THE BIOTECHNOLOGICAL PALIMPSEST:

From Evolutionary Scratches to the Synthetic Future

Department of Biotechnology
School of Life Sciences
Swami Vivekananda University

ISBN: 978-93-5890-470-3

The Biotechnological Palimpsest:

From Evolutionary Scratches to the Synthetic Future

ISBN Number: 978-93-5890-470-3

(Peer Reviewed)

Editor

Samudra Pal, Ph.D.

Department of Biotechnology
School of Life Sciences

Swami Vivekananda University

2026

Published by: Swami Vivekananda University

Imprint: Samudra Pal, Ph.D.

Publication Year: 2026

ISBN: 978-93-5890-470-3

Editor: Samudra Pal, Ph.D.
Department of Biotechnology,
School of Life Sciences,
Swami Vivekananda University,
Telinipara, Barasat–Barrackpore Road, Bara Kanthalia,Barrackpore, Kolkata – 700121,
West Bengal, India
Email: samudrageneticscu@gmail.com

Declaration by the Editor

I hereby declare that the book titled **"The Biotechnological Palimpsest: From Evolutionary Scratches to the Synthetic Future"** has been edited under my supervision. I have carefully reviewed the manuscript for clarity, coherence, structure, and language, and ensured that it meets the required academic and publishing standards.

To the best of my knowledge, the content of this book is original and has not been published elsewhere in the same form. All sources used by the contributing authors have been duly acknowledged, and any necessary permissions have been obtained where applicable.

I confirm that this book is suitable for publication and dissemination.

Samudra Pal, Ph.D.

Acknowledgements

First and foremost, I would like to express my sincere gratitude to all the contributing authors of this volume for their scholarly dedication, intellectual rigour, and timely cooperation. Each contribution has enriched the breadth and depth of this work, and this book would not have been possible without their commitment.

I gratefully acknowledge the institutional support of Swami Vivekananda University, School of Life Sciences, Barrackpore, and the Department of Biotechnology, which provided the academic environment and encouragement necessary to undertake this editorial project.

I also wish to acknowledge the members of the peer-review panel, many of whom provided their expert assessments at very short notice. Their rigorous evaluations have considerably strengthened the scientific quality of the articles contained herein.

Special recognition is due to my colleagues and mentors in the fields of Human Genetics, Genomics, Biotechnology, and Microbiology, whose stimulating discussions and collaborative spirit have continuously inspired my academic pursuits.

I further acknowledge the European Commission's recognition of the GLIDE research proposal through the Marie Curie Seal of Excellence 2025 - an honour that reinforces the translational vision underlying this volume.

Finally, I extend my deepest appreciation to all colleagues, students, and well-wishers whose encouragement, patience, and moral support have sustained this endeavour from conception to completion.

Barrackpore, Kolkata

April 2026

Samudra Pal, Ph.D.

Preface

The history of biotechnology is not a single, uninterrupted line of progress. It is, rather, a palimpsest - a layered manuscript in which older texts of evolutionary tinkering, pre-modern fermentation craft, and indigenous biological knowledge are never fully erased, but persist beneath the more recent inscriptions of molecular engineering, genomics, and synthetic biology. Each generation of biotechnologists writes its innovations on a surface already marked by what came before.

This edited volume, The Biotechnological Palimpsest: From Evolutionary Scratches to the Synthetic Future, has been curated with this layered vision in mind. The articles assembled here traverse the full arc of biotechnological thought and practice — from the evolutionary pressures that first shaped life's molecular machinery, through the classical and molecular revolutions that taught us to read and rewrite genomes, to the emerging synthetic and computational paradigms that now allow us to design biological systems from first principles.

As an editor, my objective has been to assemble contributions that are not merely descriptive surveys, but critical engagements with the field; articles that question established assumptions, highlight emerging methodologies, and identify the conceptual seams where disciplines converge or diverge. Contributors have been invited to situate their specialist knowledge within this broader palimpsestic framework, acknowledging the historical scripts that underlie their current research inscriptions.

