effective in patients with only two copies. Although exact data on the extent of SMN protein restoration and the minimal protein threshold required for normal motor neuron function remain unclear, clinical trial evidence in presymptomatic infants with two *SMN2* copies has not demonstrated major differences in motor outcomes among onasemnogene abeparvovec, nusinersen, and risdiplam (52). This suggests that the timing of early treatment—rather than the specific therapeutic mechanism—is the critical factor for achieving favorable prognosis.

Beyond genetics, the committee evaluates additional clinical and laboratory factors for all SMA therapies. Onasemnogene abeparvovec is contraindicated in patients with pre-existing immunity to AAV9, advanced SMA, hepatotoxicity, thrombocytopenia, thrombotic microangiopathy, or elevated troponin-I, and requires close pre- and post-infusion monitoring (53). Nusinersen is limited by risks associated with lumbar puncture, thrombocytopenia, coagulation abnormalities, renal toxicity, and rare hydrocephalus, but is administered in a controlled clinical setting (54). Choice of therapy considers both medical contraindications and practical factors, including parental compliance and home environment: if strict post-gene therapy isolation for onasemnogene abeparvovec or consistent daily oral administration of risdiplam may be difficult, nusinersen is often preferred, as its intrathecal administration ensures adherence, precise dosing, and close monitoring of adverse effects.

The existence of this dedicated Expert Committee is a distinctive feature of the Serbian SMA care model. Unlike many other countries where formalized decision-making bodies do not exist, Serbia's committee ensures that every child receives a personalized, evidence-based treatment plan, balancing medical, practical, and social factors. Its work goes beyond clinical decisions, providing families with guidance and support throughout the often complex treatment journey, fostering transparency, responsibility, and the highest standards of care.

Challenges, Lessons, and Future Directions

The Serbian national newborn screening program for SMA demonstrates that personalized medicine can begin at birth. Its success relied not only on precise molecular diagnostics and a validated screening algorithm but also on strategic planning, multidisciplinary collaboration, and alignment of therapy access with healthcare resources. Early engagement with families and healthcare providers, laboratory standardization, stepwise program expansion, and a dedicated Expert Committee ensured that every child's treatment was tailored to their genetic and clinical profile, maximizing therapeutic benefit while minimizing irreversible motor neuron loss.

This experience shows that precision medicine is achievable even in countries with moderate resources when scientific insight, institutional commitment, and patient-centered care converge. Presymptomatic identification of SMA transforms disease trajectories, improves long-term outcomes, and serves as a model of equitable, evidence-based public health practice.

Looking forward, ongoing advances in biomarkers, gene therapy, long-term outcome studies, and digital health integration promise to refine and expand personalized SMA care. Lessons from Serbia's program can inform early detection and intervention strategies for other rare genetic disorders, extending the impact of precision medicine at the population level.

In conclusion, Serbia's journey illustrates a powerful truth: when knowledge, coordination, and patient-centered commitment align, personalized medicine is not a distant ideal—it can begin at birth, transforming lives from their very first days.

Acknowledgments. We extend our deepest gratitude to the Association SMA Serbia and its former president Olivera Jovović for their vital collaboration, which was essential in ensuring the success of our project. Their efforts enabled us to engage with all relevant stakeholders and government bodies, significantly contributing to the establishment of newborn screening initiatives. We also acknowledge the substantial contributions of Matija Tričković, a master's student who played a crucial role in validating the newborn screening assay. Furthermore, we express our appreciation to MRC-Holland b.v. and their Technical Support team, especially Jeroen van der Steen, for their invaluable expertise and insightful discussions during the optimization and validation of the newborn screening assay. Finally, we would like to thank the Republic Healthcare Insurance Fund, Institute for Public Health of Serbia "Dr Milan Jovanović Batut", and the Government of Serbia for recognizing our efforts and expertise, and for providing final approval and funds for the national screening project for SMA.

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Gut Microbiota as a Potential Target for Improving Immunotherapy

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Abstract

Immune-mediated diseases, including cancer and autoimmune disorders, are a growing global health burden. While immune checkpoint inhibitors (ICI)s and immunogenic cells have achieved significant success in oncology, approaches involving immunosuppressive immune cells are under investigation for autoimmune diseases. Increasing evidence highlights the gut microbiota as an important factor influencing immunotherapy outcomes. Clinical and preclinical data show that greater microbial diversity and enrichment of beneficial taxa correlate with improved therapeutic responses and fewer immune-related adverse events in ICI-treated patients. Our findings demonstrated that specific members of gut microbiota associate with the differentiation of dendritic cells (DC) with more pronounced immunogenic properties, which could be leveraged to improve the efficacy of DC-based anti-cancer vaccines. On the other hand, by using the animal model of multiple sclerosis, we demonstrated that the efficacy of myeloid-derived suppressor cells (MDSC) in attenuation of the disease symptoms is followed by preservation of gut microbiota with an immunosuppressive metabolic profile. These data support the idea that interventions based on specific probiotics, dietary fibres, and faecal microbiota transplantation (FMT) could be used to improve the antitumor and anti-inflammatory effects of therapies.

Key words: immunotherapy, cancer, autoimmune diseases, gut microbiome, myeloid cells

Mikrobiota creva kao potencijalna meta za unapređenje imunoterapije

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Apstrakt

Imunski posredovane bolesti, uključujući kancer i autoimunske bolesti, predstavljaju sve veći globalni zdravstveni izazov. Dok su terapije sa inhibitorima imunskih kontrolnih tačaka (eng. *immune checkpoint inhibitors*, ICI) i imunogenim ćelijama postigle značajan uspeh u onkologiji, pristupi zasnovani na imunosupresivnim ćelijama istražuju se u terapiji autoimunskih bolesti. Sve veći broj studija ukazuje da je mikrobiota creva važan faktor koji utiče na ishod imunoterapije. Klinički i preklinički podaci pokazuju da veći diverzitet mikrobiote creva i prisustvo "korisnih" taksona koreliraju sa boljim terapijskim odgovorima i manjim brojem neželjenih efekata kod pacijenata lečenih ICI terapijom. Naša istraživanja pokazala su da pojedini predstavnici mikrobiote creva utiču na diferencijaciju dendritskih ćelija sa izraženijim imunogenim svojstvima, što se može iskoristiti za unapređenje efikasnosti vakcina protiv tumora. S druge strane, korišćenjem životinjskog modela multiple skleroze pokazali smo da je terapijska efikasnost supresorskih ćelija mijeloidnog porekla u ublažavanju simptoma bolesti praćena očuvanjem mikrobiote sa imunosupresivnim metaboličkim profilom. Ovi podaci podržavaju ideju da bi intervencije zasnovane na specifičnim probioticima, dijetetskim vlaknima ili transplantaciji fekalne mikrobiote mogle doprineti jačanju antitumorskih i antiinflamatornih efekata terapija.

Ključne reči: imunoterapija, kancer, autoimunske bolesti, mikrobiota creva, mijeloidne ćelije

Introduction

Immune-mediated diseases encompass cancer and autoimmune disorders, which lie at opposing ends of the immune spectrum. In autoimmune diseases (AD), a breakdown of self-tolerance mechanisms causes the immune system to attack the body's own tissues mistakenly. In contrast, in cancers, the immune response is suppressed or evaded by transformed self-cells, allowing tumor growth [1]. Despite this fundamental difference, both cancer and autoimmunity involve dysregulated immune response, and both diseases represent a growing global health challenge.

Cancer is recognized as a leading cause of death worldwide, responsible for nearly 10 million deaths in 2020, according to the World Health Organisation (WHO) report. The incidence of cancer continues to rise with aging populations and lifestyle changes [2]. On the other hand, ADs cumulatively affect an estimated 7-10% of people in developed countries, and their incidence is steadily increasing across the globe [3]. The rising prevalence of ADs, coupled with the high mortality associated with cancer, underscores the urgent need for a deeper understanding and more effective therapies.

Conventional therapies for cancer, such as chemotherapy and radiation, are not immune-targeted and often have limited efficacy in advanced disease [4]. There is also a problem with side effects when using standard treatments for ADs, where nonspecific immunosuppressive or anti-inflammatory drugs such as corticosteroids or tumor necrosis factor (TNF) inhibitors are used to suppress pathological immunity and help control the disease [5]. Patients often face not only drug toxicities but also an elevated risk of opportunistic infections or even secondary cancers because immune surveillance is compromised [6].

Significant progress in immunology over the past two decades has begun to transform the way we treat both cancer and ADs. Immunotherapies, such as immune checkpoint inhibitors (ICIs) in cancer and experimental tolerogenic therapies in autoimmunity, have revolutionized treatment by more directly modulating the immune system. However, responses to these therapies vary significantly among patients, and adverse effects remain a problem [7]. Therefore, there is a need to understand the factors that influence the efficacy and safety of immunotherapy to develop more targeted strategies.

Our immune system does not operate in isolation. Various environmental factors, including diet, microbial exposure, antibiotics, and pollutants, influence its function. During the lifetime, these factors interact with host genetics to determine immune tone and responsiveness, potentially altering the outcomes of both disease progression and immunotherapy [8].

Among environmental factors, gut microbiota is the emerging one. The gut microbiota represents the complex community of microbes residing on the mucosal surfaces of the intestines. While the term microbiota refers only to the population of microorganisms, such as bacteria, fungi, viruses, and protozoa that colonize specific sites without their functions, the term gut microbiome comprises a diverse community of microorganisms along with their genomes and metabolites. Within the gut microbiota, bacteria have received the most scientific attention [9].

Gut colonization by bacteria begins immediately after birth, with the mode of delivery recognized as a significant factor influencing the initial development of the infant gut microbiota. Newborns delivered via C-section are colonized predominantly by skin-associated species from the genera such as *Corynebacterium*, *Staphylococcus*, and *Propionibacterium*, rather than typical vaginal microbiota members (*Lactobacillus* and *Prevotella* species) [10]. In addition to direct microbial colonization, the maternal contribution continues through breastfeeding, which delivers secretory IgA (sIgA), lactoferrin, and free oligosaccharides that play

crucial roles in shaping the infant gut microbiota and supporting immune system development [11]. Bidirectional interactions between beneficial microbes and the host immune system during infancy enable the immune system to receive adequate training and maintain immunological tolerance [12]. During life, the composition of the gut microbiota can vary significantly between individuals. However, when observed longitudinally within a single healthy individual, only minor fluctuations are typically seen, reflecting a relatively stable microbial profile during adulthood. This intra-individual stability suggests that the gut microbiota of healthy adults maintains a resilient and homeostatic community structure over time [13].

The human gut is often considered the largest immune organ, containing a majority of peripheral immune cells and constantly interacting with microbial-derived metabolites [14]. Through these interactions, the gut microbiota plays a pivotal role in educating and regulating the host immune system. Gut commensal microbes play a crucial role in maintaining intestinal homeostasis through their metabolic contributions and immune signaling. For example, microbial metabolites, such as short-chain fatty acids (SCFAs) and specific bile acids, shape both innate and adaptive immune responses [15]. Germ-free or antibiotic-treated animals have underdeveloped immune compartments, highlighting that continuous cues from commensals are necessary for normal immune development and function [16]. Pattern recognition receptors (PRRs) are germline-encoded receptors expressed by intestinal epithelial and immune cells that can detect microbe- and pathogen-associated molecular patterns (MAMPs and PAMPs) and typically strike a balance between tolerance and activation, allowing the host to tolerate commensals while responding to pathogens [14]. There are several major PRR families, among which Toll-like receptors (TLRs) are the best-characterized PRRs. In humans, multiple TLRs are expressed either on the cell surface or within intracellular compartments [17]. For example, membrane-bound TLRs include TLR1, TLR2, TLR4, TLR5, and TLR6, each with distinct roles: TLR1 detects components of Gram-positive bacteria, TLR2 detects bacterial lipoproteins and peptidoglycans, TLR4 recognizes lipoteichoic acid and lipopolysaccharide (LPS) from Gram-negative bacteria, and TLR5 binds flagellin from motile bacteria. At the same time, TLR6 responds to Gram-positive bacterial lipoproteins [18] and TLR11 detects profilin from *Toxoplasma gondii* [19]. On the other hand, intracellular TLRs, located within endosomes, recognize nucleic acids: TLR3 senses viral double-stranded RNA, TLR7 and TLR8 detect single-stranded RNA from viruses, and TLR9 recognizes unmethylated CpG motifs found in bacterial and viral DNA [18]. Ligand-TLR complex initiates intracellular signaling cascades primarily through a conserved Toll/IL-1 receptor (TIR) domain, which interacts with adaptor molecules such as Myeloid Differentiation Primary Response 88 (MyD88) and Interleukin-1 Receptor-Associated Kinase (IRAK). The assembly of this signaling complex at the membrane leads to IRAK phosphorylation, which subsequently activates major downstream pathways, including the nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways [20]. The signaling cascade leads to the transcriptional activation of genes involved in inflammation, antimicrobial defense, and immune regulation.

One of the key mechanisms of gut defense is the mucosal epithelial barrier, primarily maintained by intestinal epithelial cells (Figure 1). Tight junctions, composed of proteins such as claudins and occludins, regulate barrier integrity and selective paracellular permeability. While claudins 1, 3, 4, 5, and 8 strengthen the barrier function, claudins 2 and 10 regulate permeability [21]. Reduced expression of barrier-forming claudins is associated with impaired integrity, allowing microbial translocation [22]. In addition to barrier maintenance, specialized epithelial cells contribute to host defense, such as Paneth cells that produce antimicrobial peptides (AMPs) and epithelial cells that secrete cytokines like IL-8 to activate immune responses [23,24].

An intact mucosal barrier and immune regulatory network, including IL-10-producing regulatory T cells (Treg), further ensure that immune tolerance is maintained toward gut microbiota under healthy conditions.

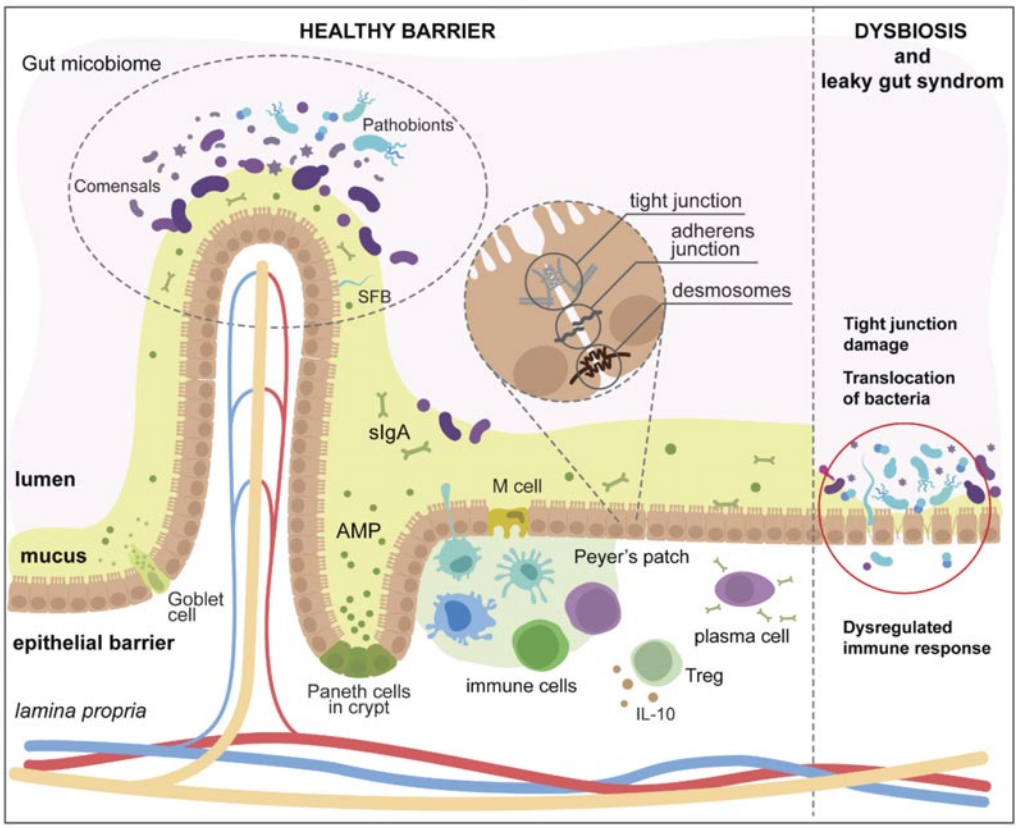

Figure 1. Intestinal mucosal barrier structure in health and disease. sIgA - secreted IgA; AMP - antimicrobial peptide; IL-10 - interleukin 10; SFB - segmented filamentous bacteria. Treg - regulatory T cell. Image adapted and modified from PhD thesis of Dušan Radojević [25].

Gut microbiota dysbiosis in cancer and autoimmune diseases

Gut microbiota dysbiosis represents a disruption of the normal gut microbial community and has been linked to the development or progression of both cancer and ADs. Alterations in gut microbiota composition can disrupt immune homeostasis and promote chronic inflammation, thereby contributing to disease pathology [26,27].

In cancer, specific microbial imbalances create a pro-tumorigenic environment. Some commensal bacteria, particularly pathobionts, have been implicated in the development of tumors. Pathobionts are typically low-abundance members of the healthy gut microbiota that can act as opportunistic pathogens when host defense mechanisms are compromised [28,29]. For example, *Helicobacter pylori* infection-driven inflammation is a well-established trigger of gastric cancer [30]. At the same time, the bacterium *Fusobacterium nucleatum* is overabundant in many colorectal cancers and drives immune evasion by recruiting immunosuppressive myeloid cells. *Fusobacterium* has been shown to promote the accumulation of myeloid-derived suppressor cells (MDSC)s in the tumor microenvironment, leading to dampened anti-tumor T cell activity [31]. *Fusobacterium*-recruited MDSCs have been linked to poorer responses to chemotherapy and immunotherapy in colorectal cancer [32]. Next-generation sequencing (NGS) technology has also identified intra-tumor microbial signatures in colorectal, lung, and prostate cancers [33–35]. Moreover, hypoxic tumor microenvironments can favor the growth of anaerobic or facultative anaerobes, such as *Clostridium*, *Bifidobacterium*, *Salmonella*, and *Bacillus species* [36–38].

In autoimmunity, gut microbiota dysbiosis can similarly skew immune responses in a pathogenic direction [39]. Several mechanisms have been implicated, including molecular mimicry, where microbial pep-

tides resemble host antigens and activate autoreactive lymphocytes, as demonstrated with *Fusobacterium* antigens in type 1 diabetes and commensal peptides in autoimmune uveitis and lupus [40–43]. Epitope spreading and post-translational modifications such as citrullination further broaden autoreactivity [44]. Chronic exposure to bacterial TLR ligands may sustain pro-inflammatory signaling, while bystander activation enables antigen-presenting cells (APCs) to present self-antigens alongside microbial ones, promoting autoimmunity [45]. Evidence from multiple sclerosis (MS) patients and experimental autoimmune encephalomyelitis (EAE) models suggests a central role for dysbiosis, with segmented filamentous bacteria (SFB) exacerbating disease through T helper (Th)17 responses [46–48]. In contrast, species such as *Bacteroides fragilis* and *Prevotella histicola* exert protective effects by inducing regulatory cells [49,50]. Probiotic interventions with *Bifidobacterium* and *Lactobacillus* have shown benefits in ameliorating EAE [51,52]. These findings highlight gut microbiota manipulation, through the use of probiotics or next-generation microbial therapies, as a promising strategy for restoring immune homeostasis in autoimmunity.

Notably, emerging evidence suggests that the gut microbiome may display opposing patterns in cancer versus autoimmunity, reflecting their opposite immune phenotypes. A recent meta-analysis identified microbial signatures that consistently move in opposite directions between cancer and AD cohorts [53]. For instance, the species *Fusobacterium nucleatum* (along with others, such as *Peptostreptococcus stomatis*) is found to be significantly enriched in many cancers, but not in AD. The study also showed that four species from the genus *Eubacterium* (*E. ventriosum*, *E. rectale*, *E. hallii*, and *E. eligens*) are decreased only in cancers. Interestingly, species *Bifidobacterium longum* and *Streptococcus salivarius* tend to be reduced in several cancers yet enriched in autoimmune conditions [53]. Some gut commensals actively induce regulatory immune mechanisms that counteract autoimmunity. For example, polysaccharide A from *Bacteroides fragilis* can expand IL-10-producing Treg cells and protect mice from autoimmune demyelination in an IL-10-dependent manner [54]. Loss of such beneficial microbes or their products may remove a brake on inflammation. On the other hand, expansion of pathobionts that breach the intestinal barrier or trigger aberrant innate immune activation (e.g. via inflammasomes or endotoxins) can lead to systemic immune dysregulation and loss of self-tolerance. Altogether, gut microbiota dysbiosis is now recognized as a common thread that can tip the balance between immune activation and suppression, thereby influencing whether the pendulum swings toward tumor progression or autoimmunity.

Microbiota-targeted interventions in immunotherapies

Given the evidence that gut microbes can profoundly affect cancer and ADs, several strategies are being explored to modulate the microbiota-immune axis for therapeutic benefit intentionally. In cancer, microbiota-targeted interventions have garnered significant interest as adjuncts to enhance ICI efficacy or mitigate immune-related toxicities. Approaches such as fecal microbiota transplantation (FMT), probiotics, prebiotics, dietary modification, and selective antibiotics are under study and have been reviewed in detail by Lei and coauthors [55]. Here, we will briefly describe several strategies. Early clinical trials in advanced melanoma have offered proof-of-concept that FMT from a responding donor can convert a checkpoint-inhibitor-refractory patient into a responder. In two independent studies, around one-third of metastatic melanoma patients who were initially unresponsive to anti-PD-1 therapy achieved clinical responses after receiving FMT from donors who had responded to immunotherapy [56,57]. These results suggest that transferring a favorable microbiome can reprogram the tumor microenvironment and T cell activity in patients, overcoming resistance to ICIs [55]. Indeed, responders in such trials exhibited increased $CD8^+$ T-cell infiltration in tumors after FMT and anti-PD-1, along with alterations in their gut bacterial composition [58]. On the

other hand, the deleterious impact of an unfavorable gut microbiome has been highlighted by studies of antibiotic use. Patients receiving broad-spectrum antibiotics around the start of ICI therapy tend to have significantly worse outcomes, presumably because antibiotics wipe out beneficial commensals that support anti-tumor immunity [55]. These findings underscore the importance of maintaining or restoring a healthy gut microbiota during immunotherapy. To that end, ongoing trials are investigating custom probiotic cocktails and high-fiber diets to boost ICI performance. For example, specific strains, such as *Bifidobacterium longum*, *Akkermansia muciniphila*, and *Faecalibacterium prausnitzii*, which are identified as more abundant in ICI responders, are being formulated into next-generation probiotics (NGPs) [59]. Sivan and colleagues have demonstrated that oral gavage of *Bifidobacterium* alone markedly enhances anti-PD-L1 therapy in tumor-bearing mice, augmenting T cell responses against the tumor [60]. Similarly, *Bacteroides fragilis,* known for producing polysaccharide A, was shown to be required for an optimal response to CTLA-4 blockade in mice. Co-administration of *B. fragilis* could improve anti-tumor T cell activity while also reducing colitic side effects in these models [61]. Such results suggest that using defined microbial agents could both enhance efficacy and mitigate the toxicity of immunotherapy.

Microbiota-targeted interventions and myeloid cell-based therapies

In parallel, myeloid cell-based therapies are being developed with an eye to the gut microbiome. In cancer, dendritic cell (DC) vaccines are being revisited in combination with immunomodulators. One could envision combining a DC vaccine with a microbiome modulator (e.g., an FMT or a prebiotic) to ensure the patient's gut microbiota supports robust DC activation [62]. Conversely, in autoimmunity, where the goal is to induce tolerance, integrating gut microbiota interventions with cellular therapies may enhance their efficacy. There is also growing interest in tolerogenic DC (tolDC) therapy for AD. These are DCs conditioned (*ex vivo* with IL-10, vitamin D3, etc.) to be anti-inflammatory and promote Tregs upon infusion [63]. While still experimental, the gut microbiota's status may influence tolDC therapy, as a non-dysbiotic gut could favor the survival and function of infused tolerogenic DC or the induction of Tregs by those DC [64]. Probiotic approaches have already shown some success in small trials for autoimmune conditions. For example, probiotic mixtures have been shown to lead to increased Treg cells in MS patients [65], suggesting that the gut microbiome can be tilted toward an anti-inflammatory state.

An important consideration for all these strategies is personalization. Each patient's gut microbiome profile is unique, and thus interventions may need to be tailored because what works for one patient's melanoma or arthritis may not work for another's. Future diagnostic tools that profile a patient's gut microbiome could help identify who might benefit from a microbiota-based adjunct or which specific microbes should be added or removed. Additionally, safety is paramount, as manipulating the gut microbiome carries the risk of infection or unintended immune effects, so approaches like FMT are being carefully refined. Using defined consortia of bacteria rather than whole stool transfer to avoid pathogens is a good option to be considered. Despite these challenges, microbiome-driven adjuvant therapy is advancing rapidly. The concept of modifying gut microbiome using live bacteria, dietary components, or microbial-derived metabolites as immunotherapeutics is becoming a reality in both oncology and autoimmunity [55].

Gut microbiota and dendritic cell immunogenicity in cancer immunotherapy

DCs represent a heterogeneous population of the most potent APCs, originating from pluripotent hematopoietic stem cells [66]. They form a crucial link between innate and adaptive immunity by enabling the activation of naïve T and B lymphocytes while maintaining immune tolerance. Their primary function is to cap-

ture and process antigens, which are then presented on Major Histocompatibility Complex (MHC) class I and II molecules to $CD8^+$ and $CD4^+$ T cells, respectively [67]. DCs exist in two states: immature (iDC) and mature (mDC), characterized by distinct phenotypic and functional profiles (Figure 2.). In homeostasis, imDCs efficiently capture antigens but express low levels of MHC class II and costimulatory molecules (CD80, CD86) [68], while activation induces CD83 surface expression and triggers irreversible maturation [69]. During this process, their antigen uptake capacity decreases, whereas expression of co-stimulatory molecules (CD80, CD86, CD40), MHC II, and the chemokine receptor CCR7 increases, enabling migration along CCL19/CCL21 gradients to draining lymph nodes [70,71]. There, mDCs engage naïve $CD4^+$ and $CD8^+$ T cells, initiating adaptive immune responses to pathogens, alloantigens, tumor-associated neoantigens, or allergens [72,73].

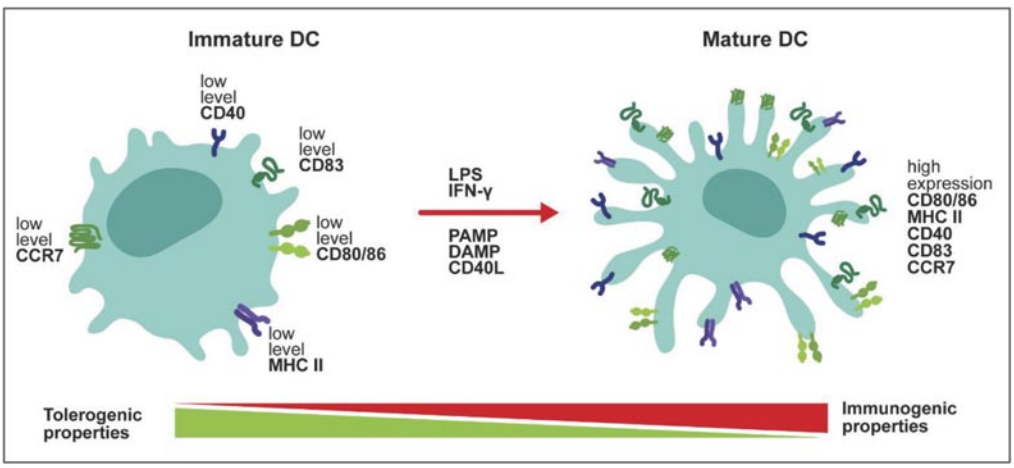

Figure 2. Phenotypic properties of immature and mature dendritic cells (DC). MHC - major histocompatibility complex; LPS - lipopolysaccharide; IFN-γ - interferon γ; PAMP - pathogen-associated molecular patterns; MAMP - microbe-associated molecular patterns. Image adapted and modified from PhD thesis of Dušan Radojević [25].

In humans, DCs are classified into several subsets based on their origin, marker expression, and function. Plasmacytoid DCs (pDCs), derived from lymphoid precursors, specialize in recognizing viral and bacterial nucleic acids through TLR7 and TLR9 [74], leading to robust type I interferon production along with IL-12 and TNF-α [75,76], but can also contribute to autoimmunity or tumor immune evasion [75,77]. Classical DCs (cDCs), of myeloid origin, are the principal APCs that activate naïve T cells and exist as tissue-resident or migratory populations, including Langerhans cells in the skin and DCs at mucosal surfaces [78,79]. Inflammatory monocyte-derived DCs (moDCs) arise during inflammation via CCR2-dependent recruitment of monocytes, antigen uptake, and subsequent maturation, ultimately migrating to lymph nodes to initiate antigen-specific T cell responses [80–82]. The protocols for generating moDCs *in vitro* are well established, their differentiation is typically induced by Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and IL-4, and they can be phenotypically defined by expression level of HLA-DR, CD11b, CD11c, CD1a, CD14, CD206, Signal Regulatory Protein alpha (SIRPα), CD64, and CCR2 [73,83,84].

DCs have been pursued as therapeutic vaccines against cancer. In DC vaccine therapy, a patient's monocytes are isolated from peripheral blood samples and differentiated *ex vivo* into moDCs, which are then loaded with tumor antigens and reinfused to stimulate tumor-specific T cells [85]. DC-based vaccines have shown promise in inducing anti-tumor immunity, with one FDA-approved example, sipuleucel-T, for prostate cancer [86]. However, one of the significant challenges is the inter-patient variability in the quality and immunogenicity of generated DCs, which can limit vaccine efficacy [87,88]. A recent study revealed that immune cells exhibit considerable inter-individual variability, as they can respond differently to stimulation by MAMPs derived from the gut microbiota [89].