The volume spans the molecular and cellular mechanisms that govern biological complexity, the biotechnological innovations transforming diagnostics and therapeutics, the genomic and epigenomic architectures being revealed by next-generation sequencing, and the synthetic and computational horizons that define biotechnology's immediate future. Throughout, the chapters collectively ask: what has been retained from the evolutionary past, what has been deliberately overwritten, and what new text are we now inscribing?

This book is intended to serve as a scholarly resource for postgraduate students, researchers, and academic professionals working at the intersection of life sciences, biomedical research, and translational biotechnology. It is also offered as an invitation to read biological history as a living document, always partially visible beneath the innovations of the present.

Furthermore, the volume pays particular attention to the ethical and socio-political dimensions that act as the binding for this metaphorical manuscript. As we move from the haphazard edits of selective breeding to the precision of CRISPR-mediated gene drives, the "ink" we use becomes increasingly permanent, carrying implications that extend far beyond the laboratory bench. The contributors explore how indigenous knowledge systems and traditional ecological practices are not merely historical footnotes, but essential marginalia that provide critical context for modern sustainability. By examining the governance of emerging technologies and the democratization of biomanufacturing, these chapters challenge the reader to consider who holds the pen in this ongoing process of biological inscription and whose stories are being prioritized in the synthetic age.

Ultimately, *The Biotechnological Palimpsest* seeks to foster a holistic literacy that is often lost in the hyper-specialization of contemporary science. By bridging the gap between the "evolutionary scratches" of deep time and the high-resolution "computational scripts" of the future, this collection encourages a synthesis of perspective. It argues that to truly innovate in fields like regenerative medicine or metabolic engineering, one must be able to decipher the underlying layers of biological logic that have been refined over eons. As you navigate these pages, we hope you find that the future of biotechnology is not found in a blank slate, but in the sophisticated, iterative, and deeply storied complexity of life itself.

Barrackpore, Kolkata
April 2026

Samudra Pal, Ph.D.

About the Editor

Samudra Pal, Ph.D.

Editor, The Biotechnological Palimpsest

Dr. Samudra Pal is an Assistant Professor of Biotechnology at Swami Vivekananda University, Barrackpore, Kolkata, specialising in Human Genetics and Genomics. He completed his Ph.D. in Human Genetics (2018 – 2024) at the University of Calcutta, under the supervision of Prof. Sujay Ghosh (Cytogenetics and Genomics Research Unit, Department of Zoology), with a thesis focused on the molecular and genetic underpinnings of male infertility, including the identification of rare and novel genetic variants associated with azoospermia and spermatogenic failure in Indian men.

Dr. Pal's broader research vision is oriented toward precision diagnostics and biomarker discovery in reproductive medicine, with a long-term goal of translating genetic insights into clinically actionable tools for the diagnosis and management of male infertility. His current work also encompasses the cytogenomics of Down syndrome, the molecular mechanisms underlying immune-mediated neuronal damage, and therapeutic bioactive compounds.

He has published 10 peer-reviewed articles in internationally indexed journals (Web of Science), accumulating 50+ citations and an H-index of 4. His publications appear in journals including Journal of Assisted Reproduction and Genetics, Reproductive Sciences, PLOS ONE, Molecular Biology Reports, and the Journal of Human Reproductive Sciences, among others.

Dr. Pal is a recipient of several prestigious national fellowships and awards, including the DST–INSPIRE Fellowship (2013), CSIR-UGC NET JRF (2017, Rank 79), the K.C. Ghose Memorial Award for highest marks in M.Sc. Zoology (University of Calcutta, 2018), the Sudev Bhusan Ghosh Young Scientist Award (2022), and the 1st Prize for Oral Presentation at the ACE 2021 Annual Conference of the Academy of Clinical Embryologists, Kolkata.