Recently, we demonstrated that the composition of a healthy individual's fecal microbiota is strongly associated with the functional properties of their moDCs [90]. Donors with increased microbial species richness, manifested by higher α-diversity and a gut microbiome enriched in SCFA-producing bacteria, tended to yield DCs with a more mature and immunostimulatory baseline phenotype. In these individuals, imDCs expressed lower levels of CD1a (key marker of DC immaturity) and higher levels of immunoregulatory ILT-3. Upon maturation, they expressed higher co-stimulatory molecules (CD86, CD40) and produced more pro-inflammatory cytokines (TNF-α, IL-6, IL-8) along with a high IL-12p70/IL-10 cytokine ratio [90]. This profile is consistent with a Th1-polarizing, immunogenic DC that can effectively activate cytotoxic T cells. Donors with lower microbiota diversity, especially those whose microbiome was dominated by taxa like *Bifidobacterium* and *Collinsella*, showed the opposite trend. DCs from these donors had a more immature starting phenotype (higher CD1a) but demonstrated a greater capacity to upregulate costimulatory markers and IL-12 relative to IL-10 when stimulated, essentially requiring a maturation stimulus to achieve an immunogenic state [90]. These findings suggest that a diverse gut microbiota primes the myeloid precursor cells towards an already partially activated, pro-inflammatory DC phenotype. In contrast, a less varied faecal microbiota might allow precursors to remain more quiescent until stimulated. Based on these results, we concluded that gut microbiota composition was a significant determinant of a donor's DC immunogenic potential in our study. This insight could be harnessed to improve DC-based cancer immunotherapies. For example, by modulating a patient's microbiota (through diet, probiotics, or metabolites) before DC vaccine generation, it may be possible to produce DCs with an enhanced ability to stimulate anti-tumor T cells. Indeed, SCFAs and other microbial metabolites could serve as natural adjuvants for DC differentiation, as suggested by the positive correlation between faecal SCFA-producing bacteria and immunogenic DC markers [90]. Overall, the gut microbiome emerges as a factor that can shape the efficacy of active immunotherapies, such as DC vaccines. Adjusting the gut microbiome could be a novel strategy to reduce inter-patient variability in vaccine responses.

Myeloid-derived suppressor cells and the gut barrier in autoimmune therapy

MDSCs are another heterogeneous population of immature myeloid cells that are gaining interest as a therapeutic tool due to their strong immunosuppressive potential. These cells are commonly known for hindering anti-tumor immunity in cancer, and this suppressive ability can be beneficial if redirected to quell autoimmune responses [91]. While their role in tumor immune evasion is well established, their function in autoimmunity and therapeutic potential remain less clear. MDSCs suppress immune responses through multiple mechanisms (Figure 3.), including the production of IL-10, TGF-β, reactive oxygen species (ROS), nitric oxide (NO), and high levels of arginase-1 (ARG-1). These pathways directly inhibit T cells, NK cells, and DCs, or indirectly promote tolerance by expanding Treg cells [92]. NO produced by inducible nitric oxide synthase (iNOS) impairs antigen presentation and T cell function, while ROS sustain MDSCs in an immature state within tumors and contribute to T cell receptor modification [93,94]. In parallel, ARG-1 depletes extracellular L-arginine, resulting in impaired T cell proliferation and cytokine production [95,96] Other immunoregulatory molecules, including prostaglandin E2 (PGE2) and indoleamine 2,3-dioxygenase (IDO), further stabilize the suppressive phenotype of MDSCs by altering amino acid metabolism and promoting Treg induction [97–99]. Together, these mechanisms highlight the multifaceted role of MDSCs in dampening immune responses and maintaining their persistence in chronic inflammation and cancer.

Studies using rodent models of ADs have shown that adoptive transfer of *ex vivo* isolated MDSCs can attenuate disease severity, although *in vitro* generated MDSCs have been less explored [100]. In our work,

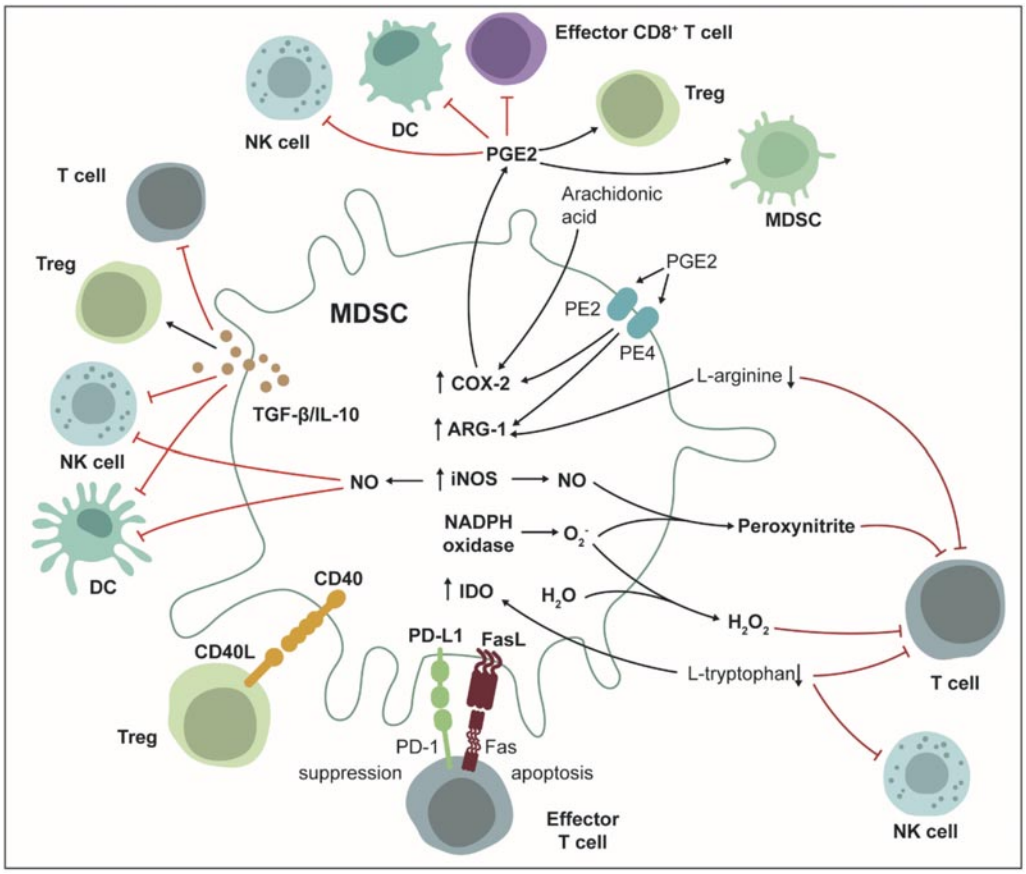

Figure 3. Main immunosuppressive mechanisms of myeloid-derived suppressor cells (MDSC). NK - natural killer; Treg - regulatory T cell; DC - dentritic cell; PGE2 - prostaglandin E2; NADPH - nicotinamide adenine dinucleotide phosphate; IDO - indoleamine 2,3-dioxygenase; ARG-1 - arginase 1; NO - nitric oxide. Image adapted and modified from PhD thesis of Dušan Radojević [25].

we applied bone marrow precursors from DA rats differentiated with GM-CSF, FLT3, and IL-6, a cytokine cocktail known to induce STAT3 signaling and confer suppressive activity [101–105]. Importantly, the addition of PGE2 further enhanced the immunosuppressive potential of these cells, consistent with our previous findings in human monocyte-derived MDSCs [106]. These results suggest that MDSCs, particularly when modulated with defined differentiation protocols, hold promise as a cell-based therapy for ADs. In detail, we generated bone marrow-derived MDSCs, with one group of cells differentiated in the presence of PGE2 to enhance suppressive function (termed MDSC-PGE2). When transferred into EAE-induced rats, the PGE2-conditioned MDSCs significantly ameliorated clinical disease severity compared to controls. Treated animals showed reduced infiltration of pathogenic Th17 cells and IFN-γ-producing NK cells into the central nervous system, along with an increase in FoxP3$^+$ Treg cells, indicating that the MDSCs dampened pro-inflammatory lymphocytes while promoting regulatory mechanisms [107]. Remarkably, the gut microbiota of these rats was also protected by the MDSC therapy. Typically, EAE induction results in a loss of gut microbial diversity and significant shifts in composition, often leading to dysbiosis, as frequently observed in MS patients [108]. MDSC-treated groups prevented the drop in microbial diversity that EAE causes, suggesting that MDSCs helped maintain a healthier, more diverse gut ecosystem even during autoimmune attack. More strikingly, the PGE2-enhanced MDSCs not only preserved diversity but also prevented the pronounced taxonomic changes seen in untreated EAE. The gut microbiota composition remained closer to that of healthy rats in these animals. Early migration of the MDSCs to gut-associated lymphoid tissues (Peyer's patches and mesenteric lymph nodes) was observed, implying that MDSCs may act locally to stabilize the gut-immune

interface. The gut microbiome of MDSC-PGE2-treated animals is characterized by enrichment of bacterial taxa with presumed immunoregulatory functions, and fecal metabolic analysis showed significantly higher levels of SCFAs (butyrate, propionate) and the polyamine putrescine [107]. SCFAs, such as butyrate, are known to improve gut barrier integrity and induce regulatory T cells [109], while putrescine exhibits anti-inflammatory effects [110]. The increase in these metabolites aligns with improved disease outcomes. Our results demonstrated a fascinating bidirectional interplay: not only can the gut microbiota influence immune cells, but immune-cell therapy can also influence the microbiota. By preventing gut barrier disruption and dysbiosis, the MDSCs likely interrupted the vicious cycle of gut-driven inflammation in autoimmunity. These findings open the door to combined strategies, pairing MDSC-based therapy with microbiome-targeted interventions or identifying which microbial changes (e.g. specific SCFAs or species increases) are mechanistically responsible for the protection. It also underlines that maintaining gut homeostasis is a key part of controlling autoimmunity. While much work remains (including ensuring MDSC therapies do not inadvertently promote infections or cancer), this approach exemplifies the therapeutic potential of leveraging the body's own suppressor cells together with the gut microbiome to recalibrate immune responses in ADs.

Future directions and therapeutic outlook

Research at the convergence of the gut microbiome and immunotherapy is still in its early days, and many open questions remain. Hence the aim of this review was to summarize current knowledge on the interplay between the gut microbiota, myeloid cells, and immunotherapy, thereby providing a framework for future studies and potential therapeutic applications.

One priority for future studies is to dissect the mechanisms by which specific microbes or their metabolites influence immune cell function. While correlations abound (e.g. particular taxa linked to responders vs. non-responders), pinpointing causal relationships and molecular pathways will enable more rational design of gut microbiota-based therapies. For example, if a bacterial metabolite like butyrate is found to enhance $CD8^+$ T cell memory responses to tumors [55] or if a *Bacteroides* species' polysaccharide is confirmed to drive IL-10 Treg expansion, these could be developed into therapeutic adjuncts or supplements to conventional immunotherapy. Harnessing microbial metabolites is especially attractive: these small molecules (like SCFAs, tryptophan derivatives and bile acid derivatives) can be synthesized or delivered in diets and might avoid some of the risks of live bacteria. Another area of interest is the tumor microbiome (microbes residing within tumors) and how it interacts with gut microbiota. Understanding this interaction could reveal additional targets to improve tumor immune infiltration or drug response [55].

From a clinical perspective, we anticipate more personalized immunotherapy approaches that integrate microbiome profiling. Just as genomic biomarkers (like tumor mutation burden or PD-L1 expression) are used to guide immunotherapy decisions, a patient's gut microbiome may become a biomarker and modifiable factor. Soon, oncologists and immunologists might screen the gut microbiome and prescribe a course of microbiota modulation such as a high-fiber diet, prebiotic, or a defined probiotic cocktail to set the setting for a more effective and safer immunotherapy outcome. Small trials are already testing whether adding a probiotic or dietary intervention can increase ICI response rates [111–113]. In ADs, microbiome-based therapeutics could shift the treatment paradigm away from generalized immunosuppression toward restoring immune tolerance. For example, NGP producing anti-inflammatory signals, or even MDSC-promoting factors, might be used in conjunction with cell therapies to sustain remission without weakening normal immunity.

Ultimately, the interplay of gut microbiota, DCs, and MDSC represents a fertile ground for therapeutic innovation. It embodies a systems immunology approach: treating the patient not just by targeting a single immune pathway, but by recalibrating the broader ecosystem of the immune system and its microbial partners. While challenges in safety, consistency, and regulatory approval remain, the prospect of enhancing cancer immunotherapy efficacy or inducing drug-free remission in ADs by gut microbiome manipulation is a compelling new frontier. As our understanding deepens, we move closer to a future where one's microbiome can be leveraged as both a predictive biomarker and a tool to engineer better immunotherapy outcomes, bringing truly personalized medicine into practice.

Acknowledgment

This work was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia under Contract No. 451-03-9/2021-14/200019, 451-03-68/2022-14/200042, 451-03-9/2021-14/200042 and 451-03-68/2022-14/200019, and by the Science Fund of the Republic of Serbia, PROMIS, #6062673, Nano-MDSC-Thera.

The authors would like to express their sincere gratitude to all colleagues from INEP, IMGGE, Medical Faculty of the Military Medical Academy, University of Defense in Belgrade and Serbian Academy of Sciences and Arts who contributed to the doctoral dissertation research from which this review paper originated.

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C9orf72–Associated ALS/FTD: From Genetic Diagnosis to Therapeutic Opportunities and Challenges

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Abstract

This review critically analyzes evidence on the shared genetic architecture of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), focusing on the *C9orf72* hexanucleotide repeat expansion–the most common genetic cause of ALS, FTD, and their overlapping presentation ALS/FTD. Although ALS and FTD were traditionally considered distinct, they are now viewed as part of a clinical and genetic continuum, termed the ALS–FTD spectrum, due to overlapping symptoms and pathology.

C9orf72–associated disease shows highly variable clinical manifestations, making the prediction of disease course challenging. Several genetic modifiers–such as intermediate CAG repeats in *ATXN1*, *ATXN2*, *HTT*, the *APOE* genotype, the minor allele A of rs1009776, *TMEM106B*, *NIPA1,* and rs12608932 in *UNC13A*–have been studied for their influence on disease phenotype and progression, though findings remain inconsistent.

Currently, no therapies specifically target *C9orf72*–related ALS/FTD. Management relies on standard ALS treatments and symptomatic care for FTD. However, promising investigational therapies are in development, particularly antisense oligonucleotides (ASOs), repurposed small molecules, and monoclonal antibodies.

Understanding genetic interactions is essential for advancing precision medicine, shaping therapeutic strategies, and interpreting clinical trial outcomes. Genetic testing for *C9orf72* and relevant modifiers is crucial–not only for accurate diagnosis and genetic counseling but also for stratifying patients in clinical trials targeting this unique subgroup.

This evolving knowledge underscores the need for a genetically informed approach to ALS/FTD, which may ultimately lead to more effective, personalized treatments.

Keywords: amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), ALS/FTD, *C9orf72*, genetic modifiers

ALS/FTD uzrokovan ekspanzijom ponovaka u genu *C9orf72*: Od genetičke dijagnoze do terapijskih izazova i perspektiva lečenja

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Apstrakt

Ovaj revijalni rad analizira zajedničku genetičku osnovu amiotrofične lateralne skleroze (ALS) i frontotemporalne demencije (FTD), sa posebnim naglaskom na ekspanziju heksanukleotidnih ponovaka u genu *C9orf72*, kao najčešćim genetičkim uzrokom ALS–a, FTD–a i ALS/FTD preklapajućeg fenotipa. Iako su ALS i FTD tradicionalno smatrani zasebnim kliničkim entitetima, savremena istraživanja ih prepoznaju kao deo kliničkog i genetičkog kontinuuma, usled značajnih preklapanja u simptomima i patološkim karakteristikama.

Ekspanzija ponovaka u genu *C9orf72* je povezana sa širokim spektrom kliničkih manifestacija. Više genetičkih modifikatora se ispituje u kontekstu fenotipskih manifestacija i progresije bolesti, uključujući intermedijarne CAG ekspanzije u genima *ATXN1*, *ATXN2*, *HTT*, genotip *APOE*, alel rs1009776, kao i varijante u *TMEM106B*, *NIPA1* i *UNC13A*. Međutim, rezultati studija ostaju kontradiktorni.

Trenutno ne postoje terapije koje specifično ciljaju ALS/FTD povezan sa *C9orf72*–. Lečenje se zasniva na standardnim terapijskim pristupima za ALS i simptomatskoj terapiji kod FTD. Ipak, u toku je razvoj obećavajućih eksperimentalnih terapija, naročito *antisens* oligonukleotida (ASO), preusmerenih malih molekula i monoklonskih antitela.

Detaljno razumevanje genetičkih interakcija je ključno za razvoj personalizovane medicine, optimizaciju terapije i interpretaciju kliničkih ishoda. Genetička analiza ekspanzija u genu *C9orf72,* kao i genetičkih modifikatora, ima ključnu ulogu u dijagnostici, genetičkom savetovanju i stratifikaciji pacijenata za kliničke studije.

Rezultati dosadašnjih istraživanja ističu potrebu za genetički zasnovanim pristupom kod obolelih od ALS/FTD spektra bolesti, što potencijalno može doprineti razvoju efikasnijih i personalizovanih terapijskih strategija.

Ključne reči: amiotrofična lateralna skleroza (ALS), frontotemporalna demencija (FTD), ALS/FTD, *C9orf72*, genetički modifikatori

1. Introduction

Genetic testing should be considered highly important even in multifactorial diseases such as the ALS/FTD spectrum, for several reasons. First, it enables patient stratification, which is the essential process for optimization of therapeutic choices. Furthermore, genetic testing enables the detailed genetic characterization of patients, which can be correlated with the highly variable clinical presentation of the ALS/FTD spectrum diseases. Equally important is the fact that identification of genetic variants that contribute to the phenotype–whether directly, as modifiers, or through their influence on treatment response–significantly advances research and deepens our understanding of pathogenic mechanisms. Characterization of the pathophysiological cascade is uncovering new potential therapeutic targets, which is particularly important given that effective disease–modifying or preventive treatments for ALS/FTD have yet to be discovered.

2. Clinical and genetic landscape of ALS and FTD

With that in mind, it is essential to reveal the broader clinical and genetic landscape of *C9orf72*–associated ALS and FTD overlapping phenotypes. Understanding both the major causative genetic variants and the role of possible genetic modifiers provides a framework for interpreting phenotypic variability and guiding future therapeutic strategies.

2.1. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disorder that affects motor neurons in the brain and spinal cord. The disease leads to muscle weakness, respiratory paralysis, and ultimately death, usually within 2–5 years after the symptom onset. ALS can be presented in several forms: spinal form, the most common form, occurs in approximately two–thirds of patients and begins with limb weakness; bulbar onset characterized by difficulties with speech and swallowing, and respiratory onset, though less common, presents initially with breathing difficulties (1). The global incidence and prevalence rate of ALS shows geographical distribution, with the incidence rate roughly ~ 2.0 per 100,000 and prevalence rate 3.44–10.88 per 100 000 in Europe (2). Based on the studies in Serbia (Belgrade county), the annual incidence rate shows 1.11 per 100 000 (3), and the prevalence of 1.07 per 100 000 (4). ALS is predominantly a sporadic disease accounting for approximately 90–95% of the cases, with the remaining 5–10% classified as familial (5). The first gene linked to a familial form of ALS was *superoxide dismutase 1* (*SOD1*), identified in 1993 (6). With more than 200 variants reported to date, the *SOD1* was the most prevalent gene identified in ALS cases until the discovery of *C9orf72* repeat expansions (https://alsod.ac.uk) (7,8). Numerous additional genes have been associated with both familial and sporadic forms of the disease, highlighting its very diverse genetic background (9). The frequency of genetic variants associated with ALS shows geographical and ethnic variability–for example, *SOD1* and *FUS* pathogenic variants are more prevalent in Japan, while *C9orf72* expansions are more common in Europe and the United States–highlighting the need for region–specific diagnostic algorithms to enable efficient patient stratification for potential gene–targeted therapies (9).

2.2. Frontotemporal dementia

Frontotemporal dementia (FTD) is the third most prevalent type of dementia across all ages, following Alzheimer´s disease (AD) and Lewy body disease (10). Among individuals under the age of 65, FTD ranks as the second most common cause of dementia after AD (11,12). In European populations, the annual incidence rate is 2.36 per 100 000 and varies between the countries. In Serbia, the incidence rate matches the

European average and amounts to 2.37 per 100 000 (13). Epidemiological data on the prevalence of FTD in Serbia are still lacking, while in Europe, the prevalence among individuals aged 45–64 varies considerably across regions, ranging from 2.7 to 22 per 100,000 (14). Clinical presentation in patients having FTD can be expressed as dysfunction in behavioral or language domain (15). Behavioral variant of FTD is characterized by predominant behavioral disinhibition, changes in diet, stereotyped or compulsive behavior, apathy, loss of empathy, and impaired executive and cognitive abilities (16). Additional clinical subtypes of FTD include progressive nonfluent/agrammatic aphasia, which is marked by motor speech impairments and agrammatism, and the semantic variant of primary progressive aphasia expressed as impaired naming and single–word comprehension (17). A positive family history consistent with autosomal dominant inheritance is observed in up to 50% of frontotemporal dementia (18). The genetic basis of FTD is primarily attributed to pathogenic/likely pathogenic variants in three major genes: *progranulin* (*GRN*), *microtubule–associated protein tau* (*MAPT*), and *chromosome 9 open reading frame 72* (*C9orf72*) (19).

2.3. ALS/FTD overlapping phenotype

Although ALS and FTD appear to be clinically distinct entities, where in ALS predominates motor and in FTD cognitive–behavioral symptoms, today these two diseases are recognized as part of a neurodegenerative disease continuum, commonly referred to as ALS–FTD spectrum, due to their overlapping clinical, pathological, and genetic features (20,21). ALS/FTD presents a rare phenotype within an already rare disease spectrum, with an incidence rate of 0.11 per 100 000 in Europe (13). The frequency of overlapping clinical phenotypes between ALS and FTD varies among individuals. Approximately 50% of patients with sporadic ALS exhibit some degree of cognitive impairment, and around 20% meet the diagnostic criteria for FTD (22). On the contrary, co–occurrence of Alzheimer's disease in ALS is uncommon, reported in fewer than 2% of cases (23). Conversely, among individuals with a clinical diagnosis of FTD, approximately 36% display features suggestive of ALS, and in 14%, a clinical diagnosis of ALS can be established (24).

From the pathological standpoint, both ALS and FTD are characterized by the accumulation of TAR DNA–binding protein 43 (TDP–43) within ubiquitinated intracellular inclusions in the brain (25,26). This shared pathological hallmark classifies these disorders as TDP–43 proteinopathies (25). Another feature of the ALS/FTD pathology is the presence of the second RNA binding protein, fused in sarcoma (FUS), which has been identified in both diseases (27,28). Along with TDP–43, FUS is a major component of the pathological inclusions found in over 90% of ALS patients and more than 50% of those with FTD. This overlap strongly suggests a shared pathological mechanism involving dysregulation of RNA processing (29). Disrupted protein homeostasis represents another key mechanism in ALS/FTD pathogenesis, involving dysfunction of the ubiquitin–proteasome system and autophagy. The proposed converging pathogenic model includes both disruption of RNA and protein homeostasis, forming a feed–forward loop towards disease progression, where a disease initiating event can occur in either RNA protein or homeostasis pathways (29).

3. *C9orf72* as the central genetic link in ALS/FTD spectrum

Given shared clinical and pathological features of ALS and FTD, a major breakthrough in understanding their common molecular basis was the discovery of a hexanucleotide repeat expansions in the *C9orf72* gene (30,31). Those intronic GGGGCC repeats disrupt normal gene expression, promote RNA foci formation, and lead to toxic dipeptide repeat protein aggregation (32).

3.1. Frequency of *C9orf72* repeat expansions in Serbia and worldwide

The *C9orf72* hexanucleotide repeat expansion represents the most common genetic cause of ALS, FTD, and the overlapping ALS/FTD phenotype. It accounts for approximately 16% of familial ALS cases and 20% of familial FTD cases. In sporadic forms, the expansion is present in about 6–8% of ALS and FTD patients. The highest expansion frequency is observed in individuals with the combined ALS/FTD phenotype, reaching up to 30% (33). The frequency of this expansion exhibits notable geographical variations, with a high rate reported in the Finnish population (34). In a recent study of the Serbian population, the overall prevalence of the *C9orf72* expansion among ALS patients was 8.09%, with a frequency of 17.86% in familial ALS and 7.12% in sporadic ALS cases (35). Among FTD patients, the overall frequency of the *C9orf72* repeat expansion was 6.98% (13.46% familial and 2.6% sporadic) (36). Interestingly, in patients with undetermined types of dementia, the expansion was detected in 4.08% of cases overall, and in 8% of those with a positive family history (36). The highest expansion rate in the Serbian population was observed in patients with ALS/FTD, with a rate of 31.82% (37). Given the variability in phenotypic expression among expansion carriers, *C9orf72* repeat expansions have also been detected in other neurodegenerative disorders, although at much lower

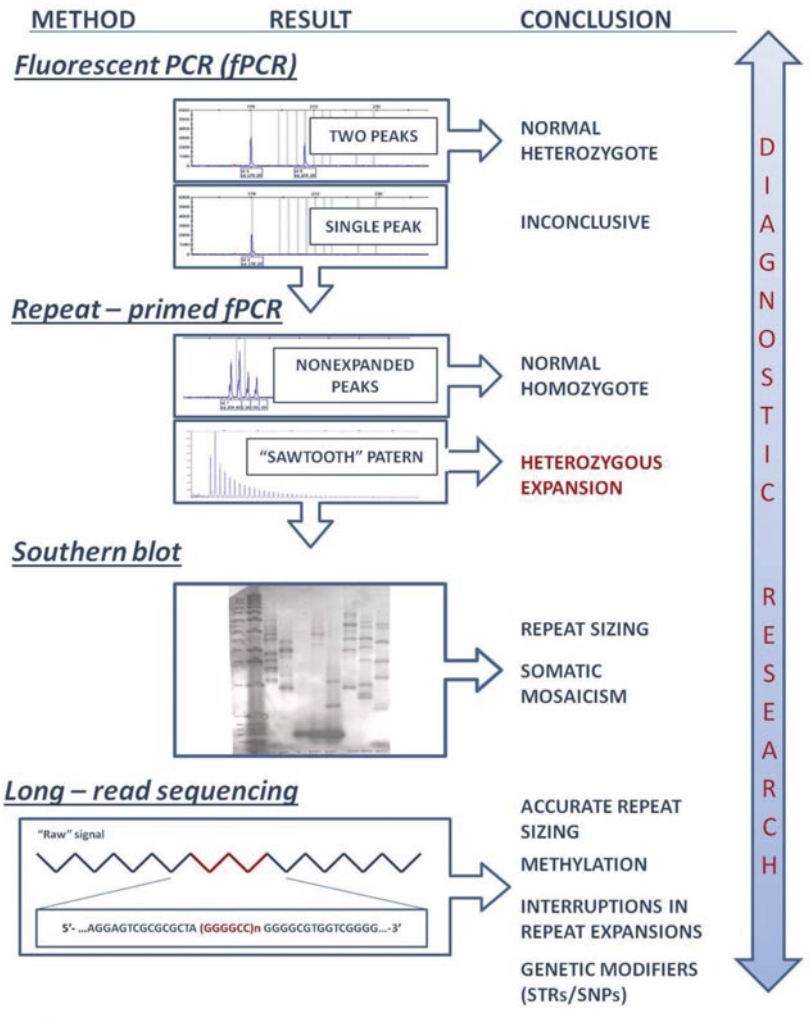

Figure 1. Diagnostic and research methodological considerations for *C9orf72* expansions. Fluorescent PCR identifies alleles in the wild–type range. If only one peak is detected, repeat–primed PCR distinguishes true homozygosity from a pathogenic expansion, visible as a "sawtooth" pattern. In the research and sometimes in clinical practice, expansions are confirmed by Southern blotting. Recently, long–read sequencing enables more accurate repeat sizing, detection of interruptions in repeat expansions and methylation status assessment. Additionally, short tandem repeats (STRs) and single nucleotide polymorphisms (SNPs) that may modify the disease are possible to be detected.

frequencies (33,38). Nevertheless, in the Serbian population, the *C9orf72* genetic screening in patients other than ALS and FTD (AD, mild cognitive impairment, multiple system atrophy, and progressive supranuclear palsy) did not reveal the presence of the expansion but only the presence of an intermediate repeat number of 20–29 (36,39) whose clinical significance is still under debate (40).

3.2. Genetic analysis of the C9orf72 repeat expansions

Genetic analysis of the *C9orf72* repeat expansions is now an established important part of the diagnostic process in patients with ALS, FTD, and ALS/FTD overlapping phenotype, particularly in those with a positive family history or an early disease onset (41). A novel family history–based model for estimating family–specific penetrance and personalized age–related risk in *C9orf72* expansion carriers, when combined with specific guidelines, enhances the accuracy of genetic counseling (42,43).

The pathogenic allele consists of an intronic GGGGCC hexanucleotide repeat expansion in the first intron of *C9orf72*. Normal alleles typically carry fewer than 20–25 repeats, whereas pathogenic expansions usually exceed several hundred repeats, although the exact pathogenic threshold remains undefined (44). Routine diagnostic testing is commonly performed using fluorescent PCR and fragment length analysis to estimate repeat size of the normal alleles, while repeat–primed PCR is used to detect the presence of large expansions and the result cannot be sized accurately (Figure 1). In research and specialized diagnostic laboratories, Southern blotting is used to estimate repeat length and assess potential somatic variability (41,45). This diagnostic algorithm of *C9orf72* repeat expansions is now also established in Serbia and performed routinely in specialized neurogenetic laboratories, ensuring that patients and families have access to comprehensive genetic evaluation.

More recently, the development of long–read sequencing technologies has provided the possibility of accurate sizing of expanded *C9orf72* alleles (46,47). Besides detailed characterization of the *C9orf72* repeat expansion length, long–read sequencing technologies may be useful in the assessment of methylation status of the expansions and detection of interruptions in the *C9orf72* repeat expansions (Figure 1). Both the methylation patterns of the *C9orf72* expanded repeat and the presence of interruptions, which may act as disease modifiers, remain largely unexplored (48).

Genetic research is also focused on elucidating the causes of phenotypic heterogeneity among *C9orf72* expansion carriers. One approach involves identifying variants in other genes that may influence whether the clinical presentation is predominantly motor or cognitive, as well as modifying disease onset and progression. Genome–wide association studies (GWAS) have been conducted to identify single nucleotide polymorphisms (SNPs) associated with ALS/FTD in *C9orf72* expansion carriers (49,50). However, short tandem repeat (STR) variations are not captured by this type of methodological approach. Traditionally, targeted fragment length analysis was the primary method for STR sizing, but advances in bioinformatics now enable their detection and sizing from short–read whole–genome sequencing data (51,52). This approach is particularly useful in *C9orf72* research for estimating repeat sizes within the range detectable by short reads, such as normal or intermediate alleles of STRs in polyglutamine disease–associated genes, as polyQ–related STRs have been proposed to influence pathways already disrupted in *C9orf72*–associated disease (53).

3.3. C9orf72 expansions: pathogenic mechanisms

Understanding the underlying disease pathology is essential for identifying potential therapeutic targets. However, in the case of *C9orf72* expansion, the pathogenic mechanisms are considerably complex and

not yet fully elucidated. Based on current evidence, three main mechanisms have been proposed to explain the pathogenicity of *C9orf72* hexanucleotide repeat expansions: 1–loss of function mechanism through haploinsufficiency of the *C9orf72* gene, 2–RNA toxic gain of function, and 3–gain of function through synthesis of toxic dipeptide repeat (DPR) proteins that accumulate in intracellular inclusions (32). At the molecular level, *C9orf72* repeat expansions disrupt multiple cellular processes, including nucleocytoplasmic transport, RNA processing, nucleolus function, formation and function of membraneless organelles, autophagy, and apoptosis, with most of the current knowledge derived from cellular and animal models, as well as post–mortem human brain tissue analyses (54,55). Edbauer and Haass suggested an amyloid–like cascade hypothesis for *C9orf72* pathogenesis in which DPR proteins and toxic RNA initiate a cascade of molecular events that affect TDP–43 many years before the clinical symptoms appear. The presence of the DPR protein aggregates could induce neuroinflammation, which further enhances TDP–43 aggregation and leads to neuronal cell death (56).

4. Genetic modifiers in *C9orf72*–associated ALS/FTD

Although *C9orf72* repeat expansions are highly prevalent in ALS and FTD, the clinical presentation in *C9orf72* repeat expansion carriers is remarkably heterogeneous and remains difficult to predict. Even within the same family, affected individuals may exhibit distinct clinical phenotypes, ranging from isolated ALS or FTD to the combined ALS/FTD presentation (31,57). The complexity of phenotypic expression is best illustrated in the studies of twins, emphasizing the pleiotropic effect of *C9orf72* repeat expansions (58,59). These findings are suggesting that genetic and epigenetic modifiers may contribute to the clinical heterogeneity observed among *C9orf72* expansion carriers.

Genetic modifiers of the clinical phenotype in patients with ALS and/or FTD, such as intermediate CAG repeats in *ATXN1*, *ATXN2* gene, and *APOE* genotype, have been extensively investigated in the last decade with conflicting findings (60–65).

APOE ε4 allele is a well known established genetic risk factor for familial and sporadic forms of AD (66). In ALS patients, the presence of the *APOE* ε2 significantly modulates the risk for FTD by 2.5–fold, while ε4 does not have that effect (64). On the other hand, in patients having *C9orf72* repeat expansion, diagnosed with ALS or FTD, *APOE* ε3/ε3 represents the most common genotype (67,68). In a case involving two siblings diagnosed with frontotemporal lobar degeneration/motor neuron disease (FTLD/MND), carrying a *C9orf72* expansion, and positive family history for FTLD/MND, the sibling with *APOE* ε4/ε4 had more pronounced cognitive–behavioral symptoms and a more abundant APOE and TDP–43 complexes in the anterior cingulate cortex compared to the sibling with *APOE* ε3/ε3 genotype (69).

Expansion of the CAG repeats in the *ATXN1* and *ATXN2* genes is causing spinocerebellar ataxia 1 and 2, respectively (70). The association between these genes and ALS has been supported by findings showing a high frequency of the intermediate length CAG repeats in both of these genes among individuals with ALS (71). Further, data have shown a strong association of intermediate *ATXN1* and *ATXN2* repeats with ALS carrying a *C9orf72* repeat expansion (62,72,73). *ATXN1* repeats in *C9orf72* expansion carriers predispose them to develop ALS, and their presence in the familial cases is almost twice as frequent than in sporadic cases (73). Within the ALS/FTD spectrum, intermediate length *ATXN2* CAG repeat expansions have been proposed as disease modifiers that may increase susceptibility to ALS (61). Experimental studies have shown that co–expression of intermediate *ATXN2* repeats alongside partial *C9orf72* depletion leads to enhanced *ATXN2* protein aggregation and increased neuronal cell death (74). A recent study that analyzed STRs linked to

polyglutamine disorders in whole–genome sequencing data from *C9orf72* expansion carriers revealed an association between CAG repeat number in *ATXN2* and the presence of ALS symptoms, and additionally associated the *ATXN3* intermediate repeats with earlier age at disease onset (53).

The same study has shown that intermediate alleles of the *HTT* gene, which encodes huntingtin, are associated with an earlier age at disease onset. On average, carriers of these alleles developed symptoms over 10 years earlier than non–carriers, highlighting the potential role of polyglutamine–related STRs as genetic modifiers in *C9orf72*–associated disease (53).

Another trinucleotide repeat expansions associated with ALS have been identified in the *NIPA1* gene. It has been found that among *C9orf72* repeat carriers, there was a higher than expected frequency of *NIPA1* longer GCG alleles (expansions >8 repeats) (75,76).

It has been shown that SNPs also may affect disease onset in *C9orf72* expansion carriers through modulating gene expression (50). Higher expression of *SLITRK,* a gene encoding a postsynaptic adhesion protein, has been associated with minor allele A of SNP rs1009776. Presence of this allele is associated with earlier dementia onset in *C9orf72* expansion carriers, and these findings suggested a potential link between *C9orf72* expansions, synaptic dysfunction, and the timing of symptom onset (50).

Van der Ende et al. have found that SNPs in *TMEM106B* gene were associated with a reduced risk of developing FTD in *C9orf72* expansion carriers, and this suggests that *TMEM106B* may act as a disease modifier in *C9orf72*–related neurodegeneration, influencing the clinical phenotype toward a cognitive rather than a motor presentation (48).

Another gene associated with modified penetrance of the *C9orf72* expansions is *UNC13A,* coding a presynaptic protein involved in neurotransmitter release. It has been shown that homozygous allele C of SNP rs12608932 in *UNC13A* increased ALS or dementia risk in *C9orf72* expansion carriers (77). Previously, the same allele has been associated with worse prognosis and reduced survival after disease onset in *C9orf72*-positive patients, especially in the motor neuron disease subgroup (78). The lithium–carbonate clinical trial, targeting ALS patients with the high–risk C/C *UNC13A* genotype, is an important example of testing a therapeutic agent stratified by specific genotype (79).

5. Therapeutic opportunities in the *C9orf72* –associated ALS/FTD

Identification of a *C9orf72* expansion has important implications not only for genetic counseling but also for stratifying patients in clinical trials targeting repeat–associated toxicity. This kind of stratification may help to reduce heterogeneity in trial populations and increase the chance of detecting treatment effects.

Currently, no therapies are specifically approved for *C9orf72*–positive ALS/FTD patients and they are treated with standard ALS drugs (riluzole, edaravone, tofersen, AMX0035) and symptomatic management for FTD. Significant investigational approaches are underway–particularly focused on antisense oligonucleotides (ASOs), repurposed small molecules, and monoclonal antibodies.

Several targeted clinical trials have been conducted in these subgroups. Antisense oligonucleotides (BIIB078 and WVE–004) aimed selectively at the *C9orf72* repeat expansion, were tested in *C9orf72*–positive ALS/FTD patients but failed to demonstrate clinical efficacy (80,81). More recently, small molecules such as metformin, TPN–101, and apilimod, as well as the monoclonal antibody AL001, are under investigation in *C9orf72* expansion carriers with ALS/FTD, showing encouraging biomarker effects in early–phase studies, although clinical benefits remain to be established (82).

Preventive or disease–modifying drugs for asymptomatic carriers of the *C9orf72* expansions are not currently approved. This reflects the reduced and variable penetrance of these variants and the potential for significant adverse effects from long–term drug administration (76).

One of the key factors in the development of possible therapeutics is the identification of specific biomarkers such as glycine–proline dipeptide repeats (polyGP) in cerebrospinal fluid of the *C9orf72* repeat expansion carriers (83).

6. Challenges and Future directions

Although no targeted therapy for the *C9orf72*–related ALS/FTD has yet been established, genetic testing remains a crucial step in precision medicine, as it enables accurate diagnosis, informs genetic counseling, and allows patient stratification for ongoing and future clinical trials.

The failure of several early–phase clinical trials in *C9orf72*–positive ALS/FTD may, in part, be explained by the still not elucidated pathological processes that begin long before clinical onset. Consequently, many studies have not targeted the most appropriate mechanisms for disease modification. Another important consideration is the influence of genetic modifiers. Variants in genes such as *ATXN2*, *TMEM106B*, *HTT*, and *APOE* have been shown to modulate age at onset, disease progression, and clinical phenotype in *C9orf72* expansion carriers. The presence of such modifiers introduces substantial heterogeneity within patient cohorts, potentially masking therapeutic effects in relatively small, early–phase studies. Furthermore, the interplay of polygenic background and epigenetic factors may influence penetrance and response to experimental therapies. These observations highlight the importance of integrating genetic stratification and biomarker–based subgroup analyses into future trial designs to improve sensitivity for detecting therapeutic benefit.

Acknowledgments

This study was supported by the Ministry of Science, Technological Development and Innovation, Republic of Serbia (grant no. 200110).

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Violacein as a Potential Agent for Sarcoma Treatment

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Abstract

Sarcomas, particularly pediatric types such as rhabdomyosarcoma and osteosarcoma, remain difficult to treat due to limited therapeutic efficacy and significant toxicity of current regimens. Natural compounds are gaining attention as potential alternatives or adjuvants in sarcoma therapy. Among them, violacein, an indole-derived pigment produced by *Chromobacterium violaceum*, has demonstrated promising antitumor effects across several cancer types. This review outlines the biological features of violacein, as well as its anticancer properties, particularly in sarcoma models. Current evidence highlights violacein's ability to selectively target tumor cells by inducing apoptosis and inhibiting cell migration. Mechanistic insights and observed effects in sarcoma models are discussed. Additionally, formulation and delivery challenges are addressed, along with strategies to enhance its therapeutic potential. By synthesizing current findings, this review positions violacein and similar compounds as compelling candidates for further investigation in the context of sarcoma therapy.

Keywords: sarcomas, osteosarcoma, rhabdomyosarcoma, violacein, natural compounds, antitumor agents

Violacein kao potencijalni agens za terapiju sarkoma

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Apstrakt

Sarkomi, posebno pedijatrijski tipovi kao što su rabdomiosarkom i osteosarkom, i dalje predstavljaju terapijski izazov zbog ograničene efikasnosti trenutnih režima lečenja i značajne toksičnosti postojećih hemioterapeutika. Prirodna jedinjenja sve više privlače pažnju kao potencijalne alternative ili adjuvansi u terapiji tumora. Među njima, violacein, indolski pigment koji proizvodi *Chromobacterium violaceum*, pokazao je obećavajuće efekte na različitim tipovima tumora. Trenutni dokazi ukazuju na sposobnost violaceina da selektivno deluje na tumorske ćelije indukujući apoptozu i inhibirajući migraciju ćelija. Ovaj pregled opisuje biološke karakteristike violaceina, kao i njegova antitumorska svojstva, posebno na modelima sarkoma. Posebna pažnja posvećena je novim uvidima u mehanizme njegovog delovanja. Pored toga, diskutovani su izazovi vezani za formulaciju i isporuku violaceina, zajedno sa strategijama za unapređenje njegovog terapeutskog potencijala. Sintezom postojećih saznanja, ovaj pregled pozicionira violacein i slična jedinjenja kao perspektivne kandidate za dalja istraživanja u kontekstu terapije sarkoma.

Ključne reči: sarkomi, osteosarkom, rabdomiosarkom, violacein, prirodna jedinjenja, antitumorski agensi

1. Introduction

Sarcomas are a rare and heterogeneous group of malignant tumors of mesenchymal origin, comprising more than 70 histological subtypes that can arise in bone, muscle, cartilage, adipose tissue, and connective tissues [1]. Although sarcomas make up only ~1% of adult cancers, they represent ~20% of pediatric solid tumors and are one of the major causes of cancer-related morbidity and mortality in young patients [2].

Despite advances in surgery and multimodal care, therapeutic progress has been modest. Anthracycline-based chemotherapy remains the backbone for most subtypes, but response rates and survival benefits are limited. Targeted and histology-specific therapies have improved outcomes in selected entities; however, transformative breakthroughs remain rare, and survival for advanced or relapsed disease is generally poor [3].

These limitations are particularly evident in pediatric sarcomas, with osteosarcoma (OS) and rhabdomyosarcoma (RMS) being the predominant types. Survival for localized disease has improved with multimodal regimens, but the prognosis for high-risk, metastatic, or recurrent cases remains below 30% [4]. Moreover, intensive treatment is associated with severe acute and long-term toxicities [5]. These challenges underscore the urgent need for safer and more effective treatments across both pediatric and adult sarcomas.

Natural compounds are of key interest in the search for alternatives. Indeed, several standard sarcoma drugs, including doxorubicin, vincristine, and actinomycin D, originate from natural sources [6]. In OS and RMS, recent work has reported promising activity of plant-derived molecules [7, 8], such as curcumin [9], hesperidin [10], and naringenin [11], as well as compounds like flavokawain B and betulinic acid in other sarcoma types [12, 13]. However, most research has centered on phytochemicals, leaving microbial metabolites comparatively underexplored.

Violacein, a purple pigment produced by *Chromobacterium violaceum* and several other bacterial species, has recently drawn attention for its therapeutic promise [14, 15]. Studies show that violacein exerts potent cytotoxicity against multiple tumor types, including sarcomas, where it induces apoptosis, impairs migration, and selectively targets malignant cells while sparing non-malignant cells [14, 16]. These effects have been achieved in *in vitro* and *in vivo* models without the systemic toxicity, characteristic for conventional chemotherapy [17, 18].

Here, we summarize the current knowledge on violacein, beginning with its source, structure, and general anticancer mechanisms, before focusing on evidence from sarcoma models. We then discuss translational challenges and opportunities, with the aim of positioning violacein as a promising yet underutilized candidate in the effort to improve sarcoma therapy.

2. Violacein: Source, Structure, and General Antitumor Activity

Violacein is a naturally occurring bis-indole pigment first described in 1882 [19] and subsequently identified in over 130 bacterial species, including members of the genera *Chromobacterium*, *Alteromonas*, *Janthinobacterium*, *Pseudomonas*, *Duganella*, and *Collimonas*, with *Chromobacterium violaceum* being the most studied source [14]. The compound has a molecular mass of 343.3 g/mol, is insoluble in water but soluble in organic solvents such as ethanol, methanol, and dimethyl sulfoxide, and absorbs strongly in the visible spectrum, giving rise to its characteristic deep purple color [20].

Violacein has been recognized for a broad spectrum of biological activities, including antibacterial, antifungal, antiviral, antiparasitic, immunomodulatory, anti-inflammatory, and anticancer properties [14,

15]. Advances in understanding its mechanism of action have enhanced its potential for pharmacological application and increased research interest [21, 22].

Violacein has shown cytotoxic activity against a wide range of human malignant cells, including those from colorectal carcinoma, breast cancer, head and neck cancer, hepatocellular carcinoma, glioblastoma, melanoma, and leukemia [23]. In most of these models, effective concentrations are within the low micromolar range (≤5-μM) (Table 1), while non-malignant cells generally tolerate higher doses (5–10 μM), as shown for ovarian cell line CHO-K1 and MRC-5 fibroblasts [24]. This differential sensitivity highlights violacein's selectivity for malignant cells, a key feature of potential antitumor therapeutics [25]. Consistently, our recent study confirmed violacein's limited toxicity in non-malignant V79-4 fibroblasts, a standard model for mutagenicity and toxicity testing [16, 26]. The IC_{50}, concentration that reduces the viability of these fibroblasts to 50%, was 1.6 μM - higher than the IC_{50} reported for most tumor cell lines.

Table 1. Violacein's half-maximal inhibitory concentrations (IC_{50}) and proposed mechanisms of action across various tumor cell lines.

Tumor type		Cell line(s)	IC_{50} (μM)	Main mechanism(s)	ROS involvement	Reference(s)
Colorectal carcinoma		CaCo2	2	Mitochondrial damage, cytochrome c release, apoptosis;	ROS-dependent	[30]
		HCT116	1.35	p53/p21 activation, cell-cycle arrest; ↓ migration	Unclear	[28, 37]
Breast cancer		MCF-7	0.42	Bax upregulation, apoptosis ↓ CXCL12/CXCR4 signaling; ↓ migration	ROS-dependent	[31, 35, 38]
Erlich tumor		EAT	5	Oxidative stress, apoptosis	ROS-dependent	[17]
Leukemia		HL60	0.7	TNF-mediated apoptosis via caspase-8, NF-κB, and MAPK	Unclear	[32]
Erythroleukemia		TF1	~2	kinome reprogramming, ER stress, Golgi collapse	Unclear	[33]
Melanoma		SKMEL-103	0.5	Autophagy modulation via AKT/AXL inhibition; ↓ migration	Unclear	[34]
Glioblastoma		U87	~1	Cytoskeletal disruption; ↓ migration	Unclear	[36]
Sarcomas	Rhabdomyo-sarcoma	HS-729, RD, SJRH30	0.66–0.88	Apoptosis via membrane-associated mechanism; ↓ migration	ROS-independent	[16]
	Osteosarcoma	HOS, MG-63, SaOS2, U-2 OS	0.35–0.72	Apoptosis via membrane-associated mechanism; ↓ migration (line dependent)	ROS-independent	[16]

The selective activity of violacein was also confirmed *in vivo* by testing its toxicity on zebrafish embryos, where it was shown that concentrations toxic to tumor cells were not toxic to embryos up to 120 hours post-fertilization [16]. Correspondingly, intraperitoneal or intratumoral administration of violacein has not caused pathological organ damage or signs of toxicity in healthy or tumor-bearing mice, while it reduced tumor volume in the latter [17, 18]. Moreover, oral administration of violacein in mice at doses up to 10 mg/kg showed no toxic effects [27].

Violacein also enhances the effectiveness of conventional chemotherapeutics. It has been shown to sensitize colorectal cancer cells to 5-fluorouracil [28] and bladder carcinoma cells to cisplatin, while also reducing cisplatin-induced genotoxicity [29]. These observations suggest a dual role for violacein as both a stand-alone cytotoxic agent and a chemosensitizer capable of reducing adverse systemic effects.

2.1. Mechanisms of Action

The mechanisms underlying violacein's antitumor effects are multifaceted and appear to be cell-type dependent (Table 1).

Oxidative stress: In colorectal CaCo2 and breast cancer cells MCF-7, violacein induces reactive oxygen species (ROS) production, leading to mitochondrial damage, cytochrome c release, and apoptosis; these effects can be reversed by antioxidants such as N-acetylcysteine [30, 31].

Mitochondrial dysfunction: In HeLa cervical cancer cells, violacein promotes apoptotic cell death via mitochondrial membrane hyperpolarization [24].

Cell cycle arrest: In HCT116 colorectal carcinoma cells, violacein blocks cell cycle progression and increases p53 and p21 expression [28].

Stress pathways activation: In HL60 leukemia cells, violacein activates TNF-mediated apoptosis via caspase-8, NF-κB, and MAP kinases [32], while in erythroleukemia TF1 cells, cell death occurred independently of caspases, through endoplasmic reticulum and Golgi stress [33].

Autophagy modulation: In metastatic melanoma, violacein inhibited AKT and AXL signaling, impairing autophagy [34].

Hypoxia sensitization: Tumor cells were more vulnerable to violacein under hypoxic conditions, suggesting synergy with the specific tumor microenvironment [35].

In addition to direct cytotoxicity, violacein disrupts tumor cell migration and invasiveness. It inhibited the invasion of SKMEL-103 melanoma cells in 3D culture [34], migration of glioblastoma U87 cells [36], as well as HCT116 and HT29 colorectal cancer cells [37]. Violacein also reduced the motility of breast cancer cell line MCF-7, partly through suppression of CXCL12/CXCR4 signaling, a key axis in metastasis [38].

2.2. Summary

Collectively, violacein demonstrates potent anticancer activity across diverse tumor types through multiple mechanisms, including apoptosis induction, oxidative stress, autophagy modulation, and suppression of migration. Its selectivity for malignant cells and tolerability in preliminary *in vivo* models distinguish it from many other natural compounds. Building on these general anticancer activities, we next review evidence specific to sarcoma models.

3. Violacein in Sarcomas

3.1. Cytotoxicity

Building on its broad anticancer activity, violacein has shown pronounced cytotoxic effects in sarcoma models. Violacein inhibited the growth of osteosarcoma cells (HOS, SaOS-2, MG-63, and U-2 OS), with IC_{50} values ranging from 0.35 to 0.72 μM − within or even below the ranges reported for cells of other tumor types, where the IC_{50} typically ranges from 1 to 5 μM (Table 1) [16]. Among these, HOS cells were the most sensitive. More recently, violacein's activity was validated in advanced osteosarcoma models: spheroids generated from U-2 OS and SaOS-2 cells responded strongly, with cytotoxicity evident at concentrations as low as 0.5 μM, confirming efficacy in 3D tumor architectures that more closely mimic clinical conditions [39].

Comparable results were reported in RMS cell lines, including RD and HS-729 (embryonal type) and SJRH30 (alveolar type), with IC_{50} values between 0.66 and 0.88 μM. For context, a systematic review of 84 studies on natural compounds in RMS reported only a few agents, derived from *Choerospondias axillaris*, *Anacolosa clarkii*, and *Macaranga barteri*, which achieved IC_{50} values equal to or lower than those of violacein [8, 40-42]. In RD cells, the violacein's IC_{50} was similar to that of vincristine [43], one of the most commonly used agents in RMS therapy, and four times lower than that of cyclophosphamide [44].

Importantly, these cytotoxic effects appear tumor-selective. Non-malignant cell lines, such as V79-4 fibroblasts, tolerated markedly higher concentrations (IC_{50}~1.6 μM). Moreover, zebrafish embryos exhibited no developmental toxicity when exposed to concentrations effective against sarcoma cells, supporting a favorable therapeutic window (8).

Interestingly, related compounds have also been tested in other sarcoma models. A violacein-like pigment (PVP) derived from *Janthinobacterium* strains caused dose- and time-dependent growth inhibition of murine UV-induced 2237 fibrosarcoma cells at 0.1–1 μM [45]. However, because this compound is not chemically identical to violacein, further work is needed to determine whether these effects reflect the activity of authentic violacein.

3. 2. Mechanisms of Action

Violacein's cytotoxic activity in sarcoma cells appears to be mediated primarily through apoptosis. Annexin V/PI staining in both OS (HOS) and RMS (RD) cells confirmed programmed cell death, while necrotic fractions were not significantly increased after violacein treatment [16]. In fibrosarcoma cell line 2237, the violacein-like pigment caused both G0/G1 and G2/M cell cycle arrest, accompanied by the downregulation of cyclin-dependent kinases, upregulation of tumor suppressors p21 and p27, disruption of the mitochondrial membrane potential, and activation of caspases, leading to apoptotic cell death [45].

Unlike carcinomas, where violacein often induces oxidative stress, OS and RMS cells showed no increase in reactive oxygen species (ROS) or lipid peroxidation, and antioxidant pretreatment failed to rescue viability, indicating that sarcoma cytotoxicity is ROS-independent [16]. Confocal imaging further revealed that violacein accumulated on the plasma membrane rather than penetrating intracellularly [16], suggesting that apoptosis may be triggered by membrane-associated mechanisms, potentially involving death receptor signaling [46]. Preliminary *in silico* studies have suggested a possible interaction of violacein with the epidermal growth factor receptor, involved in a pathway central to tumor progression [47]. However, these observations remain hypothetical and require experimental validation. In recent studies, it was shown that violacein's interaction with cell membranes can induce lipid reorganization and alter viscosity, elasticity, and structural properties without affecting permeability [48, 49]. Its impact on lipid monolayers, such as changes in tilt angles and electrostatic-dependent arrangements [50], suggests that its ability to modulate signaling pathways may partly arise from these membrane interactions.

3. 3. Anti-Migratory Effects

In addition to its cytotoxicity, violacein can also impair the motility of sarcoma cells. Migration assays showed significant inhibition in HOS and SaOS-2 osteosarcoma cells, while MG-63 and U-2 OS were unaffected [16]. In RMS, alveolar SJRH30 cells exhibited a strong reduction in migratory capacity, whereas embryonal lines RD and HS-729 displayed weaker responses. These variable effects mirror observations in different types of carcinoma cell lines, where violacein affects migration by disrupting cytoskeletal dynamics and chemokine signaling in a context-dependent manner [36, 51].

3. 4. Combination Therapy

Violacein also shows potential in combination with standard chemotherapeutics. Findings from other tumor models reinforce its combinatorial promise: violacein potentiated the activity of 5-fluorouracil in colorectal cancer [28] and cisplatin in bladder carcinoma cells, while also reducing cisplatin-induced genotoxicity [29]. In RMS, it enhanced the effect of doxorubicin, particularly in RD cells, though little effect was observed with irinotecan or vinflunine [52]. These data suggest that violacein could enhance efficacy and safety profiles of certain sarcoma regimens, particularly those incorporating doxorubicin.

3.5. Comparative Insights

Taken together, available evidence shows that violacein exerts potent, selective, and mechanistically distinct antitumor activity in sarcomas. Unlike many other tumor types, sarcomas exhibit ROS-independent cytotoxicity, suggesting alternative apoptotic pathways mediated at the plasma membrane. Migration is inhibited in at least one cell line of each sarcoma type tested, though effects vary by subtype. Finally, violacein has shown promise in combinatorial regimens, particularly with doxorubicin, and retains efficacy in 3D spheroid models, highlighting its potential to advance toward clinical translation in sarcoma therapy.

3.6 .Translational Challenges and Future Perspectives

While *in vitro* data strongly support violacein's activity in sarcomas, its translation into a therapeutic option requires addressing several biological and practical challenges.

For violacein to advance from preclinical observations to a clinically relevant anticancer agent, several key criteria must be addressed. These requirements encompass efficacy, selectivity, safety, mechanistic clarity, pharmacokinetics, stability, large-scale production, and regulatory feasibility. While encouraging progress has been made, further research is essential to translate violacein's potential into a viable therapeutic option for sarcoma patients.

3.6.1. Efficacy

The first prerequisite for drug development is robust and reproducible antitumor efficacy. Research shows that violacein and related PVP exhibit strong cytotoxicity in multiple sarcoma cell lines, originating from OS, RMS, and fibrosarcoma, with effective concentrations in the low micromolar range [16, 45]. *In vitro*, violacein reduces cell viability, induces apoptosis, and impairs migration. Activity has also been confirmed in 3D osteosarcoma spheroids [39], which more closely mimic tumor architecture. However, efficacy must now be validated in animal models, including patient-derived xenografts, to establish *in vivo* relevance and guide dosing strategies.

3.6.2. Selectivity and Low Toxicity

Selectivity for malignant over normal cells is critical to minimize toxicity. *In vitro* data show that violacein selectively kills OS and RMS cells at concentrations below those required to affect non-malignant fibroblasts. These tumor-specific effects are consistent with earlier findings and are supported by the absence of developmental toxicity in zebrafish embryos at doses relevant for therapy (8). Murine studies in non-sarcoma models have confirmed safety and tumor regression following intraperitoneal or intratumoral administration (9, 10). Therefore, confirming and extending this selectivity to sarcoma animal models will be a necessary step toward clinical translation.

3.6.3. Mechanism of Action

For violacein to be considered a viable candidate as an antitumor therapeutic, it is necessary to identify its molecular targets, determine the impact on signaling pathways, and investigate whether it can overcome mechanisms of resistance. While violacein's cytotoxic effects are well documented, its exact molecular targets remain incompletely understood. In OS and RMS cells, violacein localizes to the plasma membrane, induces apoptosis, and acts independently of oxidative stress [16], distinguishing it from ROS-dependent mechanisms reported in carcinomas [17, 30, 31]. Further research is needed to determine whether violacein influences pathways involving death receptor activation, alterations in membrane microdomains, or changes to growth factor signaling.