In 2025, Dr. Pal was honoured with the **Marie Curie Seal of Excellence 2025** by the European Commission, awarded to his MSCA Postdoctoral Fellowship proposal

(Project GLIDE) submitted through Sapienza University of Rome. This award recognises proposals of the highest scientific merit that could not be funded solely due to budgetary constraints, and certifies Dr. Pal's research as meeting the rigorous standards of the Marie Skłodowska-Curie Actions Programme.

Dr. Pal's technical expertise encompasses Next-Generation Sequencing (NGS), Sanger sequencing, PCR and RT-PCR, molecular docking and computational biology, genetic epidemiology, and bioinformatics pipeline development. He has served as a peer reviewer for internationally reputed journals including PLOS ONE and Basic and Clinical Andrology.

He has presented his research at multiple national and international conferences, including the International Conference on Down Syndrome Research (University of Calcutta, 2020), YUVA ISAR 2021, and Zoospectra 2022, where he received the award for 2nd Best Oral Presenter.

Driven by a strong commitment to scientific innovation and translational research, Dr. Samudra Pal continues to bridge fundamental genetics with clinical applications in human health and disease. Through his interdisciplinary approach, international collaborations, and dedication to academic excellence, he aspires to contribute significantly to the advancement of computational biology, precision medicine, reproductive genetics, and genomic research on Alzheimer's Biology. As an emerging scientist and educator, Dr. Pal remains committed to mentoring future researchers and developing impactful scientific solutions that address complex biomedical challenges both in India and globally.

Introduction of Contributing Authors

Dr. Semanti Ghosh

Dr. Semanti Ghosh is an Assistant Professor in the Department of Biotechnology, School of Life Sciences, Swami Vivekananda University, Barrackpore, Kolkata. She completed her Ph.D. in Biochemistry in 2018 from the University of Kalyani in the field of Computational Biology, followed by a prestigious DBT Research Associateship at the Crystallography & Molecular Biology Division, Saha Institute of Nuclear Physics, Kolkata (July 2018 – June 2021). Her primary research areas include Structural Biology & Bioinformatics, protein structure-function relationships under abiotic stress, computer-aided drug discovery against Dengue virus, and molecular regulation of neurodegenerative diseases and cancer biology. Dr. Ghosh has authored 20+ research articles in SCI international journals and contributed to 50+ book chapters. She currently supervises 3 active Ph.D. scholars, with 2 students awarded in 2025 from the Department of Biotechnology, Swami Vivekananda University.

Dr. Pritha Pal

Dr. Pritha Pal is an Assistant Professor and Head, Department of Microbiology, School of Life Sciences, Swami Vivekananda University, Barrackpore, Kolkata. Holding a Ph.D. and with teaching and research experience since 2020, her research interests encompass cancer biology, environmental toxicology, metal remediation, and microbial biotechnology. She has authored numerous scientific publications and contributed significantly to studies on microbial impacts and metal resistance mechanisms.

Dr. Priyankar Pal

Dr. Priyankar Pal is a reproductive toxicologist with over six years of research experience using rodent models, focusing on environmentally induced male reproductive disorders and their amelioration through nutritional antioxidants. He earned his Ph.D. in 2024 with a thesis on fluoride-induced reproductive toxicity and the protective roles of vitamins C and E. His research expertise includes animal handling, histology, histomorphometry, biochemical assays, and molecular techniques such as PCR, ELISA, FACS, and immunofluorescence. Dr. Pal has authored more than eight peer-reviewed research articles and contributed extensively to book chapters. He currently serves as an Assistant Professor of Biotechnology at Swami Vivekananda

University, with research interests spanning reproductive toxicology, oxidative stress, immunotoxicology, and molecular mechanisms of environmental toxicants.