3.6.4. Pharmacokinetics and Delivery

Poor water solubility poses a significant limitation to violacein's systemic administration. Nonetheless, recent formulation innovations, including the use of nanoparticles and liposomes, have improved violacein's solubility and its ability to concentrate in tumor tissues [25]. *In silico* modeling suggests that oral administration may achieve adequate bioavailability [47]. These encouraging findings should be validated *in vivo* through pharmacokinetic studies addressing absorption, distribution, metabolism, and excretion.

3.6.5. Availability and Stability

Violacein can be readily obtained from multiple bacterial strains, and it demonstrates high physical and chemical stability, remaining active after light exposure and sustained heating to 100°C [53]. These properties enhance its practical usability for research and therapeutic development.

3.6.6. Production Feasibility

Large-scale production is essential for clinical application. Natural yields from bacterial cultures are low, but advances in metabolic engineering have enabled efficient violacein biosynthesis in genetically modified *Escherichia coli* and *Saccharomyces cerevisiae* strains [14]. These developments increase scalability and reduce costs, paving the way for industrial-level production.

3.6.7. Regulatory Pathway

Available studies provide encouraging indications of the tolerability and efficacy of violacein. However, sarcoma-specific *in vivo* data remain limited. Therefore, violacein must undergo rigorous preclinical testing before entering clinical trials under the oversight of regulatory agencies such as the FDA and EMA. To date, no clinical trials have been initiated. Meeting these regulatory standards will require comprehensive toxicology, pharmacokinetics, stability data, and Good Manufacturing Practice (GMP)-compliant production.

Together, these challenges frame the path from laboratory findings to clinical application and highlight the steps necessary for violacein to reach its therapeutic potential.

4 .Conclusion

Sarcomas remain among the most challenging malignancies to treat, particularly in pediatric patients with osteosarcoma and rhabdomyosarcoma. Despite advances in surgery and chemotherapy, survival for high-risk, metastatic, and relapsed disease has stagnated for decades, while treatment-related toxicities

continue to impose significant long-term burdens. This therapeutic plateau highlights the urgent need for novel, effective, and safer strategies.

Natural compounds have historically shaped oncology, with agents such as doxorubicin, vincristine, and actinomycin D forming the backbone of sarcoma regimens. In recent years, several additional plant-derived molecules have demonstrated promising preclinical activity. Yet, microbial metabolites remain comparatively underexplored in this context.

Violacein, a pigment produced by *Chromobacterium violaceum* and related bacteria, has emerged as a compelling candidate. Its cytotoxicity spans over diverse carcinomas and extends with potency to sarcomas, including osteosarcoma, rhabdomyosarcoma, and fibrosarcoma, with activity confirmed not only in conventional 2D cultures but also in 3D osteosarcoma spheroids. Importantly, violacein exerts selective cytotoxicity toward malignant cells. In sarcomas, it induces apoptosis through ROS-independent and membrane-associated mechanisms, and inhibits migration in a cell line-dependent manner. Preliminary *in vivo* studies suggest a favorable therapeutic window, and formulation advances are beginning to address its pharmacokinetic limitations.

At the same time, several translational hurdles remain. Its precise molecular targets in sarcomas are not yet fully defined, and no sarcoma-specific *in vivo* trials have been completed. Solubility and delivery must be optimized to ensure therapeutic exposure, and large-scale GMP-compliant production will be required before regulatory evaluation.

Taken together, current evidence positions violacein as a promising natural product with unique mechanisms of action and significant potential for development as an antitumor agent in sarcomas. Meeting the criteria of efficacy, selectivity, safety, pharmacokinetics, stability, scalability, and regulatory compliance will be essential to move violacein forward. With further mechanistic and translational research, violacein may progress from a preclinical candidate to a clinically relevant therapy, contributing to the much-needed expansion of treatment options for patients with sarcoma.

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Impact of discontinuation and reintroduction of alglucosidase alpha in patients with late-onset Pompe disease

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Abstract

Late-onset Pompe disease (LOPD) is a progressive metabolic myopathy caused by *GAA* gene variants. LOPD should be considered in patients with unexplained limb-girdle weakness, isolated respiratory or trunk involvement, or asymptomatic hyperCKemia. Enzyme replacement therapy (ERT) with recombinant human acid α-glucosidase (rhGAA) is the standard treatment. However, data on the effects of treatment interruption after long-term use are scarce.

We analyzed five genetically confirmed LOPD patients treated at the Neurology Clinic, University Clinical Center of Serbia. Three patients (two female, one male) underwent enzyme testing and targeted sequencing, while two male patients were diagnosed through whole-exome sequencing (WES). All patients received rhGAA (20 mg/kg biweekly); treatment was interrupted for 54.2±8.0 days after a mean therapy duration of 4.6±2.3 years.

During long-term ERT, muscle strength measured with MRC Sum-Score improved steadily, stabilized during interruption, and increased again 2-6 months after reintroduction ($p<0.05$). The 6-minute walking test (6MWT) distance declined during interruption but recovered by month six ($p<0.05$). Forced vital capacity (FVC) deterioration accelerated during the therapy gap, with partial recovery that did not reach baseline ($p<0.01$). Quality of life SF-36 Role Physical score improved after two months of resumed therapy ($p<0.05$).

These findings confirm the sustained efficacy of ERT in LOPD – even a short discontinuation of therapy caused measurable decline with gradual recovery followed therapy reinitiation. Early molecular diagnosis and continuous treatment are essential to optimize outcomes in LODP.

Key words: Pompe disease, enzyme replacement therapy, muscle strength, walking, forced vital capacity.

Uticaj prekida i ponovnog uvođenja alglukozidaze alfa kod pacijenata sa Pompeovom bolešću kasnog početka

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Apstrakt

Pompeova bolest kasnog početka (LOPD) je progresivna metabolička miopatija uzrokovana mutacijama u genu *GAA*. Na LOPD treba posumnjati kod pacijenata sa nerazjašnjenom udno-pojasnom slabošću, izolovanom slabošću respiratorne ili trupne muskulature ili asimptomatskom hiperCKemijom. Enzimska supstituciona terapija (ERT) rekombinantnom humanom kiselom alfa-glukozidazom (rhGAA) predstavlja standard lečenja. Međutim, nema dovoljno podataka o efektima prekida terapije nakon dugotrajne primene.

Naša studija uključila je pet genetički potvrđenih pacijenata sa LOPD lečenih na Klinici za neurologiju Univerzitetskog kliničkog centra Srbije. Kod tri pacijenta (dve žene i jednog muškarca) dijagnoza je potvrđena enzimskim testiranjem i ciljanim sekvenciranjem gena, dok su dva pacijenta dijagnostikovana sekvenciranjem celog egzoma. Svi pacijenti su primali rhGAA (20 mg/kg na svake dve nedelje). Terapija je prekinuta u prosečnom trajanju od 54,2±8,0 dana nakon perioda lečenja od 4,6±2,3 godine.

Tokom dugotrajne ERT uočeno je postepeno poboljšanje mišićne snage mereno MRC-SS skalom, stabilizacija tokom perioda prekida terapije i ponovni napredak 2-6 meseci nakon njenog ponovnog uvođenja ($p<0,05$). Distanca pređena tokom šestominutnog testa hoda (6MWT) smanjila se tokom perioda prekida terapije i ranog perioda ponovnog uvođenja terapije, ali se potom produžila na inicijalne vrednosti do šestog meseca od ponovnog uvođenja leka ($p<0,05$). Pogoršanje forsiranog vitalnog kapaciteta (FVC) bilo je izraženije tokom prekida, uz kasniji delimičan oporavak koji nije dostigao početne vrednosti ($p<0,01$). Podskor Fizička uloga na upitniku SF-36, kojim se meri kvalitet života, bio je značajno poboljšan nakon dva meseca nakon ponovnog uvođenja terapije ($p<0,05$).

Ovi rezultati potvrđuju dugotrajnu efikasnost ERT kod LOPD. Čak i kratkotrajni prekid ERT dovodi do kliničkog pogoršanja, dok ponovno uvođenje terapije omogućava postepeni oporavak. Rana molekularna dijagnoza i kontinuirano lečenje su od ključnog značaja za optimizaciju ishoda bolesti kod LOPD.

Ključne reči: Pompeova bolest, enzimska supstituciona terapija, snaga mišića, hodanje, forsirani vitalni kapacitet.

Introduction

Pompe disease (PD), also known as glycogen storage disease type II (GSD-II), is a progressive disorder characterized by decreased activity of the lysosomal enzyme acid alpha glucosidase (GAA) due to mutations in the *GAA* gene [1]. The disease can manifest as infantile-onset Pompe disease (IOPD), where the disease develops in the first days or weeks of life with GAA enzyme activity less than 1%, or as late-onset Pompe disease (LOPD), where the disease develops in childhood, adolescence, or adulthood with GAA enzyme activity ranging from 1-30% compared to normal [2].

LOPD presents as a slowly progressive muscular disorder characterized by specific symptoms and signs: hyperCKemia up to 2000 IU/L, progressive weakness and atrophy predominantly affecting proximal muscles of the limbs and trunk muscles with early involvement of the respiratory musculature [3]. Additionally, patients may experience intolerance to physical exertion, fatigue, muscle pain, sleep apnea, dysarthria, dysphagia, macroglossia, hepatomegaly, hearing loss, and others. Furthermore, patients with LOPD have an increased risk of developing vascular aneurysms [4]. Identifying patients with late-onset Pompe disease (LOPD) remains challenging, and misdiagnosis is frequent [5]. LOPD should be specifically suspected in individuals with unexplained limb-girdle weakness, isolated trunk or respiratory muscle weakness, or asymptomatic hyperCKemia [6]. Given the availability of enzyme replacement therapy (ERT), which modifies disease course, timely recognition is crucial.

Enzyme replacement therapy (ERT) with recombinant human acid alpha-glucosidase (rhGAA, Myozyme®) was approved by the FDA in 2006 for IOPD and in 2010 for LOPD, becoming the treatment of choice for Pompe disease (PD) [7]. The introduction of ERT has significantly improved survival, respiratory, and muscular function in patients with IOPD [8]. It significantly enhances the quality of life and muscle function of LOPD patients, including measures such as the 6-minute walking test (6MWT), muscle strength assessments using the Medical Research Council (MRC) scale, and forced vital capacity (FVC) [9]. Long-term effects of rhGAA ERT in LOPD patients also include improvements in daily activities and psychological status [10].

It is not fully understood how temporary or permanent discontinuation of ERT affects the course of the disease. Some patients have shown stable PD status without progression even after discontinuation of therapy [11]. However, there are reports indicating worsening after prolonged periods of dosing interruption [12]. During the COVID-19 pandemic, even a brief interruption of therapy for just a few weeks lead to clear objective deterioration of the disease, which may be irreversible [11]. A Swiss study demonstrated that a multi-month interruption of therapy in LOPD lead to worsening in clinical parameters and quality of life, but the patient's clinical condition might return to its pre-discontinuation state within three years of reintroduction of the ERT [13].

The aim of the study is to prospectively monitor the efficacy of ERT with rhGAA in patients with LOPD, as well as to investigate the impact of therapy discontinuation and reintroduction.

Methods

Patients

This study included five patients evaluated at the Neurology Clinic, University Clinical Center of Serbia, who were diagnosed with late-onset Pompe disease (LOPD). Written informed consent was obtained for GAA enzyme activity analysis and/or for molecular genetic analysis. In three patients laboratory testing was conducted at the ARCHIMEDlife reference laboratory in Vienna, where GAA activity in dry blood spots (DBS)

was quantified using a tandem mass spectrometry (MS/MS) – activity levels >2 μmol/L/h were considered as normal. For individuals with reduced enzyme activity, DNA was extracted from DBS samples, and polymerase chain reaction (PCR)-based sequencing of all coding exons and flanking intronic regions of the *GAA* gene was performed. Variants were classified according to HGVS standards, and benign polymorphisms were excluded from reporting. For two patients, whole-exome sequencing (WES) was employed as part of an extended diagnostic strategy to detect disease-causing variants in a broad neuromuscular gene panel, followed by confirmatory enzymatic testing in DBS.

Clinical and sociodemographic data were systematically extracted from electronic medical records for all patients. All patients were treated with enzyme replacement therapy (ERT) using rhGAA (Myozyme®) at a dose of 20 mg/kg intravenously every two weeks at the Neurology Clinic, University Clinical Center of Serbia. Due to administrative issues, ERT was interrupted for approximately two months (April–May 2023). Patients underwent standardized evaluations at therapy initiation, immediately prior to ERT reintroduction, and at two and six months following treatment resumption (Figure 1a). The study was approved by the Ethics Committee of the University Clinical Center of Serbia, which waived the requirement for additional informed consent owing to its retrospective design. All procedures adhered to the principles of the Declaration of Helsinki.

Outcome measures

The following outcome measures were used to assess therapy efficacy (Figure 1-b): impairment measures – assessment of muscle strength using the MRC Sum-Score (MRC-SS), 6MWT), and FVC, ability measures – Rotterdam Handicap Scale (RHS) and Krupp's Fatigue Severity Scale (FSS), and quality of life (QoL) measures - SF-36 quality of life scale.

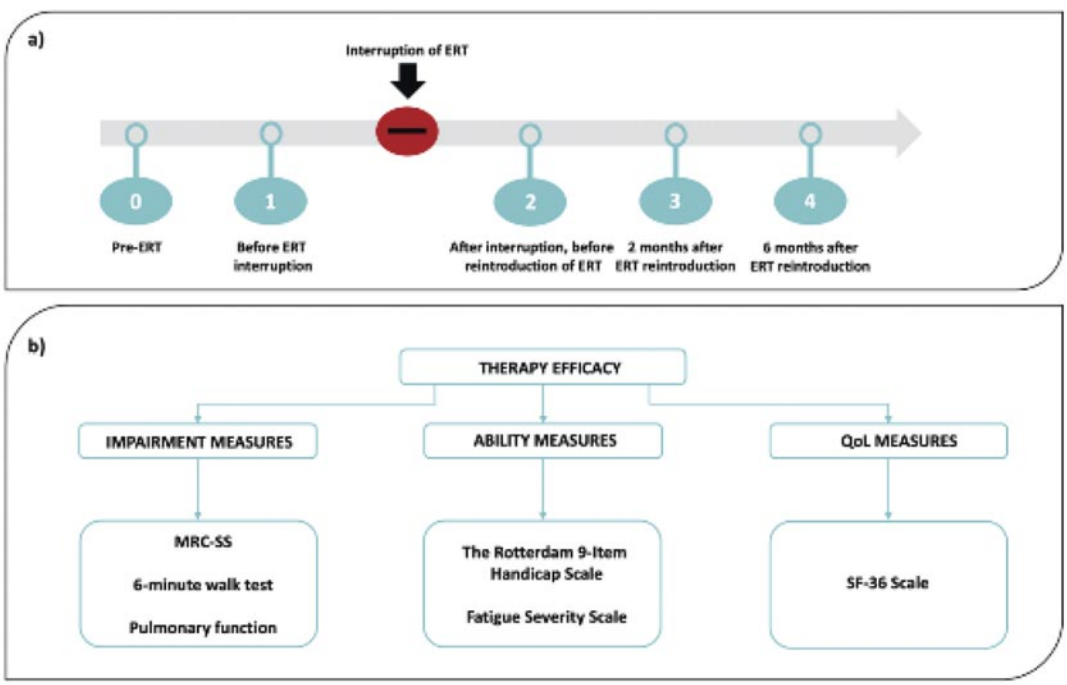

Figure 1. Overview of time course of the clinical assessments in PD patients and measures of therapy efficacy.

a) Time points: 0 – baseline (before starting ERT; pre-ERT), 1 – timepoint closest to ERT interruption, 2 – timepoint after interruption of ERT and just before reintroduction of therapy, 3 – two months after ERT reintroduction, 4 – six months after ERT reintroduction. b) Measures of therapy efficacy. ERT – enzyme replacement therapy; MRC-SS – Medical Research Council Sum-Score; QoL – quality of life.

Muscle strength was assessed through manual neurological testing according to the MRC scale, ranging from 0 = no movement to a maximum of 5 = normal strength, for six bilateral muscle groups: shoulder abductors, elbow flexors, wrist dorsiflexors, hip flexors, knee extensors, and foot dorsiflexors. The total MRC-SS ranges from 0 to 60 points [14]. 6MWT is a simple test used to assess functional capacity by measuring the distance a patient walks for 6 minutes [15]. It is a commonly used outcome measure in LOPD patients to monitor the effect of rhGAA. FVC is a spirometry measure indicating respiratory muscle function. It was performed both in sitting and in supine position. Results are expressed as percentages compared to gender-, age-, height- and weight-matched controls.

RHS is used to assess functionality in daily life activities [16]. It consists of nine questions related to indoor mobility, outdoor mobility, kitchen activities, outdoor tasks, indoor tasks, daily activities, leisure activities, travel, work, and learning. Each activity is scored on a scale from 1 to 4 (1 = unable to perform the task or activity to 4 = fully performing tasks or activities). The total RHS score ranges from 9 (unable to perform any tasks or activities) to 36 (able to perform all tasks and activities). FSS is utilized to assess the impact of fatigue on individuals [17]. It consists of a brief questionnaire with nine statements, each assessing the severity of fatigue symptoms. Respondents rate each statement on a scale of 1 to 7 based on their experience over the past week. A total score below 36 indicates a lower probability of experiencing significant fatigue, while a score of 36 or higher suggests the need for further evaluation by a physician.

SF-36 scale is a short health survey comprising eight domains: physical functioning (PF), role physical (RP), general health (GH), bodily pain (BP), vitality (VT), social functioning (SF), role emotional (RE), and mental health (MH) [18]. Additionally, physical and mental composite scores (PCS and MCS) and total SF-36 score can be calculated. Scores for all domains range from 0 to 100, with higher scores indicating better QoL [19].

Statistical Analysis

For demographic and clinical characteristics, descriptive statistics was used. The Kolmogorov-Smirnov test was used to test the normal distribution of values. The output variables of impairment measures, ability measures, as well as QoL measures, showed normal distribution. Therefore, the results were expressed as mean ± standard deviation (SD) for all parameters. Paired Student's t-test was used to evaluate differences in impairment measures, ability measures, and QoL measures within the group in different time points. The results are presented as mean ± 95% confidence interval. The criteria for the significance of statistical differences were $p < 0.05$, $p < 0.01$, or$p < 0.001$.

Results

The study included five LOPD patients undergoing ERT at our institution (3 males, mean age at onset 32.2±14.4 years, at diagnosis 42.2±14.5 years, at start of ERT 43.4±14.5 years) (Table 1).

Among the five genetically confirmed patients with LOPD, three were diagnosed through enzyme testing and targeted *GAA* gene sequencing. This subgroup included two female patients and one male patient, all of whom carried the c.-32-13T>G splice-site variant on one allele, while the second allele harbored either c.1856G>A or c.2269C>T variant. The remaining two patients were diagnosed through WES. In both cases, the c.-32-13T>G mutation was present on one allele, while the second allele carried either c.2063_2064insCGAGC or c.1655T>C.

The application of ERT until the time of discontinuation was 4.4±2.1 years and ERT was interrupted for 54.2±8.0 days due to drug unavailability. Changes in main impairment measures during time are pre-

Table 1. Demographic and clinical characteristics of LOPD patients (n=5)

Feature	LOPD
Gender (n, % females)	2 (40.0%)
Age at symptom onset (mean ± SD, years)	32.2±14.4
Diagnostic delay (mean ± SD, years)	10.0±6.5
Age at diagnosis (mean ± SD, years)	42.2±14.5
Serum CK level at diagnosis	592.6±335.8
Mutation on one allele (n, %) c.-32-13T>G	5 (100.0%)
Mutation on another allele (n, %) c.2269C>T c.1856G>A c.1655T>C c.2063_2064insCGAGC	2 (40.0%) 1 (20.0%) 1 (20.0%) 1 (20.0%)
Age at start of ERT (mean ± SD, years)	43.4±14.5
Disease duration at start of ERT (mean ± SD, years)	11.4±8.5
Time on ERT until discontinuation (mean ± SD, years)	4.4±2.1
Duration of ERT interruption (mean ± SD, days)	54.2±8.0

SD – standard devition, CK – creatine kinase, ERT - enzyme replacement therapy

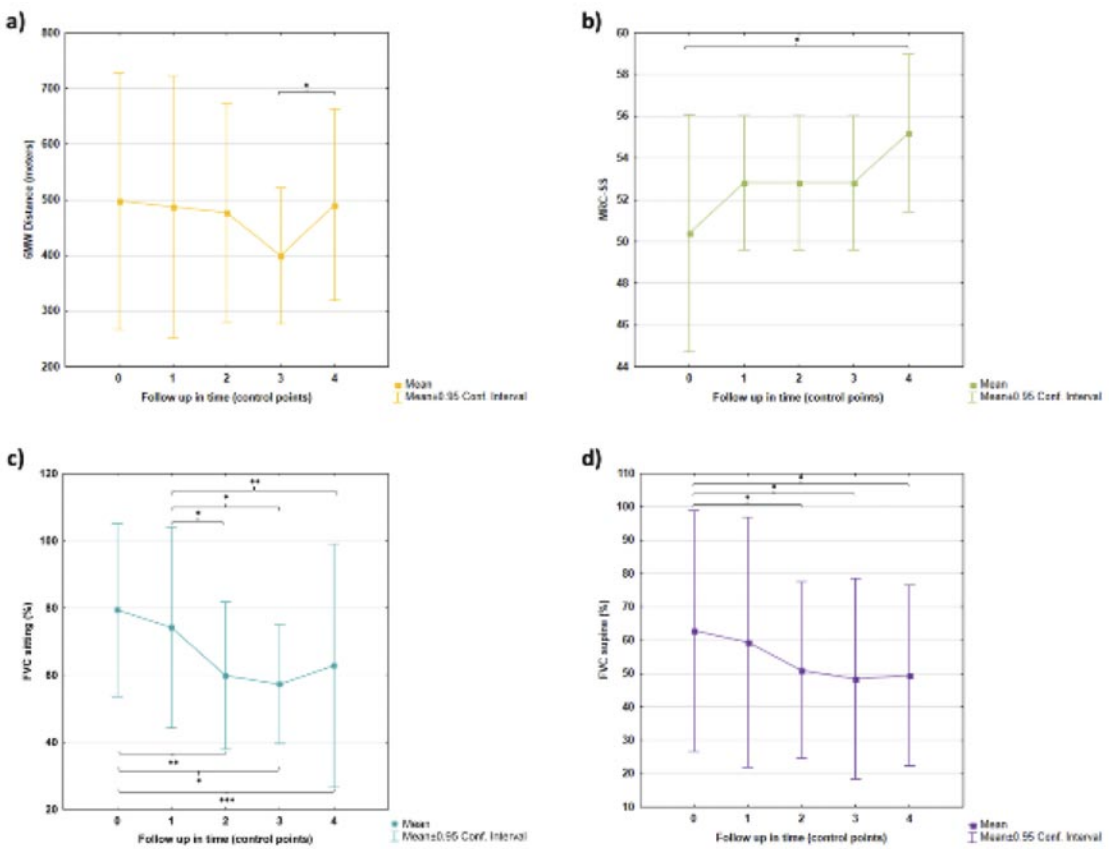

Figure 2. Impairment measures in LOPD patients on enzyme replacement therapy (ERT) over time.

a) 6MWT – 6-minute walking test; b) MRC-SS – Medical Research Council Sum-Score; c) FVC – Forced vital capacity in sitting position; d) FVC – Forced vital capacity in supine position;

Time points: 0 – baseline (before starting ERT; pre-ERT), 1 – timepoint closest to ERT interruption, 2 – timepoint after interruption of ERT and just before reintroduction of therapy, 3 – two months after ERT reintroduction, 4 – six months after ERT reintroduction.

sented in Figure 2. The decrease in 6MWT distance worsened after ERT discontinuation and during the first two months of therapy reintroduction, while it improved between month 2 and 6 after reintroduction ($p<0.05$). The mean decline in 6MWT was 34.2±20.6 m following the interruption of ERT. Furthermore, during the first two months of ERT reintroduction, there was a decrease of 77.4±72.3 meters in the 6MWT distance, which subsequently improved to an increase of 90.8±40.6 meters between two and six months after ERT reintroduction, indicating a statistically significant improvement. Slow improvement of MRC-SS was observed during ERT, followed by stabilization during ERT interruption with further improvement two to six months after ERT reintroduction ($p<0.05$). FVC decline was deepened during ERT interruption, but it slowed during the first two months of ERT reintroduction and started to improve between month 2 and 6 of ERT reintroduction although it has never reached the previous level ($p<0.01$).

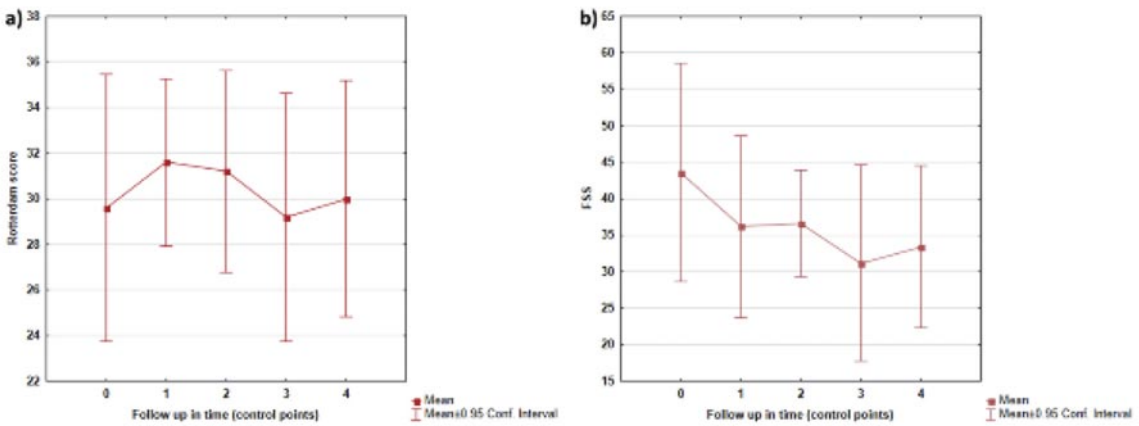

Figure 3. Ability measures in LOPD patients on enzyme replacement therapy (ERT) over time.

a) Rotterdam Handicap Scale; b) FSS - Fatigue Severity Scale. Time points: 0 – baseline (before starting ERT; pre-ERT), 1 – timepoint closest to ERT interruption, 2 – timepoint after interruption of ERT and just before reintroduction of therapy, 3 – two months after ERT reintroduction, 4 – six months after ERT reintroduction.

The main outcome ability measures are presented in Figure 3. RHS and FSS did not show statistically significant changes during the following time. Changes in QoL measures are revealed in Figure 4. RP decreased significantly after ERT interruption, and reintroduction of ERT improved the score after two months ($p<0.05$). Other SF-36 subscales did not change over time.

Since the time from ERT onset until ERT interruption differed in individual patients, we also calculated the monthly slope during ERT therapy (slope 1), during ERT interruption (slope 2), the first two months during ERT reintroduction (slope 3), and in a period of two to six months after ERT reintroduction (slope 4). The majority of scales showed increased variation during the first two months of ERT reintroduction, with a more uniform pattern in the period between month 2 and month 6 after ERT reintroduction resembling the period during the initial ERT therapy. The monthly slope in impairment measures is presented in Figure 5. Statistical difference was found in 6MWT where a monthly decrease of 38.7 m during the first two months of ERT reintroduction improved to a monthly increase of 15.2 m in a period two to six months after ERT reintroduction ($p<0.05$) (Figure 5.)

Changes in the monthly slope in ability measures did not show statistically significant difference. ERT reintroduction significantly enhanced RP score of SF-36 after a two-month period ($p<0.05$). Also, RP shows a decrease in a period of two to six months after ERT reintroduction ($p<0.05$). However, no significant changes were observed in other SF-36 subscales over the same timeframe.

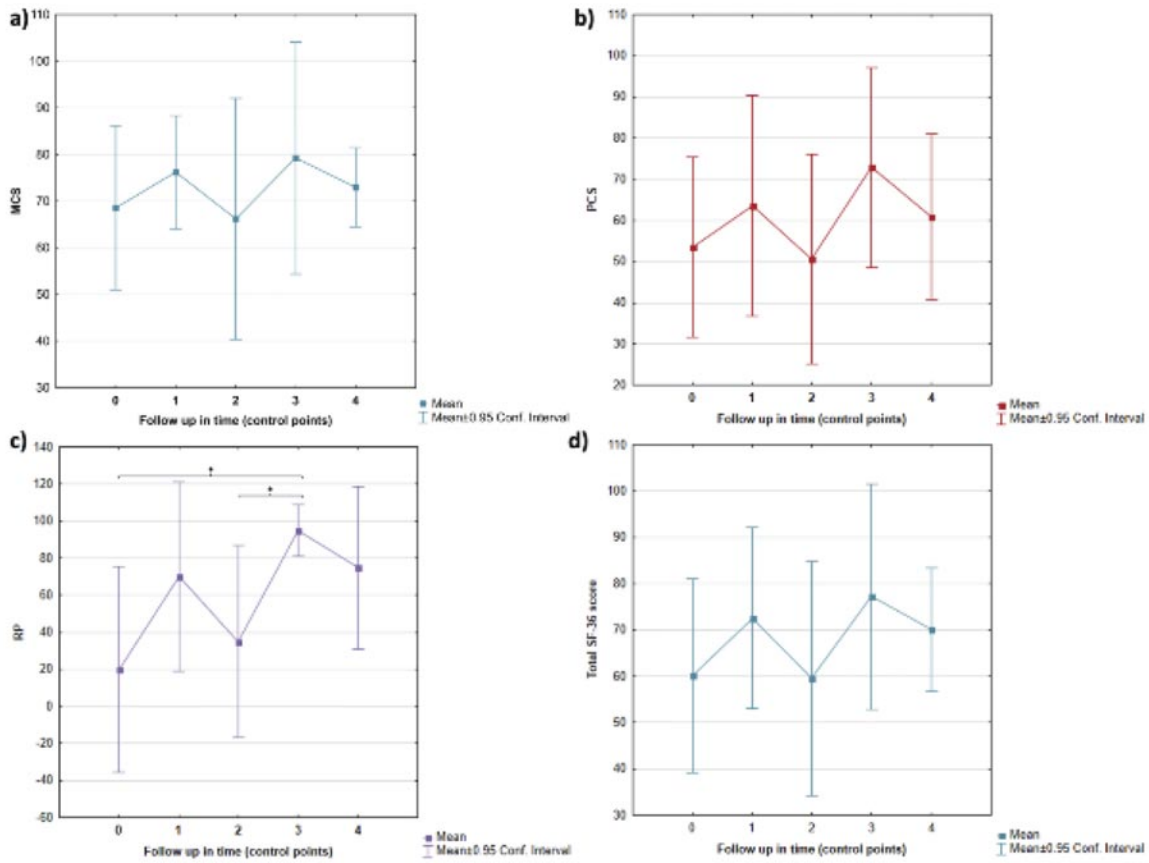

Figure 4. Quality of life measures in LOPD patients on enzyme replacement therapy (ERT) over time

a) MCS –Mental Composite score; b) PCS –Physical Composite score; c) RP – Role Physical score; d) Total SF-36 score. Time points: 0 – baseline (before starting ERT; pre-ERT), 1 – timepoint closest to ERT interruption, 2 – timepoint after interruption of ERT and just before reintroduction of therapy, 3 – two months after ERT reintroduction, 4 – six months after ERT reintroduction.