Dr. Palash Kumar Pal

Dr. Palash Kumar Pal is an Assistant Professor in the Department of Biotechnology, Swami Vivekananda University, Barrackpore. He obtained his Ph.D. in Zoology from Visva-Bharati in 2016, specialising in gastrointestinal physiology and melatonin biology in fish, and holds more than 5 years of postdoctoral research experience in neuroinflammation, toxicology, and chronobiology. Dr. Pal has published 31 international papers and is a recipient of the DST-INSPIRE Fellowship, UGC-Dr. D. S. Kothari Postdoctoral Fellowship, SERB-Research Associateship, and awards from ICAR-NASF, DBT (Govt. of India), and the North American Society for Comparative Endocrinology (NASCE, Canada).

Dr. Debjit De

Dr. Debjit De is an Assistant Professor in the Department of Biotechnology, Swami Vivekananda University. He obtained his Ph.D. in Computational Biology and Applied Microbiology from Jadavpur University, with research focused on gut microbiota in Indian Type 2 diabetic patients. His expertise spans microbial metagenomics, host–microbe interactions, machine learning applications in human disease, and NGS data analysis. Previously, he worked as a Senior Research Data Analyst at the National Institute of Biomedical Genomics. Dr. De has authored multiple peer-reviewed publications, book chapters, and a published patent, and actively mentors students while organising advanced bioinformatics training programmes.

Dr. Bidisha Ghosh

Dr. Bidisha Ghosh is an Assistant Professor in the Department of Biotechnology, School of Life Sciences, Swami Vivekananda University, Barrackpore. She completed her Ph.D. in Biophysics from Jadavpur University in 2024. Her research focuses on nanomaterials synthesis and their potential in wastewater management and biological applications. Dr. Ghosh has contributed to 37 peer-reviewed journals, 48 book chapters, and holds one filed National Patent. She has served as CO-PI in the ICSSR Vision Viksit Bharat 2047 Collaborative Research Project (funded by the Govt. of India) and is a member of the Society of Conservation Biology.

Dr. Debaprasad Koner

Dr. Debaprasad Koner earned his Ph.D. in Zoology from North-Eastern Hill University, Shillong, focusing on the protective role of nitric oxide during stress responses. He served as Research Associate-II in a SERB-funded project on hypoxia adaptation. His expertise includes NGS, RT-PCR, Western blotting, immunocytochemistry, HPLC, and primary hepatocyte culture, with research commitment to stress physiology and molecular toxicology.

Dr. Priyanka Chakraborty

Dr. Priyanka Chakraborty is an Assistant Professor in the Department of Microbiology, School of Life Sciences, Swami Vivekananda University, Barrackpore. She completed her Ph.D. in Life Science from Jadavpur University. Her research focuses on environmental microbiology, vermitechnology, and sustainable bioremediation, with particular emphasis on detoxification and valorisation of chromium-contaminated tannery sludge for safe agricultural applications. She is actively involved in DBT-funded research projects, with interdisciplinary work integrating microbiology, nanotechnology, and soil science.

Dr. Aishwarya Guha

Dr. Aishwarya Guha is a cancer immunology researcher serving as Assistant Professor in the Department of Biotechnology, Swami Vivekananda University, Barrackpore. She completed her Ph.D. in Cancer Immunology from Jadavpur University, with research focused on how tumor-educated platelets promote breast cancer progression by interacting with cancer stem cells. Her research interests include the tumor microenvironment, cancer stem cells, EMT, angiogenesis, and translational oncology. Dr. Guha has published in Breast Cancer Research, Frontiers in Immunology, and Molecular Cancer Research, and is editor of the forthcoming Springer Nature book on Platelet-Driven Oncogenesis.

Dr. Suranjana Sarkar

Dr. Suranjana Sarkar holds a Bachelor's, Master's, and Ph.D. in Microbiology. Her doctoral research centred on Drug Discovery and Rational Drug Design. At Swami Vivekananda University, she teaches UG and PG courses in Biotechnology and Microbiology, covering General Microbiology, Microbial Genetics, Biochemistry, and Medical Coding & Scribing. Her research interests include microbial pathogenesis,

antimicrobial strategies, combinational antimicrobial systems, and ML-assisted quantum chemical computational drug designing.

Dr. Sibashish Baksi

Dr. Sibashish Baksi is an Assistant Professor at Swami Vivekananda University with expertise in biomass valorisation and sustainable bioprocess development. He completed his Ph.D. in Chemical Engineering at Jadavpur University, specialising in biomass pretreatment and enzymatic hydrolysis, and also serves as a Postdoctoral Researcher at the University of Milan-Bicocca, contributing to the Agro2Circular project focused on waste upcycling and circular bioeconomy strategies. His research centres on microbial fermentation, metabolic engineering, and process optimisation for value-added bioproducts.

Mr. Rupesh Dutta Banik

Mr. Rupesh Dutta Banik is a faculty member in the Department of Microbiology, School of Life Sciences, Swami Vivekananda University, specialising in Environmental Microbiology. His research focuses on microbial community profiling, heavy metal analysis in wastewater, and microbial bioremediation. He has authored and co-authored more than ten peer-reviewed journal articles and over twenty book chapters published by Taylor & Francis and Springer Nature, and is a contributing member of the American Society for Microbiology (ASM).

Dr. Subhasis Sarkar

Dr. Subhasis Sarkar is an Assistant Professor in Microbiology, Swami Vivekananda University, having completed his Ph.D. in Biotechnology from the University of Calcutta in 2014. Over his post-doctoral journey of 12 years, he has guided UG, PG, and Ph.D. students across relevant domains of Microbiology and Biotechnology. He has contributed to 46 book chapters, 51 peer-reviewed journal articles, and 1 national patent. His Google Scholar record reflects 2108 citations, an H-index of 12, and an i-10 index of 13. He serves as a reviewer for journals including 3-Biotech and Frontiers in Pharmacology, and is a member of the American Society for Microbiology and the Society for Conservation Biology (USA).

Dr. Priyajit Banerjee

Dr. Priyajit Banerjee is an Assistant Professor in the Department of Biotechnology, School of Life Sciences, Swami Vivekananda University. His doctoral research at the UNESCO–Regional Centre for Biotechnology focused on the structural and functional

characterisation of bacterial regulatory proteins, yielding multiple Protein Data Bank depositions. He is a recipient of the David Blow Studentship Bursary, the Carl Storm International Diversity Fellowship, and the DST-SERB National Postdoctoral Fellowship. His laboratory currently focuses on Drug Discovery, Protein Engineering, and Mechanistic Toxicological Assessment, with publications in Science Advances, Molecular Aspects of Medicine, EBioMedicine, and Frontiers in Immunology.

Faculty Members of School of Life Sciences

Dr. Pritha Pal

Dr. Priyankar Pal

Dr. Palash Kumar Pal

Dr. Debjit De

Dr. Semanti Ghosh

Dr. Priyajit Banerjee

Mr. Rupesh Dutta Banik

Dr. Sibashish Baksi

Dr. Priyanka Chakraborty

Dr. Aishwarya Guha

Dr. Subhasis Sarkar

Dr. Suranjana Sarkar

Dr. Samudra Pal

Dr. Debaprasad Koner

Dr. Bidisha Ghosh

A B O U T T H E D E P A R T M E N T

School of Life Sciences • Biotechnology

The Department of Biotechnology at Swami Vivekananda University, West Bengal, is a dynamic and forward-looking academic unit committed to fostering excellence in life sciences education and research. Established within the School of Life Sciences, the department offers undergraduate, postgraduate, and doctoral programmes that blend rigorous theoretical foundations with hands-on laboratory training, equipping students with the competencies demanded by modern biotechnology, biomedical research, and allied industries. The faculty comprises experienced academicians and active researchers working across diverse domains including molecular biology, microbial biotechnology, genetic engineering, biochemistry, and bioinformatics. The department is equipped with well-resourced laboratory facilities that support both teaching and investigative research, encouraging students to engage with contemporary scientific problems from an early stage of their academic journey. Guided by the vision of Swami Vivekananda - that knowledge must serve humanity - the department places special emphasis on applied and translational research, with growing contributions to areas such as environmental biotechnology, pharmaceutical biology, and disease diagnostics. Through collaborations, seminars, and participation in national and international forums, the Department of Biotechnology at Swami Vivekananda University continues to build a culture of inquiry, innovation, and scientific responsibility, nurturing the next generation of biotechnologists who are both technically proficient and socially conscious.