Discussion

Pompe disease is most readily identified when clinicians actively consider it in the differential diagnosis, as shown by our three patients diagnosed through enzyme assays and targeted gene sequencing. However, two patients in our cohort with nonspecific limb-girdle weakness required WES to obtain the diagnosis, illustrating how LOPD can present subtly and be overlooked. These findings highlight the importance of the next-generation sequencing (NGS) in diagnosing this treatable neurometabolic disorder [20]

Our study showed clear efficacy of ERT even after 4.5 years, since therapy discontinuation of only two months led to LOPD worsening. Theunissen and colleagues examined the effects of interrupted enzyme replacement therapy (ERT) in 325 LOPD patients from 25 countries due to the COVID-19 pandemic. Around 40% of the patients reported worsening after ERT interruption [19]. Wenninger and colleagues observed that after ERT interruption, 6 of 14 experienced reduced muscle endurance and three shortness of breath [11].

ERT reintroduction caused slow improvement in our patients after two months with further improvement until six months of follow-up. We found this consistently across different impairment and QoL measures. Many outcomes did not improve to the level before ERT interruption. Hundsberger and colleagues conducted a Swiss study involving seven LODP patients, demonstrating elevated CK levels and indicating a decrease in 6MWT and FVC during the interruption of ERT, which lasted between 3.1 and 59.3 months. Even 12 months after the reintroduction of ERT, patients did not return to baseline value [23]. The 6-MWT was monitored in six patients during the interruption of ERT. All of six patients exhibited a decrease in 6-MWT

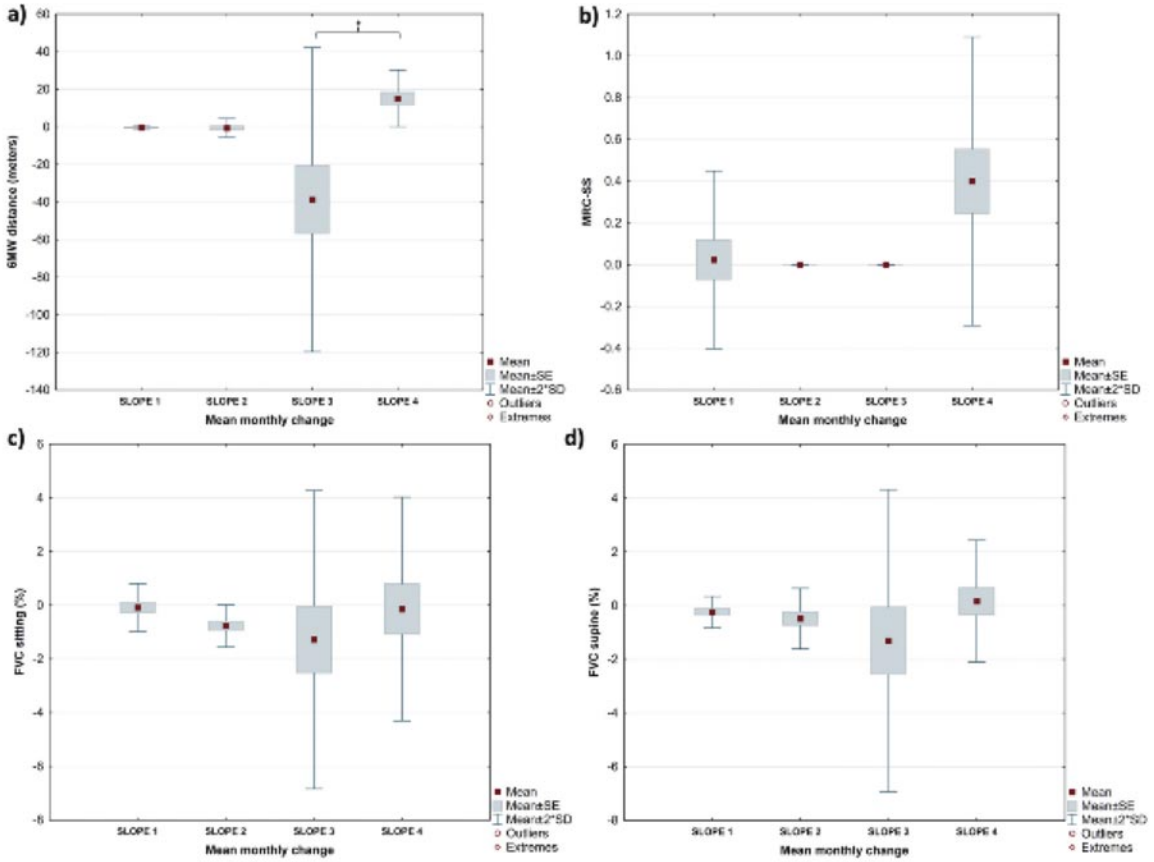

Figure 5. Monthly slope in main impairments measures in LOPD patients on enzyme repleacement therapy (ERT) over time

a) 6MWT – 6-minute walking test; b) MRC-SS - Medical Research Council Sum-Score; c) FVC - Forced vital capacity in sitting position; d) FVC - Forced vital capacity in supine position. The slope was interpreted as the change in 6MWT, MRC-SS, FVC in the sitting position, and FVC in the supine position, expressed as mean ± SE, as well as the deviation of 2 SD throughout the months between two control points. Mean monthly change: Slope 1 – change in variables between control points 0 and 1; Slope 2 – change in variables between control points 1 and 2; Slope 3 – change in variables between control points 2 and 3; Slope 4 – change in variables between control points 3 and 4. Time points: 0 – baseline (before starting ERT; pre-ERT), 1 – timepoint closest to ERT interruption, 2 – timepoint after interruption of ERT and just before reintroduction of therapy, 3 – two months after ERT reintroduction, 4 – six months after ERT reintroduction.

distance, ranging from 41 to 104 m which is in line with our resulst with mean decrease of 34 m. Upon reintroduction of ERT, four patients showed an improvement in 6-MWT distance (34-102 m), while two patients remained stable. In accordance with this, our patients showed 77-90 m monthly improvement after ERT reintroduction. However, none of the patients in the Swiss study achieved a complete recovery of walking capability [23].

Our observation was a significant declining trend in 6-MWT and FVC values due to the interruption of ERT. On the other hand, muscle strength showed no significant change, consistent with recent literature [24–27]. Our study, in line with other published studies, revealed a significant decline in pulmonary function parameters after ERT interruption, which did not return to baseline values even six months after the reintroduction of ERT. Wenninger and colleagues conducted a study on 13 patients with a mean ERT interruption of 49 days [11]. The findings suggested that interruption of ERT in LOPD should be avoided or minimized, as the cohort showed a significant decline in maximum inspiratory pressure (MIP), MRC score, and a trend towards clinical deterioration in FVC and 6-MWT [11]. Results from our study also can be compared with a French study examining the impact of ERT interruption in 31 patients. The duration of ERT therapy was 86±43 months, with interruptions lasting 2.2±0.8 months. On average, patients experienced a significant deterioration of 37 meters in the 6MWT and a loss of 210 ml in FVC after 2.2 months, without full recovery after three months of ERT reintroduction [24].

Ability measures did not show a statistically significant difference during the observation period in our cohort. On the other hand, Scheidegger and colleagues demonstrated a decrease in FSS values over time during ERT, reflecting a reduced subjective sense of fatigue [13]. This observation underscores the benefits of long-term ERT as evidenced by patient-reported outcome measures. Opposite to this findings, we observed no change in FSS which can be explained in two ways. First, our results showed trend of improvement of fatigue during ERT but statistical significance was not reached probably due to the small number of patients. Second, in the case of neuromuscular disorders, bot peripheral and central fatigue are present. Peripheral fatigue is a direct result of muscle fatigability, while central fatigue is an experienced lack of energy and feeling of tiredness not related to muscle weakness or pain [27]. Fatigue is common and non-specific symptom in all chronic disorders. Thus, one must conclude that fatigue can be improved by treatment of the underlying condition only to a minor degree [27–29]. Fatigue in many diseases may be a reactive disorder on a chronic and debilitating condition which requires prolonged and burdensome treatment. This fits with the data that fatigue is associated with knowledge of having had or having a disease of any type [27].

Our study assessed QoL, revealing a discernible decrease in RP domain following the interruption of ERT. This is expected in LOPD which is mostly muscular disease, not affecting the brain so patients are able to cope well with their disease. Additionally, our findings indicated an incomplete return of RP score to baseline values even six months after the reintroduction of ERT. The decline in RP was further illustrated by tracking the mean monthly change, calculated by determining the slope. In a 2016 study, Güngör and colleagues examined the efficacy of ERT in 174 patients over a 10-year follow-up [16]. Before starting ERT, PCS score decreased significantly by 0.73 points per year [17]. Following ERT initiation, there was a significant improvement in PCS by 1.49 points per year in the first two years, with stabilization thereafter. MCS remained stable throughout the follow-up. The most significant improvement in this study was observed in RP, which is in line with our results were RP showed difference during time.

Conclusion

Our study demonstrated the evident efficacy of ERT persisting even after a 4.5-year, since discontinuation of therapy for merely two months led to a deterioration in LOPD. The reintroduction of ERT initiated a gradual improvement observed as early as two months post-reinstatement, with further enhancements noted up to six months of follow-up. Some measures failed to return to baseline values even six months after ERT reintroduction. These findings underscore the pivotal role of ERT in mitigating the progression of LOPD. These insights underscore the crucial importance of maintaining continuous ERT therapy to prevent the rapid progression of the disease that would occur without treatment.

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NAPREDNE METODE MOLEKULARNE BIOLOGIJE

ADVANCED METHODS IN MOLECULAR BIOLOGY

Recent advances in isothermal strategies for biomedical molecular diagnostics

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Abstract

The rapid evolution of molecular diagnostics is driving a shift toward decentralized, fast, and highly sensitive methods for nucleic acid detection. Isothermal amplification techniques, which eliminate the need for thermal cycling, have emerged as strong, clinically viable alternatives to traditional PCR. These approaches enable sensitive detection of pathogens causing infectious diseases as well as disease biomarkers and genetic alterations, including single-nucleotide variants and short non-coding RNAs such as microRNAs, providing insights into disease at the molecular level. This places isothermal molecular technologies at the forefront of early detection in biomedicine. Techniques such as Loop-mediated Isothermal Amplification (LAMP), Rolling Circle Amplification (RCA), and Exponential Amplification Reaction (EXPAR) offer key advantages in target versatility, analytical sensitivity, and integration into portable, point-of-care diagnostic platforms. Moreover, their compatibility with CRISPR/Cas systems further enhances specificity and allows for high-throughput multiplexing. We provide a comprehensive review of isothermal amplification methodologies, focusing on their use in diagnostic purposes in biomedicine, assessing their integration with emerging molecular tools, and evaluating their potential applications in personalized therapies, point-of-care diagnostics, and longitudinal disease monitoring.

Keywords: Isothermal amplification, LAMP, RCA, EXPAR, CRISPR-Cas, point-of-care diagnostics, personalized therapies, genetic testing

Primena najnovijih dostignuća u izotermalnim tehnologijama u biomedicinskoj molekularnoj dijagnostici

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Apstrakt

Brzi razvoj molekularne dijagnostike teži ka decentralizovanim, brzim i visoko osetljivim metodama za detekciju nukleinskih kiselina. Tehnike izotermalne amplifikacije, koje eliminišu potrebu za termičkim ciklusima, pojavile su se kao moćne i klinički primenljive alternative tradicionalnoj metodi PCR. Ovi pristupi omogućavaju precizno otkrivanje patogena koji izazivaju zarazne bolesti, kao i biomarkera bolesti i genetičkih promena, uključujući varijante jednog nukleotida i kratke nekodirajuće RNK kao što su mikroRNK, pružajući uvid u bolesti na molekularnom nivou. Ovo stavlja izotermalne molekularne tehnologije u prvi plan za rano otkrivanje bolesti u biomedicini. Tehnike kao što su *Loop-mediated Isothermal Amplification (LAMP), Rolling Circle Amplification (RCA), i Exponential Amplification Reaction (EXPAR)*, nude značajne prednosti u različitosti ciljeva koji se mogu detektovati, analitičkoj osetljivosti i njihovoj integraciji u prenosive „point-of-care" dijagnostičke platforme. Štaviše, njihova kompatibilnost sa CRISPR/Cas sistemima dodatno poboljšava specifičnost i omogućava sveobuhvatnu dijagnostiku brojnih genetičkih markera. Ovaj rad predstavlja sveobuhvatan pregled metodologija izotermalne amplifikacije, fokusirajući se na njihovu upotrebu u dijagnostičke svrhe u biomedicini, procenjujući njihovu integraciju sa novim molekularnim alatima i procenjujući njihove potencijalne primene u personalizovanim terapijama, „point-of-care" dijagnostici i longitudinalnom praćenju bolesti.

Ključne reči: izotermalna amplifikacija, LAMP, RCA, EXPAR, CRISPR-Cas, „point-of-care" dijagnostika, personalizovane terapije, genetičko testiranje

1. Introduction

Technological advancements and the increasing demand for rapid, decentralized testing solutions are driving a constant and significant growth in the global point-of-care (POC) diagnostics market. In 2025, the POC molecular diagnostics market was valued at approximately USD 64.08 billion and is expected to reach USD 82.78 billion by 2034 (1). The primary reason for this rapid growth is the increasing prevalence of chronic diseases and the necessity for their timely diagnosis (2). For example, statistical predictions suggest that the number of cancer cases will reach 35 million globally by 2050 (3,4). Moreover, the push towards personalized medicine is setting new requirements for diagnostic solutions, emphasizing that they need to not only be accurate but also accessible and quick. The traditional approach to disease diagnostics has involved sequencing or performing a series of PCR-based laboratory tests, which are expensive, labor-intensive, infrastructure-heavy, and time-consuming (5,6). As advances in molecular technologies unfold, their applicability in addressing the need for more efficient and reliable testing methods has never been more pronounced (7,8). For instance, during the COVID-19 pandemic, home-based and easy-to-use diagnostic tests became a necessity, prompting the development of several POC-based strategies (9,10). POC testing enables the determination of visual results, rapid sample-to-result times, and testing at or near the site of patient care, which is especially crucial during pandemics or for vulnerable populations in rural areas (2,9,11).

Over the past decade, isothermal amplification (IA) techniques have emerged as powerful tools for nucleic acid detection, offering several advantages over conventional thermal cycling methods, including simplified assay design, reduced processing times, and enhanced sensitivity. For example, Loop-Mediated Isothermal Amplification (LAMP) is an isothermal nucleic acid amplification methodology that has been discussed as the next gold-standard technique for DNA amplification, potentially replacing classical PCR-based nucleic acid amplification (12). When the need for thermal cycling and sophisticated equipment is eliminated, on-site testing, minimal sample processing, and real-time decision-making are enabled. This change is especially significant in resource-limited settings, particularly within the context of the One Health approach that recognizes the interconnectedness of people, animals, plants, and their shared environment to achieve optimal health outcomes for all - the full spectrum of zoonotic disease control (13,14). Moreover, IA techniques meet the ASSURED criteria introduced by the World Health Organization (2,6).

There have been several reports in recent years on the development of IA-based tests for detecting bacterial and viral pathogens, cancer biomarkers, and even genetic mutations (3,6,15). Parallel to the development of IA techniques, the advancement of microfluidic devices and lab-on-a-chip technologies is facilitating miniaturization and automation, enabling high-volume analyses even in resource-limited settings (2).

IA-based tests and related ongoing technological advancements in molecular diagnostics have significantly improved diagnostic capabilities, both for the detection of pathogens that cause infectious diseases and for the detection of disease biomarkers, allowing early diagnosis, as well as monitoring of disease progression.

Infectious diseases continue to be a leading cause of morbidity and mortality worldwide, presenting major challenges to both public health and socioeconomic stability (16). Despite remarkable progress in medical science, these diseases are still responsible for millions of deaths yearly, with the heaviest burden falling on low- and middle-income countries. The emergence and re-emergence of pathogens such as SARS-CoV-2, multidrug-resistant bacteria, and arboviruses highlight the dynamic and unpredictable nature of these threats, emphasizing the necessity for robust surveillance and rapid-response systems (16–18). In

addition, globalization, climate change, and intensifying human–animal interactions further accelerate the spread of novel infectious agents, complicating both prevention and control strategies (19). In this context, rapid and reliable detection of pathogens related to infectious diseases has become crucial for accurate diagnosis, timely treatment initiation, and effective control of disease transmission, especially in resource-limited settings (20,21).

The detection of molecular markers of diseases, such as microRNAs (miRNAs), has garnered significant attention in the realm of disease diagnosis and prognosis. MicroRNAs are small, non-coding RNA molecules that regulate gene expression and are involved in a broad array of biological processes. Due to their stability in bodily fluids and their disease-specific expression profiles, miRNAs are emerging as promising biomarkers for conditions like cancer, cardiovascular diseases, and neurodegenerative disorders (22,23). Compared to traditional protein-based biomarkers, miRNAs offer several advantages, including the potential for earlier detection and a more accurate reflection of disease dynamics (24). However, detecting circulating miRNAs remains a challenge due to their small size, sequence similarity, and low abundance in biological samples. Conventional techniques, such as reverse transcription quantitative PCR (RT-qPCR), microarrays, and next-generation sequencing (NGS), have proven valuable in studying miRNAs. However, they often come with high costs, lengthy turnaround times (TAT), and the need for specialized laboratory infrastructure (25).

To overcome these limitations, novel IA methods have been developed as rapid, high-sensitivity alternatives that offer compatibility with POC platforms, facilitating the detection of miRNAs in clinical settings (26,27). The integration of IA technologies with miRNA detection holds significant potential for early disease diagnosis and prognostic monitoring, making these tools highly relevant in the evolving landscape of personalized medicine (24).

In parallel with advancements in miRNA detection, another critical area in molecular diagnostics is the detection of genetic variations. Genetic variations, including single-nucleotide polymorphisms (SNPs), small insertions or deletions (*indel*s), as well as larger structural changes like copy number variations (CNVs) and chromosomal rearrangements, play a key role in both disease development and human diversity. As our ability to detect and understand these genetic changes improves, it opens up new possibilities for diagnosing and treating a wide range of conditions, particularly in fields like oncology, neurology, and prenatal medicine (28).

This shift toward a deeper understanding of genetic underpinnings is reflected in the rise of IA-based genetic testing, especially in newborn screening programs, where early identification of inherited conditions can significantly impact long-term health outcomes (29). In oncology, detecting specific mutations enables the use of targeted therapies that improve treatment efficacy and reduce side effects compared to traditional options like chemotherapy (30,31). However, despite these advances, many patients still do not have access to or do not undergo the necessary molecular testing due to the complexity, processing (long TATs) and high cost of current diagnostic methods, such as next-generation sequencing (NGS) and multiplex PCR. This is where isothermal nucleic acid amplification methods present a clear advantage. By eliminating the need for nucleic acid isolation, thermal cycling and simplifying the equipment requirements, these techniques make genetic testing faster, more affordable, and suitable for decentralized settings, such as POC testing. With the ability to easily integrate into routine newborn screening or clinical genetic testing, IA methods are poised to revolutionize the detection of genetic conditions, including congenital infections and monogenic disorders (28).

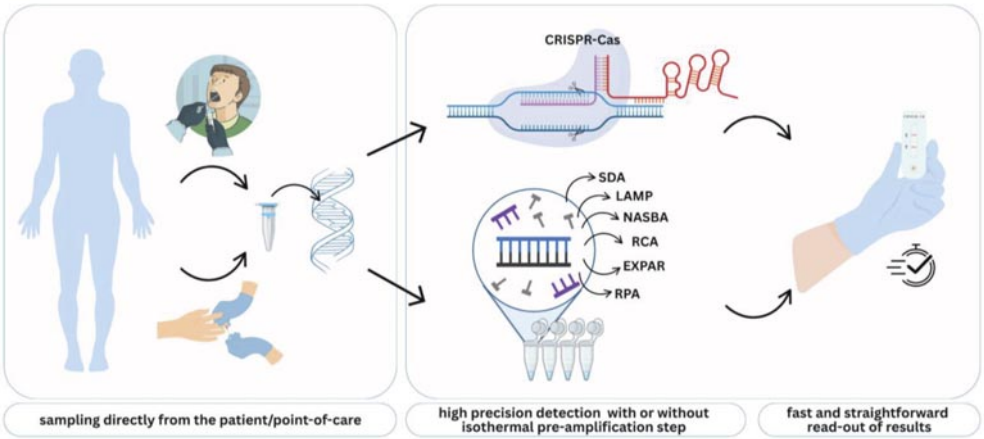

Figure 1: Workflow schematic of IA–based rapid and personalized genetic testing

2. Principles of Isothermal Amplification

2.1. Concept and mechanisms of isothermal amplification

Isothermal nucleic acid amplification methods encompass a variety of techniques that enable DNA/RNA amplification at a constant temperature, eliminating the need for thermal cycling as required in conventional PCR (32). The underlying technology behind these IA techniques, which enables the isothermal conditions, is the enzyme activity of strand-displacing DNA polymerases or unwinding enzymes (e.g., helicases), harnessing their ability to separate DNA strands (Table 1). This further facilitates primer annealing without high-temperature denaturation. By avoiding repeated heating and cooling steps, isothermal amplification can be performed with simpler equipment and is well-suited for POC diagnostics and resource-limited settings (12). While some isothermal nucleic acid amplification methods are still in a proof-of-concept phase, many have already been commercialized, such as LAMP, strand-displacement amplification (SDA), rolling-circle amplification (RCA), recombinase polymerase amplification (RPA), nucleic acid sequence-based amplification (NASBA), and helicase-dependent amplification (HDA) (6,12).

2.2. Comparison with conventional methods (PCR): Speed, simplicity, and energy efficiency

The main differences between isothermal nucleic acid amplification methodologies and traditional PCR were also the main reasons for the development of a "new generation" of amplification techniques. Isothermal amplification techniques eliminate the need for precise thermal cycling to achieve denaturation, annealing, and extension steps via a thermocycler (6,15). This drastically reduces instrument complexity and energy consumption. The streamlined thermal profile often translates into shorter reaction times. For example, LAMP can detect low-abundance targets in under 30 minutes, compared to several hours for conventional PCR. Moreover, the minimal instrumentation and more straightforward setup make isothermal methods particularly suitable for POC and field diagnostics (6).

2.3. General workflow: Primer design, enzymes, and detection strategies

The general workflow of isothermal amplification begins with careful primer design, which is often a complex or limiting step due to requirements for multiple primers and specific secondary structure motifs

(e.g., in LAMP, 4–6 primers targeting 6–8 regions) (12). This step directly increases the likelihood of primer-dimer formation, nonspecific amplification, and reaction accuracy and efficiency. For example, it has been shown that the efficiency of LAMP can be increased if using loop primers, and in some cases, it is simply not possible to design them (33). Moreover, there is a limited database of primer sets for IA methods, necessitating the design of primers in many cases (34,35).

Enzyme selection is crucial and varies depending on the method. Each IA method utilizes one or a set of enzymes, including strand-displacing DNA polymerases (e.g., Bst DNA polymerase in LAMP, Phi29 DNA polymerase in RCA), recombinases, helicases (in HDA), or combinations thereof, depending on the specific mechanism. NASBA, for example, incorporates reverse transcriptase and RNase H for RNA targets, while RPA utilizes SSB proteins to ensure ssDNA stabilization (12).

Once the target is amplified and accumulated in the reaction vessel, it can be detected using various detection strategies. The array of detection strategies not only increases the adaptability of IA techniques but also opens up new possibilities. The visualization strategies include turbidity measurements from magnesium pyrophosphate precipitates, colorimetric indicators such as pH-sensitive dyes like phenol red and metal-ion indicators like hydroxynaphthol blue, fluorescent intercalating dyes, immunoassays, and even CRISPR-Cas-based readouts for enhanced specificity (36,37).

3. Isothermal Amplification Techniques

3.1. Loop-mediated Isothermal Amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is currently the most thoroughly researched isothermal amplification technique, widely recognized for its rapidity and ease of use in nucleic acid amplification. First developed in 2000 by a group of Japanese scientists (38), LAMP has since found a broad range of applications, including POC diagnostics, healthcare, environmental monitoring, and food safety testing (39–46).

LAMP reaction is performed at a constant temperature (usually between 60–65 °C), eliminating the need for thermal cycling, which is facilitated by the use of Bst DNA polymerase, an enzyme exhibiting strand-displacement activity and derived from *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*). Bst DNA polymerase has both strong strand displacement and 5' - 3' polymerase activity, which allows it to separate the DNA strands during amplification without the need for a separate denaturation step (47,48). This property allows the polymerase to synthesize, displace, and release single-stranded DNA during the amplification process, enabling the LAMP reaction to proceed under isothermal conditions. This makes LAMP fundamentally different from PCR, which requires high temperatures to denature the DNA and initiate amplification (38,47,49,50).

The LAMP reaction utilizes a set of four to six primers that bind to distinct regions of the target DNA sequence, ensuring high specificity. These primers consist of two types: outer primers (F3 and B3) that bind to the outer regions of the target DNA, and inner primers (FIP and BIP) that bind to the inner regions. The inner primers consist of both a forward and a reverse primer, forming a complex that facilitates the formation of a dumbbell or stem-loop structure. This structure then serves as a template for subsequent rounds of amplification (38,51). Additionally, the inclusion of loop primers (LoopF and LoopB), which bind to the loop regions of the stem-loop structure, further accelerates the reaction by increasing the number of amplification sites. This, in turn, leads to a rapid and efficient amplification process, producing large quantities of DNA within a short period. The amplification often results in the formation of characteristic cauliflower-like structures, which are visible as large concatemeric DNA products (33,52–54).

Thanks to its high specificity and rapid amplification capabilities, LAMP can generate up to 10^9 copies of DNA in less than an hour, making it especially valuable for time-sensitive applications like POC diagnostics and field testing. Its isothermal nature enables efficient DNA amplification in a short timeframe, which is particularly advantageous in resource-limited environments or regions with minimal laboratory infrastructure (55,56). In addition, LAMP produces detectable by-products, including magnesium pyrophosphate and hydrogen ions, which can be identified using various monitoring methods such as turbidimetry, fluorometry, and colorimetry. Over the past decade, advanced detection techniques—including optical, electrochemical, and magnetoresistive sensors—have further enhanced the versatility of LAMP. These features contribute to LAMP's adaptability for both laboratory and field diagnostics, particularly in remote or low-resource settings where traditional lab equipment may not be available (54,55,57,58).

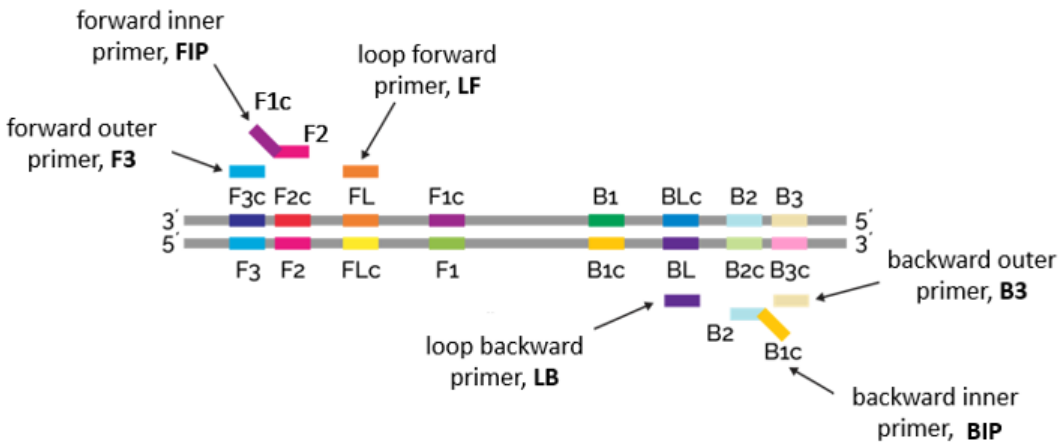

Figure 2. Mechanism of LAMP reaction: schematic representation of the generation of primary LAMP products (dumbbell-like structure)(51).

3.2. Rolling Circle Amplification (RCA)

Rolling Circle Amplification (RCA) is a powerful isothermal nucleic acid amplification method that leverages circular DNA or RNA templates to generate long single-stranded products containing thousands of tandem repeats. RCA operates at a constant temperature and relies on polymerases with strong strand-displacement activity, such as phi29 DNA polymerase or Bst-derived variants (59). Processivity and adaptability of RCA make it a versatile tool for bioanalysis in solution, on surfaces, and even in living cells (60–62).

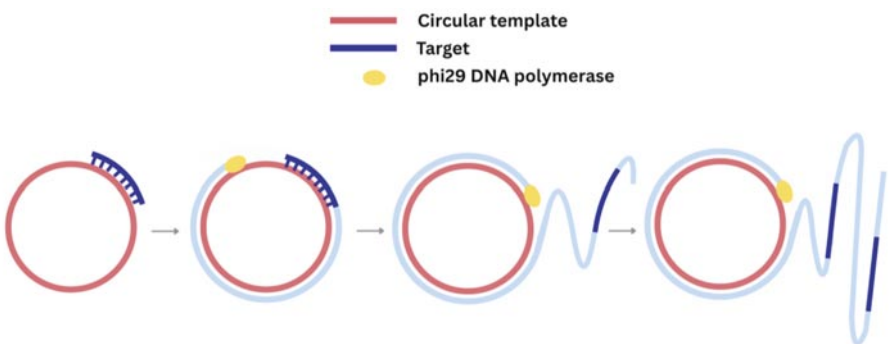

Figure 3. The mechanism of RCA. Reprinted and modified from (63).

A hallmark of RCA-based assays is the use of padlock probes, linear oligonucleotides that are ligated into circular templates only upon achieving target recognition (64). Conventional padlock probes consist of two target recognition arms flanking a functional sequence. When the probe is ligated in the presence of a complementary nucleic acid target, the resulting circular template supports efficient RCA, enabling target-specific signal amplification (65). Importantly, rational design of the padlock's functional region allows for diverse signal outputs, such as fluorescence via intercalating dyes, luminescence through DNAzyme or G-quadruplex formation, colorimetric changes with chromogenic substrates, or electrochemical signals in biosensor formats (66).

The sensitivity and specificity of RCA are among its most significant advantages. Linear RCA typically offers femtomolar detection limits, while exponential formats such as hyperbranched RCA (HRCA) or circle-to-circle amplification (C2CA) further improve sensitivity by generating branched amplicons or new cycles of circular templates (59). These strategies are beneficial for targets present at low abundance, such as circulating microRNAs (miRNAs), which act as critical disease biomarkers. However, conventional padlock-based RCA can be limited by ligase fidelity and difficulty distinguishing single-base mismatches, leading to challenges in detecting single-nucleotide variants (SNVs) or discriminating homologous RNA sequences (67).

To address these issues, newer approaches integrate toehold-mediated strand displacement, high-fidelity ligases (e.g., SplintR), or probe innovations such as "iLock" padlocks, which allow highly specific detection of RNA isoforms (66,68,69). The adaptation of RCA to RNA templates, often referred to as rolling circle transcription (RCT), expands its capacity for direct RNA detection, enabling *in vitro* assays for short non-coding RNAs and even *in vivo* detection of microRNAs using nanoparticle delivery systems (70).

The integration of RCA with nanomaterials, CRISPR/Cas systems, and biosensing platforms has further pushed its diagnostic potential. For example, RCA-triggered CRISPR/Cas cleavage cascades allow signal amplification with high specificity, while RCA products immobilized on surfaces or nanoparticles facilitate electrochemical and colorimetric readouts suitable for point-of-care devices (61,71,72). These advances collectively demonstrate that RCA is not only a robust amplification method but also a modular platform for precision diagnostics.

By offering high sensitivity, flexible probe design, and compatibility with diverse detection modes, RCA is uniquely positioned for personalized genetic testing, particularly in the detection of SNVs (73), circulating tumor DNA, and small RNAs, such as miRNAs and circular RNAs. As probe chemistries and amplification strategies continue to evolve, RCA will likely remain central to the next generation of isothermal molecular diagnostics.

3.3. Exponential Amplification Reaction (EXPAR)

The Exponential Amplification Reaction (EXPAR) is one of the isothermal nucleic acid amplification techniques still in the proof-of-concept phase, with a strong potential to reach the commercialization phase (12). The method is based on amplifying short oligonucleotide "trigger" sequences exponentially, thereby achieving remarkable speed, with 10^6 to 10^9-fold amplification within less than 30 minutes under constant temperature conditions (74–76). The reaction employs a single-stranded trigger probe (X) and a single-stranded functional template (X–X), where X and X have complementary sequences (77). After the primer (which contains a nicking enzyme recognition site) binds to the target sequence, Bst DNA polymerase extends the strand (12). This is followed by the activity of a nicking endonuclease, which cleaves the upper

strand, and the strand displacement activity of the Bst polymerase, leading to the release of the newly generated "trigger" sequences and driving exponential signal growth (76). The rapid kinetics of the EXPAR reaction are derived from a cyclical mechanism, resulting in short reaction times of 20-40 minutes. Consequently, EXPAR is particularly well-suited for detecting short nucleic acid targets, such as microRNAs, which are typically too small for efficient PCR amplification (78). Another advantage of the EXPAR method is that the reaction can be carried out using a ready-to-use mix available for the LAMP reaction, as demonstrated by Qian et al. in 2022 (79). Some of the main challenges of EXPAR are non-specific or background amplification and specificity issues, often arising from self-priming events with templates, especially under high template concentrations. Optimization strategies include the use of additives in the reaction mix to reduce template–polymerase affinity (77).

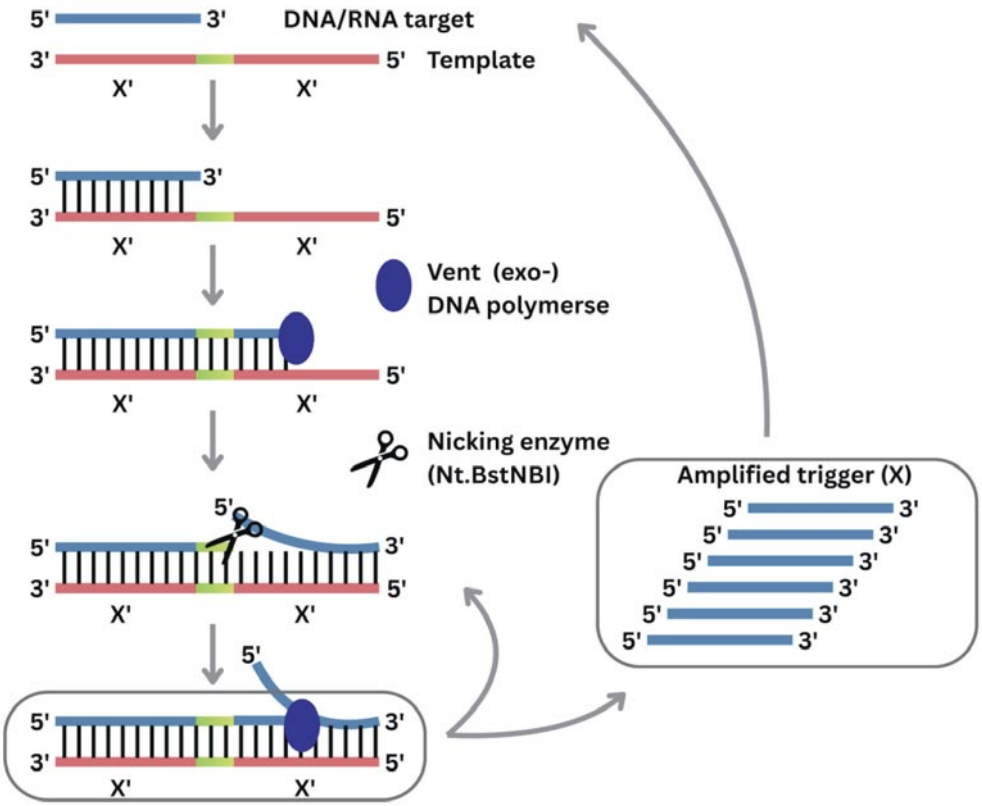

Figure 4. The mechanism of EXPAR. Reprinted and modified from (76), under the Creative Commons CC BY license.

3.4. Brief overview of other IA methods: NASBA, RPA, SDA

Nucleic acid sequence-based amplification (NASBA) is an isothermal, transcription-driven, RNA-specific method inspired by the replication process of retroviruses (12). The reaction is designed to detect gRNA targets, and uses a set of 2 primers and three enzymes: reverse transcriptase (copies RNA into complementary DNA (cDNA)), RNase H (removes the original RNA strand from the RNA–DNA hybrid), and T7 RNA polymerase (produces many RNA copies from the cDNA template) (80). The temperature of 41°C meets the requirements for the activity of the three enzymes (12). Reaction results can be visualized by employing molecular beacons to produce a fluorescent signal upon binding to RNA amplicons (80).

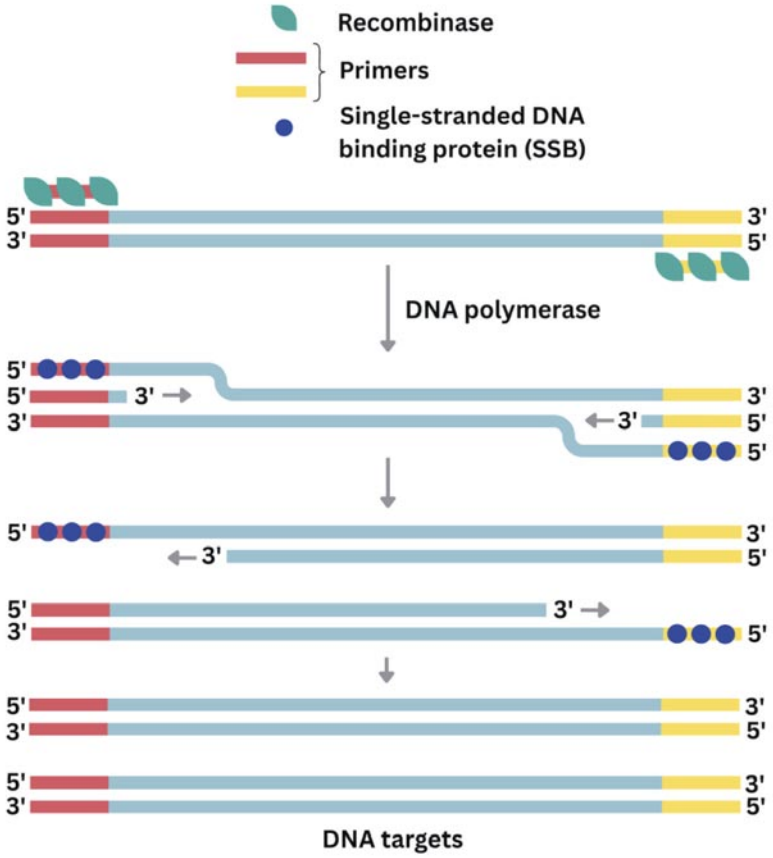

Figure 5. The mechanism of RPA. Scheme adapted from publicly available brochure created by New England Biolabs, Inc. (82).

Recombinase polymerase amplification (RPA) is an isothermal method for DNA amplification. The reaction utilizes a set of forward and reverse primers, along with a set of proteins, to achieve high sensitivity and specificity at 37-42°C in under 30 minutes (35). First, recombinase proteins UvsX bind to primers to form a recombinase-primer complex (stabilized by a crowding agent) and invades double-stranded DNA at the matching sequence (81). Single-stranded proteins bind and stabilize the forming single strands. Next, as the recombinase is disassembled and detached, a DNA polymerase (*Bacillus subtilis* Pol I) with a strand displacement activity binds to the 3' ends of the primers and elongates the complementary chain (81).

Strand displacement amplification (SDA) is another isothermal nucleic acid amplification technique that utilizes a DNA polymerase with strand-displacement activity (exonuclease-deficient Klenow (exo-klenow) polymerase) coupled with a nicking endonuclease. The mechanism of SDA consists of two stages: 1) target generation and 2) exponential target amplification (83). Moreover, the two pairs of primers (bumper primers and amplification primers) used in the reaction are designed in a specific way: the bumper primer binds upstream of the amplification primer, thereby supporting the subsequent displacement of the new amplification product (12,84). After DNA polymerase extends from the amplification primer (which contains a nicking recognition site), the new strand is cleaved and separated, followed by elongation from the bumper primer. The displaced synthesized strand serves as the template for the second phase of the reaction (12,84). This method has been utilized for clinical diagnosis of *Chlamydia trachomatis*, *Neisseria gonorrhoeae,* and herpes simplex virus (12).

Table 1. Overview of isothermal nucleic acid amplification methods, their characteristics, development stage, and representative commercial kits.

Method	Target (DNA/RNA)	Key Reference	Enzymes Involved	Development phase	Available products/kits	Clinical samples / Typical use cases
SDA	DNA/RNA	(85)	Strand-displacing DNA polymerase, restriction endonuclease	Commercialization	Ready-to-use kit by Becton Dickinson (BD ProbeTec™); Reaction components by NewEngland Biolabs	Cervical swabs, urine / *Chlamydia trachomatis*, *Neisseria gonorrhoeae* (86,87)
NASBA	RNA	(88)	Reverse transcriptase, RNase H, T7 RNA Polymerase	Commercialization	Ready-to-use kit by amsbio (*NASBA Lyophilized Kit*); bioMérieux (NucliSENS EASYQ®); Reaction components by NewEngland Biolabs	Plasma, serum, whole blood, Cerebrospinal fluid (CSF), stool / HIV, HCV, enteroviruses (89,90)
RPA	DNA (RNA with RT-RPA)	(91)	Recombinase, SSB, Bsu DNA polymerase (strand-displacing)	Commercialization	Ready-to-use kit by TwistDx™ (*TwistAmp® Basic kit*); ThermoFisher Scientific (*Lyo-ready RPA Kit*); Reaction components by NewEngland Biolabs	Nasal swabs, saliva, whole blood, serum, plasma, urine / SARS-CoV-2, Zika, *Plasmodium* spp. (92–94)
LAMP	DNA (RNA with RT-LAMP)	(38)	Bst DNA Polymerase (strand-displacing)	Commercialization	Ready-to-use kit by NewEngland Biolabs (*WarmStart® LAMP Kit (DNA & RNA)*; Eiken Chemical (Loopamp®), Abbott (Panbio)	Sputum, nasal/NP swabs, saliva, stool, urine / *Mycobacterium tuberculosis*, SARS-CoV-2, *Schistosoma*, *Strongyloides* (95–100)
HDA	DNA	(101)	DNA helicase, SSB, DNA polymerase	Commercialization	Ready to use kit by BioHelix Quidel (IsoAmp®); NewEngland Biolabs (**Catalog # H0110 was discontinued on December 15, 2024*; Reaction components by NewEngland Biolabs)	Vaginal/cervical swabs / bacterial vaginosis, *Trichomonas vaginalis* (102)
RCA	DNA/RNA	(103)	Phi29 DNA Polymerase (high processivity, strand displacement)	Commercialization	Ready-to-use kit by New England Biolabs (*phi29-XT RCA Kit*)	Tissue, plasma / experimental pathogen detection (e.g., *Borrelia* plasmids), Biomarker diagnostics (61,104–106)

EXPAR	Short DNA (oligonucleo tides)	(107)	DNA polymerase with strand displacement, nicking endonuclease	Proof of concept	/	Serum, plasma / miRNAs (prostate cancer, lung cancer, other oncology biomarkers) (108–110)
SMART	RNA	(111)	None (signal amplification via RNA hybridization)	Proof of concept	/	serum / HIV-1 RNA, HCV RNA (proof-of-concept, early diagnostic assays) (111–113)
HCR	DNA	(114)	None (enzyme-free amplification via DNA hairpin hybridization)	Proof of concept	/	Tissue sections (FFPE, frozen), whole-mount samples, biopsy specimens / multiplexed RNA imaging, in-situ hybridization, spatial transcriptomics (research use only) (115–118)

4. Analytical Performance and Technical Comparisons

4.1. Analytical sensitivity and specificity

In terms of analytical sensitivity, EXPAR has been generally shown in recent reviews to be the most analytically sensitive of the major isothermal amplification strategies. Its mechanism, based on rapid extension of short DNA triggers, enables exponential growth within minutes and supports detection limits in the zeptomolar to attomolar range (10^{-21}–10^{-18} M), sometimes corresponding to only a few template molecules per assay. This ultrafast amplification capacity makes EXPAR particularly suited for applications requiring rapid and highly sensitive detection of low-abundance nucleic acids. However, the same properties that enable extreme sensitivity also predispose EXPAR to nonspecific background reactions, since short oligonucleotide triggers can initiate unintended amplification pathways. Accordingly, several reviews emphasize the need for careful sequence design or integration with orthogonal specificity enhancers, such as CRISPR-based recognition, to fully harness EXPAR's analytical power while mitigating false signals (6,12,15,119).

Loop-mediated isothermal amplification (LAMP) is widely regarded as a highly sensitive method, with detection limits frequently reported in the attomolar to femtomolar range (10^{-18}–10^{-15} M). Its use of four to six primers targeting multiple regions of a sequence, coupled with strand-displacing polymerases, enables the rapid accumulation of amplicons and the generation of strong signals. The analytical sensitivity of LAMP is often comparable to that of the PCR in clinical diagnostics, making it a practical alternative in POC contexts. Nevertheless, the complexity of primer design increases the risk of nonspecific amplification, which can slightly compromise sensitivity under suboptimal assay conditions. Despite this limitation, LAMP is consistently described as a robust method that balances high analytical sensitivity with relative ease of application, explaining its widespread adoption in diagnostic workflows (6,12,15,119).

Rolling circle amplification (RCA) offers distinct advantages in terms of fidelity and specificity, but typically exhibits lower analytical sensitivity compared to LAMP and EXPAR. Reported limits of detection generally fall within the femtomolar to picomolar range (10^{-15}–10^{-12} M), reflecting the slower amplification kinetics inherent to its reliance on circularized DNA templates. While this template requirement confers strong specificity and reduces nonspecific amplification, it also constrains speed and sensitivity, limiting the method's standalone diagnostic competitiveness. Nonetheless, RCA's performance can be substantially enhanced when integrated into cascade amplification schemes (e.g., RCA–LAMP or RCA–EXPAR), where it contributes specificity and signal stability while benefiting from the superior sensitivity of companion amplification systems. Thus, RCA is most often positioned not as a frontline diagnostic method, but as a complementary amplification strategy within multi-step assay designs (6,12,15,119).

4.2. Quantitative vs. qualitative outputs

Loop-mediated isothermal amplification (LAMP) yields robust qualitative outputs that can be easily monitored through changes in turbidity, fluorescence, or colorimetric properties, making it well-suited for point-of-care diagnostics. Fluorescence offers the highest sensitivity and dynamic range, while colorimetric methods provide rapid, equipment-free visualization at the expense of precision (120). Quantitatively, LAMP typically achieves limits of detection in the attomolar to femtomolar range, corresponding to just a few target copies per reaction, with amplification times of 30–60 minutes. These characteristics provide LAMP sensitivity comparable to that of PCR, while its qualitative versatility enhances its diagnostic applicability (6,12).

Table 2. Analytical sensitivity (LoD), time-to-signal, and qualitative vs. quantitative outputs of three major isothermal amplification methods: loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), and exponential amplification reaction (EXPAR).

Method	LoD (typical range)	Time-to-signal	Common qualitative outputs	Quantitative outputs	References
LAMP	Attomolar–femtomolar (10^{-18}–10^{-15} M); often a few copies per reaction	~30–60 min	Turbidity (Mg^{2+} precipitation), colorimetric pH dyes, lateral flow, fluorescence	Real-time fluorescence; quantitative threshold times (Tt) comparable to qPCR	(6,12,120)
RCA	Femtomolar–picomolar (10^{-15}–10^{-12} M) standalone; improved to attomolar levels in RCA-LAMP or RCA-EXPAR cascades	60–120 min (slower, linear amplification)	Fluorescence probes, hybridization-based signals; some cascade systems enable lateral flow/colorimetric outputs	Signal proportional to concatemer length and probe binding; slower quantitative kinetics	(12,15,119,121)
EXPAR	Zeptomolar–attomolar (10^{-21}–10^{-18} M); single-digit copies per reaction	<10 min in optimized assays	Primarily fluorescence; endpoint readouts possible but less reliable due to background	Real-time kinetic fluorescence curves with very steep amplification slopes	(6,15,119,122)

Rolling circle amplification (RCA) generates long, repetitive DNA concatemers, producing qualitative outputs that are best visualized via fluorescence or hybridization-based probes. Unlike LAMP or EXPAR, RCA's readouts are less amenable to rapid visual inspection. However, cascade combinations with other methods (e.g., RCA-LAMP) have introduced colorimetric or lateral flow possibilities (121). Quantitatively, standalone RCA is slower and less sensitive, with limits of detection typically in the femtomolar to picomolar range, reflecting its linear amplification kinetics. However, when integrated into cascaded amplification systems, RCA contributes strong specificity and fidelity while boosting overall assay sensitivity to near-attomolar levels (12,15,119).

Exponential amplification reaction (EXPAR) yields predominantly quantitative fluorescent outputs, with rapid signal accumulation enabling real-time kinetic monitoring. Its extreme amplification speed can also support endpoint qualitative detection, though non-specific background often complicates simple yes/no readouts. Quantitatively, EXPAR exhibits the greatest sensitivity among the three methods, with reported detection limits in the zeptomolar to attomolar range and reaction times of less than 10 minutes (122). This performance surpasses both LAMP and RCA, however, nonspecific amplification and narrow sequence design tolerances can undermine its practical reproducibility, necessitating auxiliary specificity controls (6,15,119).

4.3. Sample preparation and contamination control

Sample preparation for LAMP is relatively straightforward compared to other amplification methods, which has contributed to its widespread adoption in point-of-care diagnostics. While conventional workflows still rely on nucleic acid extraction from clinical specimens such as nasopharyngeal swabs or blood, LAMP has demonstrated robustness in the presence of crude lysates, enabling direct testing from saliva or minimally processed samples (6,12). Simplified approaches, such as heat lysis or magnetic bead-based purification, are often employed to strike a balance between speed and performance. However, inhibitors present in unprocessed specimens can compromise sensitivity and reproducibility (120). This tolerance to simplified preparation allows LAMP to be more compatible with decentralized and low-resource settings than most other isothermal methods. Contamination control is a critical concern in LAMP, as its high sensitivity can lead to false positives from carry-over amplicons. The most widely implemented strategy involves the incorporation of dUTP in place of dTTP, followed by pretreatment with uracil-DNA glycosylase (UDG/UNG) to degrade any contaminating products from previous reactions (123,124). Additional physical measures, such as closed-tube detection, mineral oil overlays, and spatial workflow separation, are commonly used to further minimize aerosolized contamination (123). It should be noted that LAMP assays are prone to false-positive results, particularly when reactions are allowed to run for extended periods. Studies have shown that the likelihood of false positives increases with reaction time, with the earliest cases observed after about 45 minutes. Time-gating strategy can be employed, i.e., limiting duration to < 45 minutes, to minimize this risk and help ensure reliable results (44).

Collectively, these measures are essential for ensuring the reliability of LAMP assays, particularly in point-of-care testing, where simplified sample handling increases the risk of cross-contamination.

When it comes to RCA, this method generally requires more stringent sample preparation due to its dependence on a circularized DNA template. For nucleic acid detection, the target sequence must first be captured by padlock probes and enzymatically ligated into a circular form before amplification can proceed (121). This additional ligation step typically follows standard RNA or DNA extraction, as crude samples rarely provide the necessary purity or structural accessibility for efficient probe hybridization and ligation (12,119).

Although RCA offers exceptional specificity due to the circular template requirement, its reliance on precise pre-amplification processing makes it less amenable to simplified workflows, unless combined with other amplification strategies that help mitigate sample quality issues (15). Rolling circle amplification is less prone to aerosol-based carryover contamination than LAMP; however, contamination control remains crucial due to the possibility of false signals from unligated or misligated padlock probes. To address this, most workflows incorporate exonuclease digestion steps to remove linear, unligated probes before amplification, thereby ensuring that only correctly circularized templates undergo amplification (74). Nuclease protection strategies, probe design optimization, and physical separation of ligation and amplification steps in microfluidic devices have also been reported to reduce spurious amplification products (105). These measures, although adding complexity to the workflow, are essential for maintaining the high specificity of RCA, especially in clinical or multiplexed assay formats.

Lastly, EXPAR is highly sensitive to input purity and therefore typically requires conventional nucleic acid extraction before amplification. Unlike reverse transcription-dependent assays, RTF-EXPAR has been demonstrated to bypass cDNA synthesis by directly converting RNA targets into short DNA triggers. However, this still relies on extracted RNA rather than crude lysates (122). Nonspecific amplification and background noise are significant risks when inhibitors or non-target nucleic acids are present, making extraction and clean-up steps critical for assay fidelity(6,15,119). Thus, while EXPAR achieves ultrafast and ultrasensitive detection, its stringent sample preparation requirements currently limit its use in fully extraction-free diagnostic workflows. Considering its high sensitivity, EXPAR is particularly vulnerable to nonspecific amplification and background noise, making contamination control a significant design challenge. Mechanistic studies have shown that spurious template interactions can trigger exponential amplification even in the absence of a target, leading to false positives (110). Careful sequence design, hot-start approaches, buffer additives, and chemical gating of triggers are necessary to suppress nonspecific initiation (77,125). Additional innovations include the use of CRISPR-Cas systems to selectively confirm true amplicons and eliminate background signals, further improving specificity (126). Such combined biochemical and readout-level controls are crucial for harnessing the ultrafast and ultrasensitive capabilities of EXPAR for reliable diagnostic use.

5. Integration with CRISPR/Cas and other molecular diagnostic systems

A new class of molecular diagnostics tools, CRISPR-Cas, was initially discovered as a part of the prokaryotic immune defense system, enabling bacteria and archaea to recognize and defend against invading genetic elements such as bacteriophages and plasmids. CRISPR-Cas-based detection utilizes the programmable nucleic acid recognition ability of CRISPR-associated proteins such as Cas12, Cas13, and Cas9 (127,128). Guided by a short RNA sequence, Cas enzymes can identify specific genetic targets and, upon activation, cleave reporter molecules to produce a detectable signal (128). Several CRISPR-Cas-based platforms, including SHERLOCK (Cas13-based) and DETECTR (Cas12-based), have demonstrated rapid, highly sensitive, and specific detection of pathogens and genetic variants, often without the need for thermal cycling (127,128). Moreover, the CRISPR-Cas system is often coupled with a preamplification step, such as RCA, RPA, and LAMP, to achieve even higher sensitivity and specificity (129). As this setup aligns with the requirements needed for POC testing, CRISPR-Cas-based systems are being increasingly integrated into POC platforms. For example, there are numerous reports on one-pot reactions that combine preamplification and CRISPR-Cas-based detection, with collateral cleavage co-occurring with target amplification (129). Because these assays are fast, inexpensive, and adaptable to point-of-care formats such as lateral flow strips, they

offer a practical alternative to conventional PCR in infectious disease surveillance, outbreak response, and genetic testing (130,131).

5.1. CRISPR-based detection systems (SHERLOCK, DETECTR)

DETECTR (DNA endonuclease-targeted CRISPR *trans* reporter) is a platform developed by Chen et al., which utilizes the Cas12a enzyme. In this method, the target DNA is first amplified, typically using isothermal amplification techniques such as RPA (130). The RNA target binding of this enzyme unleashes nonspecific single-stranded DNA cleavage, which is harnessed to report the presence of specific DNA or cDNA targets (130). In the context of molecular diagnostics, there are reports on DETECTR for the efficient identification of HPV16 in 25 of 25 cases and HPV18 in 23 of 25 cases in crude DNA extracts, where the entire reaction is completed within 1 hour (130). The system has also been customized for SARS-CoV-2, with a readout form through an LFA, with readable results in 40 minutes (132).

SHERLOCK (Specific High-Sensitivity Enzymatic Reporter UnLOCKing) employs the Cas13 enzyme to detect target nucleic acids and leverages the enzyme's RNA-activated collateral RNase activity to cleave labeled reporters for signal generation (131).

In its original format, SHERLOCK combined isothermal pre-amplification (RCA or RT-RPA-based) with Cas13 detection to achieve attomolar sensitivity and single-base discrimination (129). In 2018, Myhrvold et al. demonstrated a system based on SHERLOCK, which they named HUDSON (Heating Unextracted Diagnostic Samples to Obliterate Nucleases). The system successfully detected ZIKV and DENV in the bodily fluids of patients, and the detection was coupled with a simple colorimetric readout (133).

Numerous studies have reported on an integrated CRISPR-Cas system for detecting miRNAs as biochemical markers of diseases and pathogens (134–136). Recently, De Silva et al reported a 3D-printed 96-well microfluidic plate fabricated with Cas13a–crRNA complexes immobilized in specific wells to enable amplification-free, multiplex detection of plasma miRNAs (miR-34c-5p, miR-30e-5p, miR-200c-3p). In their assay, detection relied on the collateral cleavage of a quenched fluorescent RNA reporter, yielding highly sensitive fluorescent signals ranging from 0.7 to 7.4 fg/mL, as quantified by a plate reader (137). Next, Bagi et al reported a fluorescent CRISPR-Cas assay that directly detects miRNA-21 via collateral cleavage of a fluorescent reporter. The system utilized LbuCas13a achieved a detection limit of ~75 aM with a fast readout (~30 min), illustrating ultrasensitive and label-free fluorescence detection (138). Moreover, in 2020, Wang et al reported the CASLFA system (CRISPR/Cas9-mediated lateral flow nucleic acid assay). They achieved a rapid and specific identification of pathogens (Listeria monocytogenes and African swine fever virus (ASFV) in under 1 hour at low copy numbers (139).

5.2. Enhancing specificity and multiplexing

The specificity of CRISPR-Cas systems is provided by the careful design of guide RNA, but the choice of nucleases also plays a critical role. For instance, Cas12 and Cas13 variants differ in their collateral cleavage activities, which researchers strategically select to match the diagnostic application and the right choice of nucleases. However, when CRISPR-Cas is coupled with isothermal amplification methodologies, the specificity increases even more. For example, CRISPR-Cas12/Cas13 is easily coupled with RPA, because the working temperature conditions are matched (37°C). Similarly, the LAMP method can be coupled with the AapCas12b, which is stable at 65°C, a required temperature for LAMP (140). Alternatively, LAMP can also be combined with CRISPR-Cas12a/CRISPR-Cas12b. In that case, due to the different working temperatures, the

process is typically divided into two stages: LAMP amplification and CRISPR-Cas12 cleavage of the LAMP amplicons (141,142).

5.3. Combining isothermal methods with lateral flow or fluorescence readouts

The integration of isothermal amplification methods has significantly enhanced the accessibility and adaptability of CRISPR-based diagnostics (141). For instance, by incorporating sophisticated molecular techniques into lateral flow assays, the diagnostic test can be simplified. Lateral flow assays offer a rapid, equipment-free format that is particularly well-suited for point-of-care testing and environments with limited resources (143). By incorporating labeled nucleic acid reporters that are cleaved upon CRISPR activation, these assays enable the visual confirmation of target sequences within minutes, making them highly user-friendly (143).

In contrast, fluorescence-based readouts provide greater sensitivity and the ability to conduct quantitative analyses (142). These assays utilize fluorophore-quencher reporters that generate strong signals upon cleavage, enabling real-time monitoring of reactions. Fluorescence detection can also be adapted for use with portable devices, such as smartphones and handheld fluorimeters, effectively bridging the gap between laboratory-grade sensitivity and field-deployable formats (142).

The choice between lateral flow assays and fluorescence detection largely depends on the required balance between simplicity, sensitivity, and the specific application at hand.

5.4. Microfluidics and lab-on-a-chip integration

While many of the recent research efforts focus on improving specificity and readout formats, integrating CRISPR-based diagnostics with microfluidic / lab-on-a-chip platforms remains a burgeoning area. The multiplexed Cas12a cis-cleavage-mediated LFA (cc-LFA) platform, for example, is combined with portable devices to automate amplification and strip detection, indicating the potential for fully integrated systems (144).

6. Clinical and Diagnostic Applications

6.1. Infectious disease and pathogen detection

Nucleic acid sequence-based amplification (NASBA) has been demonstrated as an effective method for detecting infectious agents, particularly RNA viruses. One of the earliest demonstrations of its clinical application was in 1991, when the method was optimized for HIV-1 detection (145). Later, NASBA was successfully adapted for the hepatitis C virus (HCV), where the introduction of quantitative NASBA (NASBA-QT) provided high sensitivity and reliability. Importantly, NASBA-QT achieved over a tenfold increase in sensitivity compared with widely used commercial assays, including the HCV branched DNA assay and the HCV MONITOR assay (146). Later advances in NASBA technology enabled multiplex detection of HIV-1 and HCV (Mohammadi-Yeganeh et al., 2012) and facilitated the broad identification of respiratory viruses, including influenza A, influenza B, RSV, and coxsackievirus (147). NASBA has also been successfully adapted for emerging pathogens, such as Zika virus, achieving a clinical sensitivity of 97.64% (Reed et al., 2019), and SARS-CoV-2, where real-time NASBA targeting the RdRp and N genes reached a detection limit of 200 copies/ml with 97.64% clinical sensitivity, offering a cost-effective alternative to PCR in resource-limited settings (148,149).

Despite the advantages NASBA offers for pathogen detection, the method also presents certain limitations. The reaction relies on thermally unstable enzymes and must be carried out at temperatures below 42°C, which increases the likelihood of primer dimer formation and nonspecific amplification, potentially leading to false-positive results. In addition, efficient amplification requires the target RNA fragment to be between 120 and 250 nucleotides in length (150,151). Nevertheless, integration with molecular beacons, microfluidics, and CRISPR-based systems is expected to further enhance its sensitivity, specificity, and applicability, supporting the development of rapid and low-cost diagnostic platforms for infectious disease detection (152–154).

In the context of clinical and diagnostic applications, **Loop-mediated Isothermal Amplification (LAMP)** has emerged as one of the most extensively adopted isothermal amplification strategies for infectious disease detection since its initial development (155,156). Its broad applicability encompasses neglected tropical diseases (NTDs), respiratory infections, foodborne pathogens, and viral outbreaks, underscoring its versatility across diverse clinical and epidemiological settings (157). Notably, LAMP has been successfully employed to detect *Mycobacterium leprae* with high specificity despite genomic similarity to *M. tuberculosis* (157), low parasite loads of *Plasmodium* in malaria (Selvarajah et al., 2020), and a wide range of bacterial and parasitic pathogens including *Escherichia coli*, *Salmonella typhi*, *Clostridium perfringens*, *Campylobacter jejuni*, *Enterococcus faecalis*, and *Entamoeba histolytica* (158–163). It has also proven valuable in diagnosing respiratory infections such as pneumonia caused by *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Stenotrophomonas maltophilia* (157,164). This versatility is exemplified by WHO's endorsement of TB-LAMP as a diagnostic kit for tuberculosis, given its short assay time (<1h) and suitability for resource-constrained settings (165).

LAMP has also been extensively explored in viral diagnostics. During the COVID-19 pandemic, RT-LAMP emerged as a robust and cost-efficient alternative to qRT-PCR for SARS-CoV-2 detection (97,166). Beyond coronavirus, LAMP assays have been validated for adenovirus, influenza A, herpesvirus, varicella zoster virus, and cytomegalovirus (167–170). Furthermore, fungal pathogens that are traditionally diagnosed by labor-intensive culture techniques—including *Candida albicans*, *Cryptococcus neoformans*, *Mucor racemosus*, *Trichosporon asahii*, *Aspergillus fumigatus*, and *Pythium insidiosum* — have also been successfully targeted with LAMP (171–174).

Recent methodological advances have further expanded the diagnostic potential of LAMP. Multiplex RT-LAMP, coupled with microfluidic POC platforms, has enabled the simultaneous detection of SARS-CoV-2, influenza A/B, and RSV within 30 minutes and at a low detection limit (1 copy/μL) (175). Similarly, the combination of LAMP with lateral flow biosensors (LAMP-LFB) has achieved rapid and accurate detection of the *Mycobacterium tuberculosis* complex, outperforming bacterial culture and Xpert MTB/RIF assays (176). Integration with self-driven polydimethylsiloxane (PDMS)-based chips has further allowed multiplex detection of HBV, HCV, and HIV in <50 minutes with performance comparable to qPCR (177). Similar success has been reported using polymethyl methacrylate (PMMA)-based platforms for *E. coli*, *Enterococcus* spp., *Vibrio parahaemolyticus*, and *Salmonella enterica* detection, with limits of detection as low as four copies per reaction (178–180). Among sexually transmitted diseases (STDs), HPV infections are the leading cause of cervical, anal, vaginal, vulvar, penile, and oropharyngeal (throat and mouth) cancer (181). Thus, the rapid and convenient LAMP method for HPV detection without DNA extraction has been developed (182).

Despite its advantages, LAMP also has important limitations. The requirement for four to six primers increases assay complexity and the likelihood of nonspecific amplification, while its high efficiency makes

the method prone to carryover contamination and false-positive results, as already mentioned (168). In addition, quantitative applications remain less reliable compared to PCR-based approaches, limiting their use in precise viral load monitoring (47). Nevertheless, integration with microfluidics, CRISPR-based systems, and digital formats is expected to overcome these challenges, enhancing sensitivity, specificity, and field applicability in pathogen detection (183).

Recombinase Polymerase Amplification (RPA) has rapidly gained recognition as a versatile tool for pathogen detection in clinical diagnostics. Particularly, RPA combined with lateral flow assay (RPA-LFA) enables rapid, equipment-free visual readouts, thereby representing an attractive format for point-of-care diagnostics (125). For example, Ji et al. established an RPA-LFS assay for *Staphylococcus haemolyticus* with amplification achieved in 8 min and visual interpretation in just 1 min, showing excellent agreement with qPCR (100%) and culture methods (98.73%). Comparable success has been reported for *Streptococcus pneumoniae* (3.32 CFU/μL sensitivity, 98.18% concordance with PCR) (184). In bacterial diagnostics, additional assays have been developed for *Klebsiella pneumoniae* (185), *Vibrio parahaemolyticus* (186), and multiplex panels covering five common foodborne pathogens (187), with further applications in antimicrobial resistance gene surveillance (188). Similarly, real-time RPA formats allow rapid fluorescent monitoring of amplification and have been validated for *Yersinia enterocolitica* in intestinal samples as well as for high-risk HPV16/18 subtyping (189).

In the context of viral diagnostics, RT-RPA combined with lateral flow assays and microfluidic chips enables rapid and cost-effective detection of SARS-CoV-2 within ~30 minutes (190), and has also been validated for influenza A/B (191), Coxsackievirus A6 (192), and HBV/HEV with detection limits as low as 10–100 copies/mL (193,194).

Beyond bacterial and viral applications, RPA-LFA has also been tailored for fungal pathogens, enabling rapid identification of *Cryptococcus neoformans* in cerebrospinal fluid (195) and *Candida albicans* with enhanced specificity through probe and primer modifications (196), while demonstrating utility in detecting *Candida krusei* in low-resource environments (197). Parasitic pathogens, such as *Trichinella* (198), *Clonorchis sinensis* (199), and *Toxoplasma gondii* (200), have also been successfully targeted, underscoring the broad clinical relevance of RPA.

Despite its clear advantages — rapid kinetics, minimal instrumentation, compatibility with DNA and RNA targets, and adaptability to diverse readout modalities (35,201)—RPA has several inherent limitations. Amplification is generally restricted to 100–500 bp fragments, which constrains applications requiring longer sequence analysis. Primer design is technically demanding, as suboptimal structures increase the risk of nonspecific amplification. Moreover, the isothermal conditions of RPA can predispose to false positives, necessitating integration with other technologies to achieve higher specificity. Recent efforts combining RPA with CRISPR/Cas12a or Cas13a have demonstrated improved discrimination, such as simultaneous detection of HPV16/18 with a sensitivity of 10 copies/μL (202). In addition, limitations in multichannel parallelization currently hinder its scalability for high-throughput screening. Nonetheless, continued refinement and integration into microfluidic and biosensor platforms position RPA as a promising candidate for next-generation molecular diagnostics (183).

Although **Rolling Circle Amplification (RCA)** was primarily developed for the detection of genetic biomarkers, several studies also report its successful application in pathogen diagnostics. For example, Kundu et al. summarized diverse RCA-based assays targeting viruses, including *Ebola virus, SARS-CoV-2, HIV, Zika virus, adenovirus, dengue virus, HPV-16/18,* as well as parasitic agents such as *Plasmodium spp.*,

highlighting the method's broader diagnostic potential (15). Moreover, RCA has been successfully combined with LAMP to further improve sensitivity in pathogen detection. This integrated approach has demonstrated effectiveness in identifying viral pathogens such as cotton leaf curl virus in low-titer plant samples where conventional PCR and LAMP assays were insufficient (203). Such examples underscore the promise of RCA, both alone and in combination with LAMP, as a valuable tool for infectious disease diagnostics.

6.2. Detection of miRNA as diagnostic and prognostic biomarkers

Isothermal amplification methods, such as EXPAR and RCA, have emerged as particularly well-suited for miRNA detection due to their efficiency in amplifying short nucleic acid targets and potential for integration into compact diagnostic devices. For instance, in 2016, Liu et al. developed a hairpin-probe mediated EXPAR assay, where the binding of a target miRNA to a linear template initiates EXPAR and signal amplification via catalyzed hairpin assembly. Their assay achieved a detection limit of approximately 3×10^{-15} M (3 fM) within an hour, demonstrating a promising approach for rapid, point-of-care diagnostics (204). Moreover, Wang et al. introduced a two-stage EXPAR combined with a time-resolved fluorescence sensor, increasing the sensitivity to attomolar levels, with detection limits as low as 0.1 aM and strong selectivity for miRNA family members (205). In another strategy for integrating nanomaterials, Morales et al. combined EXPAR with magnetic and gold nanoparticles to achieve high specificity without the need for a pre-amplification step. In their assay, the target miRNA is simultaneously complementary to both the oligonucleotide-functionalized magnetic nanoparticle and the reporter oligonucleotide-functionalized gold nanoparticle. The assay can be used to discriminate single-nucleotide polymorphisms (206).

Further optimization of EXPAR for enhanced specificity and minimized background amplification was demonstrated by (207), who enhanced EXPAR specificity and reduced background amplification by designing a custom exp-Hairpin template with symmetric trigger sites and nicking enzyme recognition sequences on both arms, thereby enabling amplification efficiencies greater than 2 . By optimizing the stem length to 8 bp, they achieved high discrimination between let-7a and homologous miRNAs (let-7b/7c/7f/7g/7i), with sensitive detection in diluted human serum. The assay's modular design also allowed easy adaptation for other targets, such as miR-200b, demonstrating its versatility for broader miRNA profiling.

Recent advances in RCA-based strategies have introduced innovative approaches that offer both high sensitivity and remarkable specificity in miRNA detection. By integrating complementary molecular systems, RCA enables efficient signal amplification and improved analytical precision under isothermal conditions. Namely, Fang et al. developed a dual-amplification platform for miRNA-21 detection by integrating RCA with an allosteric deoxyribozyme system (MNAzyme) (208). In this system, a padlock probe hybridizes to the target miRNA and is ligated to form a closed circular template. RCA then generates long single-stranded DNA products containing repeated sequences that promote the formation of catalytically active MNAzymes. These MNAzymes cleave fluorophore-quencher-labeled substrates, resulting in a significant amplification of the fluorescence signal. The platform achieved a low detection limit of 4 pmol/L and demonstrated excellent selectivity, even among closely related miRNA family members differing by a single nucleotide.

Further expanding the capabilities of RCA, several studies have combined it with other amplification and detection technologies to enhance performance. For example, Jieng et al. combined RCA with CRISPR-Cas12a technology and gold nanoparticles to develop a colorimetric assay for miR-143 detection (209). This approach enables visual detection at femtomolar concentrations and achieves attomolar sensitivity with UV–Vis spectroscopy, highlighting its clinical diagnostic potential. Similarly, Zhou et al. introduced RS-CRISPR, a multiplexed detection system integrating RCA with SDA and CRISPR/Cas12a (210). This platform uses RCA

products as templates for SDA, amplifies multiple miRNAs simultaneously, and utilizes Cas12a's trans-cleavage activity for signal readout. RS-CRISPR demonstrated a sensitivity of 57.8 fM and was validated in clinical colorectal cancer samples, successfully distinguishing tumor from normal tissue and detecting elevated miRNA levels in patient serum.

Despite these advancements, RCA-based methods still face notable technical challenges. As highlighted by Han et al., standard RCA workflows typically involve two enzymatic steps—ligation and polymerization—requiring phosphorylated probes and increasing assay complexity and cost (211). Furthermore, RCA's dependence on specific DNA template binding makes it vulnerable to nonspecific interactions from contaminants in unpurified biological samples, potentially leading to false-positive signals. Addressing these issues remains a key priority, with ongoing efforts focused on simplifying reaction workflows, improving specificity, and developing robust diagnostic platforms suitable for clinical application.

Although LAMP was initially developed for pathogen detection and point-of-care diagnostics, recent studies have demonstrated its effective application for miRNA detection. One of the earlier notable applications was presented by Tian et al., who combined RCA with LAMP in a single-step assay (212). In this method, the target miRNA acts as a template for ligating a padlock probe, triggering RCA to produce long DNA repeats that then serve as templates for LAMP amplification. This integration significantly improved sensitivity, enabling detection of miRNAs at attomolar concentrations, while maintaining a simplified workflow by performing both amplification steps simultaneously. Wang et al. further advanced miRNA detection by merging asymmetric PCR with LAMP (213). The asymmetric PCR generates single-stranded DNA that forms the necessary stem-loop structures for LAMP, using miRNAs as loop primers. This approach achieved an impressive detection limit of 10 amol/L within 90 minutes, demonstrating enhanced specificity and rapidity. More recent innovations include the ligation-based LAMP system developed by Wu et al., which uses two stem-loop DNA probes hybridizing to the target miRNA (24). The miRNA template enables SplintR ligase-mediated ligation, forming a dumbbell-shaped DNA structure that initiates LAMP amplification. Fluorescent detection with EvaGreen dye facilitates quantification, reaching detection limits as low as 100 fM across a wide dynamic range, offering a highly sensitive and rapid assay suitable for clinical applications. Additionally, Chua et al. introduced a cost-effective, multiplexed POC testing device employing a split-LAMP strategy (214). This method breaks loop primers to detect multiple miRNAs simultaneously and integrates their signals through a logical AND-gate output, with a pH-sensitive dye providing a colorimetric readout in under 30 minutes. Such developments illustrate LAMP's expanding role in rapid, multiplexed, and on-site miRNA diagnostics. Collectively, these advances demonstrate how LAMP-based approaches have evolved and diversified, addressing the challenges of miRNA detection by enhancing sensitivity, specificity, and usability. The integration of LAMP with complementary amplification methods and innovative detection strategies continues to expand its potential as a valuable tool in molecular diagnostics.

6.3. Genetic disorders and newborn screening

The practical application of isothermal nucleic acid amplification techniques is well demonstrated by recent advances in genetic variation detection. Cierzniak et al. developed a LAMP-based assay optimized for point-of-care use that can detect as few as 250 to 1000 copies of synthetic DNA template per reaction (28). The assay runs for approximately 50 minutes and utilizes modified primers with specific alterations at the 3' end of either the F2 or B2 primers, ensuring selective amplification of mutant sequences while excluding wild-type DNA. This design enables high sensitivity and specificity, even in samples containing a mixture of mutated and wild-type alleles.

Supporting the robustness of LAMP in genetic testing, several studies have leveraged conventional LAMP assays for genotyping single-nucleotide polymorphisms (SNPs) and detecting gene rearrangements. For instance, LAMP has been successfully applied to screen for mutations in the survivin gene (*BIRC5*), an inhibitor of apoptosis-related proteins implicated in cancer, with a sensitivity capable of detecting as few as ten copies per reaction (215). Kuzuhara et al. introduced a proofreading LAMP (PR-LAMP) assay for SNP detection in the *ALDH2* gene, using genomic DNA extracted from hair follicles (216). This single-step reaction, completed within 30 minutes, utilizes fluorescently labeled primers and an exonuclease-enhanced DNA polymerase, demonstrating strong potential for high-throughput screening and POC applications in clinical diagnostics and pharmacogenomics. Furthermore, LAMP assays have been developed and clinically validated as CE-marked *in vitro* diagnostic methods for thrombophilia mutation screening. Namely, LAMP can detect common thrombophilia SNPs without prior DNA extraction (similar to Direct PCR), showing 100% concordance with standard genotyping methods (217). For instance, the LaCAR MDx thrombophilia tests are fully IVDR compliant, rapid, less time-consuming than PCR sequencing and the Real Time PCR approaches since they do not require a DNA isolation step (218). Also, the LAMP Human HLA-B27 Direct IVD-CE KIT enables qualitative detection of most HLA-B27 alleles, including the European most AS associated subtypes B*2702 and B*2705, Asian subtype B*2704 and African subtype B*2703 from the EDTA whole blood or extracted DNA. Moreover, there are several CE registered molecular tests on the market in the area of nutrigenetics, pharmacogenetics, hematological diseases, cancer genetics and similar. For instance, LAMP has also been used for identifying gene translocations such as *IgH/BCL2*, *SYT-SSX*, and *EML4-ALK*, which are relevant in various hematological malignancies and solid tumors (219). Additionally, LAMP-based approaches have been explored for analyzing lymph node metastasis in lung cancer, further underscoring its clinical utility (220). LAMP-based methods have also shown strong potential in newborn screening. Park et al. developed a quantitative LAMP (qLAMP) assay for detecting congenital cytomegalovirus (CMV) infection, offering faster results and a broader detection range compared to conventional qPCR (29). While qPCR detects CMV within 10^6–10^2 copies/μL in about 90 minutes, qLAMP achieves detection across 10^8–10^3 copies/μL in just 15 minutes. Additionally, a colorimetric LAMP version enables visual detection down to 10^3 copies/μL within 30 minutes, making the assay suitable for both centralized labs and low-resource POC settings.

7. Toward Personalized Genetic Testing

Advances in genomics and biotechnology in the last few decades are rapidly transforming the landscape of healthcare, enabling personalized genetic testing to become an integral part of modern medicine. Unlike conventional diagnostic approaches, personalized genetic testing employs high-throughput sequencing technologies and bioinformatics tools to decode complex genetic variations. Precision medicine genetic testing involves analyzing an individual's unique genetic makeup to provide tailored insights into disease risks, drug responses, and preventive health strategies (221). This allows clinicians to move beyond one-size-fits-all treatments toward precision interventions that consider an individual's genetic predisposition, environmental exposures, and lifestyle factors.

Applications of personalized genetic testing include identifying inherited risk for conditions such as cancer, cardiovascular diseases, and thrombophilia, informing pharmacogenomic decisions for optimal drug efficacy and safety, and guiding nutritional and preventive measures through nutrigenomics. As polygenic risk scoring methods improve, tests that integrate multiple SNPs provide increasingly accurate risk assessments tailored to each patient (222,223). Despite the power of the precision medicine approach, numerous challenges remain, including costly analysis, sophisticated and expensive equipment, long TAT,

highly trained and specialized molecular diagnostic personnel, many ethical considerations, data privacy, the need for standardized interpretation frameworks, and ensuring equitable access to testing. However, ongoing research and clinical integration efforts promise to expand the use of personalized genetic testing, ultimately enhancing disease prevention and patient outcomes (224).

Thus, since isothermal amplification involves rapid diagnostics (short TAT) without sophisticated laboratory equipment, this approach for nucleic acid amplification testing (NAAT) offers faster, simpler, and affordable POC testing compared to traditional PCR, making it useful in various clinical and field settings. Moreover, being able to amplify tiny amounts of DNA or RNA to detectable levels, isothermal methods can aid early disease molecular diagnosis, reliable prognosis or preventive measures for mutation carriers (225).

7.1. Potential for real-time monitoring of disease progression

According to studies, the lack of real-time data exchange between patients and healthcare providers often delays personalized care adjustments (226). Innovations in microfluidics and nanotechnology provide the foundation for real-time detection of molecules, converting the enhanced signal into a digital output, as seen in personal glucose monitors that communicate the detected levels to a smartwatch (227). Isothermal nucleic acid amplification techniques, such as LAMP and RPA, are being gradually modified for real-time molecular monitoring. This adaptation involves integrating signal transduction methods (such as fluorescence, electrochemical, or colorimetric signals) with microfluidic systems or digital platforms (228). For example, in a digital microfluidics platform, LAMP was used to detect the *c-MYC* oncogene in droplets, offering absolute quantification within an integrated chip in under 60 minutes (229). The integration of digital health tools, such as mobile applications, wearables, and remote monitoring platforms, would fuel the development of personalized genetic testing by providing real-time physiological and molecular data into electronic health records. Furthermore, the integration of molecular diagnostic readouts, such as those from isothermal amplification assays, could enable automatic alerts when biomarker thresholds are crossed, triggering timely clinical review or intervention (226). Aside from on-site real-time patient monitoring, robustly integrated molecular diagnostic and digital tools could also support remote monitoring, while enhancing one-health surveillance by feeding de-identified data into public health systems (226).

7.2. Integration with digital health tools and electronic medical records

Integration may be reciprocal, as digital health platforms can not only receive molecular data but also guide the development and application of molecular tests. By embedding digital health APIs (Application Programming Interfaces) into molecular devices, clinicians could receive alerts when amplification thresholds exceed pathogenic cutoffs, view longitudinal biomarker trends, and correlate molecular test outcomes with other clinical data (230). Integration demands also require robust software-to-software communication frameworks to mediate between laboratory information systems and health records, while maintaining data fidelity (231). However, many challenges, such as versioning of genomic tests, data provenance, and ensuring secure transmission of patient-genetic data, must be addressed to preserve clinical trust and compliance (226).

8. Conclusion

Isothermal nucleic acid amplification methods represent a significant advancement in molecular diagnostics, providing rapid (low TAT), sensitive, and equipment-light alternatives to traditional PCR. These

techniques can detect genetic variants, cancer biomarkers, and other disease-related nucleic acids, making them essential tools for personalized genetic testing, especially in point-of-care settings and resource-limited environments. When integrated with digital health infrastructures, these assays can deliver real-time, actionable insights that enhance individualized patient management and support population-level monitoring. This integration is mutually beneficial; digital platforms can also assist in refining molecular assays by establishing data standards, implementing security protocols, and developing clinical implementation strategies. Together, these converging innovations are paving the way for a new era of decentralized, interconnected, and personalized diagnostics, which are expected to improve outcomes in oncology, infectious diseases, and chronic health management.

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DNA Barcoding in Animal Forensics: Applications of Cytochrome c oxidase subunit 1 and Cytochrome b genes

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Abstract

Accurate species identification is a cornerstone of forensic investigations involving wildlife crime, illegal trade, and food fraud. Mitochondrial DNA (mtDNA) markers, particularly cytochrome c oxidase subunit I (*COI*) and cytochrome b (*cyt b*), have been widely applied due to their high interspecies variability, simple methodology, and robust amplification from degraded samples. *COI* has achieved global standardization through the Barcode of Life Database (BOLD), while *cyt b* has demonstrated superior discriminatory power in mammalian forensics. However, limitations remain, including incomplete reference databases, misannotation of sequences, and the inability of mtDNA to differentiate hybrids or subspecies. To address these challenges, multilocus approaches and integration with nuclear DNA markers (STRs, SNPs) are increasingly recommended. Together, these methods strengthen evidentiary value, enhance species resolution, and support both biodiversity protection and public health.

Keywords: forensic genetics, mitochondrial DNA, cytochrome b, COI, species identification, wildlife crime

DNK barkodiranje u forenzici životinja: primena gena za podjedinicu 1 citohrom c oksidaze i gena za citohrom b

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Apstrakt

Forenzičke istrage koje uključuju zločine nad divljim životinjama, nelegalnu trgovinu životinjskim vrstama i prevare u prometu životinjske hrane se baziraju na tačnoj identifikaciji vrsta. Mitohondrijski DNK (mtDNK) markeri, posebno gen koji kodira podjedinicu 1 citohrom c oksidaze (*COI*) i gen koji kodira citohrom b (*cyt b*), široko se primenjuju zahvaljujući velikoj međuvrsnoj varijabilnosti, jednostavnoj metodologiji i pouzdanom umnožavanju iz degradiranih uzoraka. Upotreba *COI* je globalno standardizovana putem baze podataka Barkod života (BOLD), dok je *cyt b* pokazao izuzetno veliku diskriminatornu moć u forenzičkim analizama sisara. Ipak, određena ograničenja i dalje postoje, uključujući nepotpune referentne baze podataka, pogrešnu anotaciju sekvenci i nemogućnost razlikovanja hibrida ili podvrsta upotrebom mtDNK markera. Kako bi se prevazišli ovi izazovi, preporučuje se upotreba većeg broja lokusa i integracija sa jedarnim DNK markerima (STRs, SNPs). Udruženo, ove metode povećavaju dokaznu vrednost, unapređuju preciznost u identifikaciji vrsta i doprinose očuvanju biodiverziteta i zaštiti javnog zdravlja.

Ključne reči: forenzička genetika, mitohondrijska DNK, citohrom b, citohrom c oksidaza podjedinica 1, identifikacija vrsta, krivična dela protiv divljih životinja

1 Introduction

Forensic science, in the broadest sense, refers to the application of knowledge and technologies from various scientific disciplines to resolve legal issues [1]. It draws on advances in chemistry, physics, biology, medicine, psychology, technology, and information science to identify and analyze different types of traces that may be used as evidence in legal proceedings against perpetrators of various crimes. While traditional forensic disciplines focus on human cases, animal forensics is increasingly relevant for detecting and prosecuting crimes such as poaching, illegal wildlife trafficking, animal cruelty, and food/meat fraud. Accurate identification of animal species enables law enforcement to determine whether seized material belongs to a protected or illegally traded species, to uncover fraudulent food products, or to assess risks of zoonotic pathogen transmission. Molecular genetics, particularly mitochondrial DNA (mtDNA) markers such as *cytochrome b* (*cyt b*) and *cytochrome c oxidase subunit I* (*COI*), have become standard tools in forensic species identification. This review focuses on their application, advantages, limitations, and integration with multilocus and nuclear approaches.

2 Animal forensics and crimes against animals

Animal forensics is a specialized branch of forensic science that applies scientific methods to investigate crimes involving animals. This includes criminal acts in which animals are the victims, as well as those in which animals injure or kill a human or dismember human or animal remains, or cases where animal traces (such as blood, hair, feathers, or skin) are found at crime scenes and can be used as evidentiary material.

The most common crimes in which animals are the victims are poaching and the illegal harvesting of protected wildlife species. Illegal hunting of wild animals results in the capture of millions of tons of wildlife, primarily mammals, each year [2]. This problem is particularly associated with the bushmeat trade, which is most prevalent in West and Central Africa and other tropical regions [3]. Although traditionally practiced as a subsistence activity, in recent decades overexploitation has turned this practice into a serious threat to biodiversity. It is generally accepted that illegal hunting and wildlife trafficking have devastating effects on the population sizes of many iconic species, such as tigers, rhinoceroses, and elephants. For example, populations of the tiger (*Panthera tigris)* and the black rhinoceros (*Diceros bicornis)* have declined by approximately 90% and 96%, respectively, over the past few decades [4].

Illegal hunting and trade of protected species, particularly numerous exotic species, are largely driven by the demand for such species as pets. In addition, hunting for the use of animal organs or body parts in traditional medicine targets many mammalian species currently recognized as endangered [5]. Interpol has identified the illegal trade of protected species as the second most widespread form of international crime, following drug trafficking. Excluding domestic black markets, global annual revenues from illegal wildlife trade are estimated to be approximately USD 20 billion [6].

Most frequently trafficked mammals belong to the family Manidae (pangolins). Pangolins are the only mammals covered with keratinized scales and are represented by eight species: three of which are critically endangered (*Manis culionensis*, *Manis pentadactyla*, and *Manis javanica*), three are endangered (*Phataginus tricuspis*, *Manis crassicaudata*, and *Smutsia gigantea*), and two (*Phataginus tetradactyla* and *Smutsia temminckii*) are listed as vulnerable on the IUCN Red List of Threatened Species. Pangolins are primarily threatened by their exploitation in traditional medicine, particularly the use of their scales as unfounded remedies [7].

Members of the families Cervidae and Bovidae are also frequently hunted, as their horns and horn powders are used in traditional medicine. A well-known example is the saiga antelope (*Saiga tatarica*), a pro-

tected migratory ungulate inhabiting Central Asia and southeastern Europe, whose horns are one of the principal ingredients in *Lingyangjiao*, a traditional Chinese remedy [8].

In addition to their use in food and traditional medicine, parts of certain animals are used to produce sculptures, ornaments, and clothing. Ivory is commonly carved into statues, while rhinoceros horn is used for crafting knife and firearm handles [7].

Other crimes in which animals are victims include animal cruelty (intentional abuse and neglect), illegal commercial exploitation (e.g., dog fighting), and sexual abuse. A growing concern also lies in food fraud investigations, cases in which illegal meat (often originating from poaching and wildlife trafficking) is sold as legitimate products at significantly higher prices. Such fraud not only has serious economic implications but also poses major risks to public health due to potential exposure to zoonotic pathogens [9]. Since the 1970s, more than 60% of emerging infectious diseases affecting human populations have been zoonotic in origin, with 71.8% of those events resulting from contact with wildlife species [10]. Certain wildlife groups, particularly bats, non-human primates, ungulates and rodents, are historically recognized as having a higher inherent risk of zoonotic disease transmission. In recent decades, Uganda has reported numerous zoonotic outbreaks resulting from contact with wildlife, including anthrax, Ebola virus, Marburg virus, rabies virus, yellow fever, and HTLV/STLV-1. In northern Uganda, although consumption of bats and primates is not widely accepted culturally, hunters report that these species are often captured and sold as more culturally desirable species such as antelope or warthog, thus increasing the risk of unrecognized exposure to zoonotic pathogens [11].

3 Identification of Animal Species in Forensic Investigations

The first step in solving the aforementioned crimes is the accurate identification of the animal species involved. Traditionally, species identification was based on the comparison of morphological characteristics, which involves analyzing the shape, size, and structure of certain animal body parts. For morphological identification to be reliable, the species must possess distinct morphological traits that clearly differentiate it from others, the examiner must have extensive taxonomic expertise, and most importantly, the diagnostic morphological features must be present in the sample under examination [9].

These requirements illustrate the inherent limitations of morphological identification. Many species have not been examined in a forensic context, and therefore no accurate morphological reference data exist, particularly in the case of cryptic species. Furthermore, in wildlife trafficking cases, the available evidence often consists of small body parts, powders, extracts, or oils, making unambiguous identification impossible using morphology alone [5].

The advent of DNA-based technologies has significantly improved species identification in wildlife forensics by overcoming the above limitations. Microgenomic identification systems, which rely on short segments of the genome, represent a highly efficient approach. These sequences can serve as genetic "barcodes" embedded in every cell [12].

Mitochondrial DNA (mtDNA) markers occupy a central place in the forensic identification of animal species. The mitochondrial genome in eukaryotes contains 37 genes: 22 encoding tRNA molecules, 2 encoding rRNA molecules, and 13 encoding proteins primarily involved in oxidative phosphorylation [7]. For forensic species identification, the most commonly used mitochondrial genes include cyt b, COI, 12S rRNA, and 16S rRNA. However, the use of ribosomal genes (12S rRNA and 16S rRNA) is sometimes limited due to the presence of insertions and deletions (indels), which complicate sequence alignment [12].

The application of mtDNA markers offers several advantages over nuclear DNA markers. First, the presence of several thousand mtDNA copies per cell increases sensitivity, which is critical when dealing with degraded samples or those containing very small amounts of DNA. Second, the nucleotide substitution rate in mtDNA is five to ten times higher than in the nuclear genome, which enhances sequence variability and thereby increases the discriminatory power of microgenomic mtDNA markers. Another major advantage is the use of universal primers, which allow for the amplification and analysis of the same target sequence across a broad range of species, thus simplifying methodological procedures [9]. Specifically, the nucleotide composition of mtDNA markers used in species identification contains conserved regions (with minimal sequence variation) that serve as primer-binding sites, flanking polymorphic regions that provide the informative sequence variation used to differentiate species.

To serve as a reliable marker for species identification, an mtDNA gene fragment should exhibit low intraspecies variability and high interspecies variability [7].

4 Biological Material and Methodological Workflow

The biological material used in wildlife forensic investigations is highly diverse, ranging from whole animals (captured or intentionally killed) to animal skins, skeletal elements, or body parts (e.g., meat, horns, teeth, and internal organs). In other cases, blood, hair, feathers, and other biological traces may be used as evidentiary material [5].

Forensic techniques such as Forensically Informative Nucleotide Sequencing (FINS) have been successfully applied to species-level identification [5]. The standard workflow for species identification generally involves the following steps [7]:

1 DNA extraction

2 DNA quantification

3 PCR amplification of target mtDNA sequences

4 Purification of PCR amplicons

5 DNA sequencing of the amplicon (in both directions)

6 Purification of sequencing products

7 Capillary electrophoresis and data analysis

8 Comparison of the obtained sequence with reference databases such as GenBank

Bioinformatic tools such as the Basic Local Alignment Search Tool (BLAST) compare the target sequence fragment with sequences in the reference database and generate a similarity score for the 100 most closely matched sequences, ranked from highest to lowest similarity. The ideal result is a 100 % match to a known species. However, there is currently no universal consensus regarding the number of nucleotide differences or the sequence length threshold that reliably distinguishes intra from interspecies variation [7].

5 COI and its Forensic Application

The mitochondrially encoded *cytochrome c oxidase subunit 1* (MT-CO1 or COI) gene is a protein-coding region that, according to the revised Cambridge Reference Sequence (rCRS, NC_012920) is located on the heavy strand of human mtDNA, spanning nucleotide positions 5904 to 7445. Cytochrome c oxidase, a large

transmembrane enzyme complex highly conserved across species utilizing oxidative phosphorylation, functions as the terminal electron acceptor in the mitochondrial respiratory chain. [13].

Hebert *et al*. [14] were the first to propose the use of the *COI* sequence as a molecular marker for species identification. Two major features make *COI* particularly suitable for this purpose: the availability of highly robust universal primers, and the high frequency of substitutions at third-codon positions, which results in an evolutionary rate approximately three times greater than that of *12S rDNA* or *16S rDNA*. These characteristics render *COI* sufficiently variable to discriminate not only between closely related species but also among phylogeographic groups within species.

Using a universal primer set designed by Folmer *et al*. [15], Hebert and colleagues amplified a 648 base pair (bp) fragment of the *COI* gene, which served as a standard barcode region. Application of this marker enabled the successful identification of 200 lepidopteran specimens, a taxonomic group characterized with high species diversity [14].

In a subsequent large-scale study, Hebert *et al*. [16] examined *COI* sequence divergence among more than 13320 congeneric pairs spanning 11 phyla, including Annelida, Arthropoda, Chordata, Mollusca, and Nematoda. Their analysis demonstrated that intraspecies variation rarely exceeded 2% (typically <1%), whereas mean interspecies divergence reached 11.3%. These findings support the earlier conclusion that COI provides reliable resolution for routine species-level identification.

Recognizing its utility, the Consortium for the Barcode of Life (CBOL) designated *COI* as the standard marker for animal DNA barcoding [17]. A DNA barcode is defined as a short nucleotide sequence from a standardized genomic region that allows species-level identification. An effective barcode must exhibit sufficiently low intraspecific variability while maintaining high interspecific divergence. Additionally, it should be easily amplified, contain minimal insertions/deletions, and allow for straightforward sequence alignment [13].

The Barcode of Life Data Systems (BOLD) (http://www.boldsystems.org) project was established to catalogue *COI* barcodes for all animal species and provide an openly accessible reference platform. Through BOLD's Identification System (BOLD-IDS), users can compare query sequences against the curated database. Typically, a match is assigned at the species level if sequence divergence from a reference is <1%, and at the genus level if <3% [17]. Unlike other international sequence databases (such as EMBL and GenBank), BOLD has a built-in quality control system. To be included in BOLD, samples must be properly voucherized according to protocols defined by the Global Registry of Biodiversity Repositories (http://grbio.org) and must meet data standards for BARCODE records. Additionally, sample details must include collection date and location with GPS coordinates and the PCR primers used to generate the sequences [5].

Given its universality and discriminatory power, *COI* DNA barcoding has become a key tool in forensic science. Dawnay *et al*. [18] conducted a validation study assessing its application in forensic casework using samples from *Bos taurus* (cow), *Gallus gallus* (chicken), and *Gadus morhua* (cod). They tested reproducibility across variables such as heteroplasmy, mixed DNA sources, concentration, chemical treatment, substrate type, environmental degradation, and thermocycling parameters. Their findings confirmed that *COI* reliably enables accurate species identification, with occasional misclassifications attributable to reference database errors rather than methodological limitations.

The utility of *COI* has also been demonstrated in wildlife forensics. Suresh Kumar *et al*. [19] applied *COI* sequencing to resolve a poaching case involving sambar deer (*Rusa unicolor*). Cooked meat and dried skin samples were analyzed via NCBI-BLAST, revealing >98% identity with reference *Rusa unicolor* sequences (Accession No. DQ989636), thereby confirming species origin.

Vieira de Carvalho [9] reported on the use of *COI* barcoding in 20 forensic case samples analyzed by the Brazilian Federal Police. Tissue samples from domesticated and wild mammals, birds, fish, and reptiles were tested against the BOLD database. Correct species-level identifications were obtained for 12 of 20 samples, while ambiguous results were associated with unresolved taxonomy, hybridization events, or limited database representation.

Collectively, these studies underscore the robustness of the *COI* gene as a molecular marker for species identification in forensic contexts. Its high resolution, broad applicability, and integration into global reference databases make it an indispensable tool in modern forensic science.

6 Cyt b as a Forensic Marker

The *cyt b* encodes the cytochrome b protein, a key component of the mitochondrial electron transport chain. The *cyt b* gene is 1141 bp in length [20] and, in the human mitochondrial genome, is located between nucleotide positions 14747 and 15887 (according to the revised Cambridge Reference Sequence, rCRS, NC_012920). Unlike the *COI* gene, which has a universally defined DNA barcode region, no standardized fragment has been established for *cyt b*. Instead, numerous studies have evaluated *cyt b* fragments of varying lengths as reliable forensic markers for species identification.

Using a universal primer set, Naidu *et al*. [21] successfully sequenced the complete *cyt b* gene in 44 mammalian species, with the goal of expanding the number of reference sequences available in GenBank. Verma *et al*. [22] employed a newly designed universal primer set to amplify a 421 bp *cyt b* fragment in 221 animal species. Hsieh *et al*. [ref 20], using a universal primer set previously developed by Kocher *et al*. [23], amplified a 402 bp fragment of the *cyt b* gene, which was subsequently applied to the identification of 19 endangered animal species in Taiwan. Comparative analysis demonstrated low intraspecies sequence diversity (0.25%–2.74%) and high interspecies diversity (5.97%–64.83%), supporting the utility of this fragment as a reliable forensic marker.

Parson *et al*. [24] applied a universal primer pair to amplify a 358 bp fragment of the *cyt b* gene across 44 vertebrate species. When reference sequences for the examined species were available in databases, species-level identification was achieved with >99% sequence similarity, demonstrating the strong discriminatory capacity of the selected fragment.

Muangkram *et al*. [25] designed a novel universal primer pair that successfully amplified a 154 bp fragment of the *cyt b* gene, providing an effective marker for identifying protected and endangered species in Thailand. Similarly, Lopez-Oceja *et al*. [26] developed a universal primer set for amplifying a short 148 bp *cyt b* fragment. The discriminatory power of this sequence was validated in 63 vertebrate species, and its forensic applicability was further confirmed using 40 degraded samples.

The forensic relevance of *cyt b* has also been demonstrated in casework. Caine *et al*. [27] reported two investigations in which the fragment described by Parson *et al*. [24] was applied to biological evidence recovered from crime scenes. In the first case, bone fragments suspected to belong to an abducted and murdered 8-year-old girl were discovered in a pigsty. Conventional STR profiling yielded no results; however, analysis of a *cyt b* fragment of the remains revealed a 100% match with the reference sequence of *Sus scrofa domestica* (domestic pig). In combination with other investigative findings, this evidence led to the conclusion that the remains had not been consumed by pigs, but rather that the case involved homicide.

In the second case, intestinal remains were discovered in a forest. Morphological examination failed to determine their origin, but *cyt b* analysis followed by BLAST comparison revealed a 100% match with wild

pig (*Sus scrofa)*. This finding excluded the possibility of human origin and confirmed the remains were of animal source.

The *cyt b* sequence was employed in the study by Dell *et al*. [11] to resolve cases of unlawful trade and meat fraud in markets in northern Uganda. A total of 229 bushmeat samples were analyzed using a *cyt b* fragment, and subsequent BLAST searches identified 34 species. The most frequently detected were waterbuck (*Kobus ellipsiprymnus*, 31.5%), warthog (*Phacochoerus africanus*, 13.7%), and black rat (*Rattus rattus*, 5.9%). These findings highlight a significant public health risk for bushmeat consumers in northern Uganda, as they are unable to accurately assess species-related risks when purchasing bushmeat and therefore cannot take appropriate precautions against potential zoonotic pathogen exposure.

7 Evaluating and combining cyt b and COI markers

Among vertebrates, mammals are the only group for which a detailed comparative study has been conducted between *cyt* b and *COI* loci to determine their relative effectiveness in species identification and their propensity to yield false-positive results. In a study by Tobe *et al*. [28], the two loci were evaluated for their ability to reconstruct mammalian phylogeny across multiple taxonomic levels, as well as their accuracy in species assignment and the frequency of false-positive identifications.

Sequence analysis of 217 mammalian species demonstrated that *cyt b* more accurately reconstructed phylogenetic relationships than *COI* when compared with established molecular and morphological evidence at the superorder, order, family, and genus levels. *Cyt b* correctly assigned 95.85% of species to superorder, 94.31% to order, and 98.16% to family, compared with 78.34%, 93.36%, and 96.93%, respectively, for *COI*. Moreover, *cyt b* contained 21.3% more variable nucleotide positions than *COI*, produced less than half the false-positive rate, and had a higher positive predictive value. These results strongly support the use of *cyt b* over *COI* as the standard locus for mammalian phylogeny reconstruction and forensic species identification.

In forensic investigations, the reliability of species identification increases when multiple markers are analyzed. Wilson-Wilde *et al*. [29] therefore recommended that both *COI* and *cyt b* should be used in wildlife crime investigations. There are well-documented examples of their combined use in resolving forensic cases. For instance, Dalton *et al*. [30] reported two cases: in the first, a suspect was arrested for possession of game meat, and samples collected from the seized material and a carcass found at the crime scene were both identified as impala (*Aepyceros melampus*) with 99.5% (*COI*) and 99.7% (*cyt b*) confidence. In the second case, a suspect was found in possession of two carcasses of unknown origin; forensic analysis identified both as eland (*Taurotragus oryx*) with 99.8% (*COI*) and 100% (*cyt b*) confidence.

The power of a multilocus approach was further demonstrated by Gaubert *et al*. [2], who analyzed 302 bushmeat samples from nine mammalian orders and 59 species collected across five African countries (Ghana, Guinea, Nigeria, Cameroon, and Equatorial Guinea). Species identification was based on a multilocus strategy incorporating *COI*, *cyt b*, *12S rRNA*, and *16S rRNA*, amplified with universal primers. Independent amplification of these four mtDNA genes enabled accurate species-level assignment by overcoming taxon-specific gaps in GenBank coverage. Using these data, the authors developed a new reference database, DNABUSHMEAT (http://mbb.univ-montp2.fr/MBB/DNAbushmeat), to provide a framework for DNA typing of African forest bushmeat. Importantly, the project incorporated multiple haplotypes per species, thus offering comprehensive coverage of intraspecies variability. This initiative also contributed to filling significant gaps in the representation of African mammals in international sequence repositories such as NCBI and BOLD.

8 Case Study: Serbia

To date, the only published study on the application of the *cyt b* gene in animal forensics in Serbia is that of Andrejević *et al*. [31]. In this work, the authors designed a novel set of universal primers for the amplification of a short 127 bp fragment of the *cyt b* gene, specifically optimized for forensic identification and species discrimination in mammals and birds. To the best of our knowledge, this represents the shortest *cyt b* fragment reported to date for forensic species identification. The study included 30 animal species (17 mammals and 13 birds), with a particular focus on domestic, wild, and captive species legally protected within the Republic of Serbia, categorized as either protected or strictly protected.

The tested fragment exhibited strong discriminatory power, with intraspecies variability ranging from 0 to 4.72% and interspecies variability from 8.36% to 42.52%. All analyzed samples were successfully identified, and the short sequence demonstrated sufficient resolution to distinguish between closely related species, including American bison (*Bison bison)* vs. cow (*Bos taurus)*, wolf (*Canis lupus)* vs. jackal (*Canis aureus)*, and Goffin's cockatoo *(Cacatua goffini)* vs. Major Mitchell's Cockatoo *(Cacatua leadbeateri)*, among others.

The study also described a notable case of misidentification based on morphology. One submitted specimen was originally classified as Fischer's turaco *(Tauraco fischeri)*. However, sequence comparison of the obtained *cyt b* fragment with reference data in the NCBI database revealed a 100% match with red-crested turaco *(Tauraco erythrolophus)*, while similarity with Fischer's turaco was only 88.98% (14 nucleotide differences). Given the high morphological similarity between the two species, this case underscores the risk of misclassification when relying exclusively on morphology.

In summary, this study not only introduced the shortest validated *cyt b* sequence suitable for forensic species identification, but also reinforced the clear advantage of molecular genetic approaches over morphological methods for achieving accurate species-level identification.

9 Current limitations and future improvements

A major limitation in forensic species identification is the insufficient representation of mtDNA reference of animal species sequences in public databases. Incomplete taxonomic coverage and poorly annotated records often hinder accurate matching, resulting in inconclusive or unreliable identifications. Addressing this gap requires several measures. First, expansion of taxonomic coverage in databases such as GenBank and BOLD through coordinated international efforts and mandatory data deposition from published studies would substantially increase reference diversity. Second, standardization of sequence quality control and metadata annotation (e.g., verified taxonomic identification, collection locality, voucher specimens) would reduce errors and improve reliability. Finally, complementary approaches, including complete mitochondrial genome sequencing and integration with nuclear DNA markers, may provide higher resolution for species-level discrimination.

A further drawback of mtDNA lies in its maternal inheritance. In cases where a male from a protected species mates with a female from a non-protected species, viable offspring will not be genetically recognized as belonging to the protected lineage, since mtDNA is exclusively maternally inherited. This issue was highlighted during the 2019 International Society of Forensic Genetics (ISFG) congress in Prague. While many examples of such hybridization are either geographically implausible (e.g., African lion (*Panthera leo)* and Indian tiger (*Panthera tigris tigris)*) or of limited forensic relevance (e.g., horse (*Equus caballus)* and donkey (*Equus asinus)*), hybridization may represent a genuine forensic challenge in certain contexts. For example, in South America, wild camelids such as vicuñas (*Vicugna vicugna*) and guanacos (*Lama guanicoe*) coexist

with domesticated llamas (*Lama glama*) and alpacas (*Vicugna pacos*). These taxa interbreed freely, producing fertile offspring. In Chile, hunting of guanaco and llama is illegal, but hybrids are not legally protected, creating opportunities to sell meat from protected species as though it originated from legally obtained animals. In one case, Chilean investigators resolved this problem by combining *cyt b* sequence data with a diagnostic polymorphism in the *melanocortin 1 receptor* (*MC1R*) gene associated with coat color. The integration of mtDNA barcoding with nuclear markers enabled both species identification and hybrid detection [7].

Another well-recognized limitation of mtDNA analysis is its inability to reliably distinguish subspecies, such as domestic pig (*Sus scrofa domestica*) from wild boar (*Sus scrofa*), or domestic dog (*Canis lupus familiaris*) from wolf (*Canis lupus*). Domestication of wolves is estimated to have occurred approximately 15000–30000 years ago [32], while pig domestication is thought to have begun around 10000 years ago [33]. From an evolutionary perspective, these time intervals are relatively short, and despite the relatively high mutation rate of mtDNA, insufficient nucleotide substitutions have accumulated within commonly analyzed mitochondrial genes to permit unequivocal subspecies discrimination.

Integration of nuclear DNA markers, particularly short tandem repeats (STRs), provides a solution to this limitation. STR loci are highly polymorphic, offering strong discriminatory power, and their application in forensic analyses allows individual identification with defined statistical confidence. For instance, Lorenzini *et al*. [34] optimized and validated a multiplex system targeting 22 canine STR loci, enabling reliable discrimination between pure wolves and dogs. When coupled with Bayesian assignment methods, this approach also allowed statistical classification of admixed individuals into hybrid categories with different levels of dog ancestry. Similarly, Lorenzini *et al*. [35] developed a porcine-specific STR multiplex in combination with real-time PCR assays of polymorphisms in the *NR6A1* and *MC1R* genes. This combined system demonstrated high diagnostic power for distinguishing wild boar, domestic pig, and their hybrids, thereby providing a valuable tool for forensic investigations.

10 Conclusion

Mitochondrial DNA markers, particularly *cyt b* and *COI*, remain central to forensic animal species identification. While *COI* has achieved global standardization through the BOLD database, *cyt b* demonstrates superior discriminatory power in mammals. Multilocus approaches, supported by well-curated databases, offer improved reliability and are increasingly recommended in forensic casework. Integration of nuclear markers further resolves subspecies and hybridization challenges, thereby strengthening evidentiary value. Forensic genetics is indispensable for combating wildlife crime, protecting biodiversity, and mitigating public health risks associated with illegal wildlife trade and meat fraud. Continued expansion of reference databases, validation of multilocus assays, and incorporation of nuclear DNA tools represent key directions for future development.

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Analysis of Variants in Cytochrome P450 Superfamily Genes as Predictive Markers for Neoadjuvant Chemoradiotherapy in Rectal Cancer

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Abstract

Colorectal cancer (CRC) is the third most common malignancy worldwide, with incidence projected to reach 2.2 million cases by 2030. While surgical resection remains the gold standard, patients with locally advanced rectal cancer (LARC) often undergo neoadjuvant chemoradiotherapy (nCRT) to reduce tumor size and improve resectability. However, therapeutic response varies, highlighting the need for predictive biomarkers. In recent study, two genetic variants with potential clinical relevance were identified. The rs149012039 variant, located in the *CYP2D7* pseudogene, represents a frameshift mutation that may enable the formation of a functional transcript. This could enhance metabolic capacity and indirectly improve drug processing and thereby a better response to 5-FU therapy. The rs3093200 variant, found in *CYP4F2* gene, is predicted to impair protein function, leading to increased vitamin K levels. Given vitamin K's radiosensitizing and synergistic effects with 5-FU, this variant may also contribute to improved therapeutic outcomes.

Keywords: rectal cancer, genetic polymorphisms, Cytochrome P450, Neoadjuvant chemoradioterapy

Analiza varijanti u genima superfamilije Citohrom P450 kao prediktivnih markera za neodjuvantnu hemioradioterapiju u karcinomu rektuma

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Apstrakt

Kolorektalni karcinom (CRC) predstavlja treći najčešći malignitet u svetu, a očekuje se da će do 2030. godine broj obolelih dostići 2,2 miliona. Iako je hirurško uklanjanje tumora zlatni standard terapije, pacijenti sa lokalno uznapredovalim karcinomom rektuma (eng. locally advanced rectal cancer - LARC) često se podvrgavaju neoadjuvantnoj hemoradioterapiji (eng. Neoadjuvant chemoradiotherapy - nCRT) radi smanjenja veličine tumora i njegovog lakšeg uklanjanja. Međutim, odgovor na terapiju značajno varira kod različitih pacijenata, što ukazuje na potrebu za prediktivnim biomarkerima. U nedavnoj studiji identifikovane su dve genetičke varijante sa potencijalnim kliničkim značajem. Varijanta rs149012039, locirana u *CYP2D7* pseudogenu, predstavlja mutaciju koja menja okvir čitanja i potencijalno može omogućiti formiranje funkcionalnog transkripta. Ovakav mehanizam bi mogao povećati metabolički kapacitet i indirektno poboljšati metabolizam leka, i samim tim bolji odgovor na terapiju 5-FU. Varijanta rs3093200 u genu *CYP4F2* verovatno remeti funkciju proteina, dovodeći do povišenih nivoa vitamina K. S obzirom na to da vitamin K povećava osetljivost na radioterapiju i deluje sinergistički sa 5-FU, ovaj polimorfizam takođe može doprineti boljem odgovoru na terapiju.

Ključne reči:
karcinom rektuma, genetički polimorfizmi, citohrom P450, neoadjuvantna hemioradioterapija

According to data from the International Agency for Research on Cancer (IARC), colorectal cancer is the third most common malignancy worldwide [1]. It is estimated that by 2030 the number of diagnosed cases will reach 2.2 million, representing an increase of approximately 20% [2]. The most significant risk factor for the development of this disease is age, as it is most frequently diagnosed in individuals over 50 years of age. However, due to lifestyle changes in modern society, colorectal cancer is increasingly being detected among younger populations. Additional risk factors include obesity, diets rich in red meat prepared at high temperatures, smoking, alcohol consumption, sedentary lifestyle, and reduced physical activity [3]. The treatment of colorectal cancer relies on different approaches selected according to the tumor characteristics (localization and number of metastases, degree of regression, presence of carcinoembryonic antigen – CEA) as well as the patient's overall condition (liver and kidney function, levels of alkaline phosphatase, bilirubin, albumin, and other parameters) [4]. Surgical removal of the tumor remains the gold standard of therapy. However, in patients with locally advanced rectal cancer (LARC), where tumor size prevents resection, neoadjuvant chemoradiotherapy (nCRT) is applied. This treatment aims to reduce tumor mass to an operable size and is most commonly based on the administration of fluoropyrimidines in combination with leucovorin [5, 6].

In the context of personalized medicine, increasing attention has been directed toward the study of polymorphisms in the cytochrome P450 (CYP) gene superfamily [7]. This gene family represents one of the largest in the human genome, and the enzymes it encodes are involved in about 90% of all enzymatic reactions, including the metabolism of numerous therapeutic agents [8, 9]. For this reason, CYP genes have become the focus of research as potential predictive biomarkers of therapeutic response.

In recent study, two genetic variants were highlighted as having potential to serve as predictive biomarkers for response to chemoradiotherapy: rs149012039 located within the *CYP2D7* pseudogene and classified as a frameshift variant, and rs3093200, located in the first exon of the *CYP4F2* gene and predicted to have a damaging effect on the protein [10].

The rs149012039 variant is located in the *CYP2D7* pseudogene, derived from *CYP2D6*, which metabolizes many therapeutic agents [11]. The *CYP2D6* locus is highly complex and polymorphic, making genotyping difficult with short-read sequencing; long-read methods are being developed to address this [12]. Normally, *CYP2D7* is inactive due to a T insertion in exon 1 [11], but rs149012039 (c.632dup) introduces a frameshift that may create an open reading frame, potentially enabling transcript formation. If functional, carriers could exhibit accelerated metabolism, indirectly improving drug processing and therapeutic response.

The rs3093200 variant is located in the *CYP4F2* gene and results in a nonsynonymous amino acid substitution (Leu519Met), predicted by bioinformatics tools to be damaging. The *CYP4F2* enzyme is involved in vitamin K catabolism, catalyzing its oxidation and facilitating its elimination through urine or bile [13]. A damaging variant could reduce this catabolic activity, thereby increasing vitamin K levels in the organism. Vitamin K has antitumor effects through modulation of signaling, reduced inflammation, ROS generation, and oncogene suppression [14]. It also acts as a radiosensitizer and shows synergy with 5-FU [15, 16]. Thus, carriers of the rs3093200 variant, with higher vitamin K levels, may exhibit improved response to 5-FU–based therapy.

Studies have demonstrated that CYP enzymes are involved not only in carcinogenesis but also in the activation and inactivation of anticancer drugs [17]. Furthermore, research is ongoing into their role in therapy resistance, the potential of targeting CYP enzymes as an innovative therapeutic approach, and

their application as predictive and prognostic biomarkers [17]. These findings indicate that understanding CYP gene variability could significantly improve treatment optimization and advance the field of personalized medicine.

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Štampa
Curent print, Beograd

Periodičnost izlaženja publikacije
Godišnje

Tiraž
20 primeraka

Autori

CIP - Каталогизација у публикацији
Народна библиотека Србије, Београд

577.2

TRENDOVI u molekularnoj biologiji = Trends in Molecular Biology. - 2021, br. 1 (sep.)- . - Beograd : Institut za molekularnu genetiku i genetičko inženjerstvo, 2021- (Beograd : Curent Print). - 28 cm

Godišnje. - Tekst na srp. i engl. jeziku.
ISSN 2787-2947 = Trendovi u molekularnoj biologiji
COBISS.SR-ID 45105929