Table of Contents

14	Environmental Stress and the Dynamics of Operon Switching in Bacteria: Mechanisms, Models, and Adaptive Significance	Sibashish Baksi	235
15	From Genotype to Metabolic Phenotype: A Multi-Omics Protocol for Systems-Level Analysis of Down Syndrome	Ankana Ghosh, Anushree Guchhait, Misha Sarkar, Debabrata Paria, Saswata Roy, Trisha Dutta Banik, Debjit De, Priyankar Pal, Rupesh Dutta Banik, Palash Kumar Pal, Debaprasad Koner, Samudra Pal	252

Chapter 1

The shift from clotting to cancer: The role of platelets

Aishwarya Guha[1]*

[1]*Department of Biotechnology, Swami Vivekananda University, Barrackpore, West Bengal- 700121, India*

**Corresponding email: aishbrabourne@gmail.com*

Abstract

Platelets, long recognized for their indispensable role in hemostasis and thrombosis, have emerged as critical participants in tumor biology. This chapter explores the multifaceted transition of platelet function from guardians of vascular integrity to active facilitators of cancer initiation, progression and metastasis. We examine how platelets interact with circulating tumor cells to shield them from immune surveillance, promote epithelial-to-mesenchymal transition and establish pre-metastatic niches. Key molecular mediators including TGF-β, PDGF, VEGF and platelet-derived microparticles are discussed in the context of tumor angiogenesis and cancer cell survival. Furthermore, the chapter addresses how the tumor microenvironment reciprocally activates platelets, creating a feed-forward loop that sustains malignant progression. Clinical implications, including antiplatelet strategies as adjunct oncological therapies and platelets as liquid biopsy biomarkers, are evaluated critically. Understanding the dual identity of platelets offers novel therapeutic windows and diagnostic opportunities in modern oncology.

Keywords: Platelets; Cancer; Tumor metastasis; Thrombosis; Antiplatelet therapy

1. Introduction

Platelets are small, anucleate cytoplasmic fragments derived from megakaryocytes in the bone marrow. For much of modern medicine's history, their significance was confined to their well-characterized role in primary hemostasis the rapid aggregation and plug formation that prevents blood loss following vascular injury. Yet over the past two decades, a compelling body of evidence has

reframed platelets as multifunctional effectors whose influence extends far beyond coagulation cascades and wound healing [Gasic G.J. et al, 1968].

The observation that many cancer patients exhibit elevated platelet counts a condition termed paraneoplastic thrombocytosis and that thrombotic complications are among the leading causes of mortality in oncology patients, prompted investigators to examine whether platelets might be more than bystanders in malignant disease. What emerged from this inquiry was a paradigm-shifting understanding: platelets actively participate in virtually every stage of cancer biology, from the initial neoplastic transformation and angiogenesis, to invasion, systemic dissemination, and establishment of distant metastases.

This chapter traces the conceptual and mechanistic journey from platelet physiology to platelet oncology. Beginning with a review of classical platelet biology, we then systematically examine how tumor cells co-opt platelet functions, how platelets reciprocally reprogram the tumor microenvironment, and how this bidirectional crosstalk accelerates cancer progression. We conclude by evaluating the emerging clinical implications both therapeutic and diagnostic that arise from this reframing of platelet identity.

The significance of this topic transcends academic interest. As cancer remains the second leading cause of death globally, and as resistance to conventional therapies continues to pose formidable clinical challenges, the platelet-cancer axis represents an underexplored but increasingly tractable frontier for intervention. Understanding how the body's own hemostatic machinery is subverted to serve malignancy may open novel avenues for drug development, biomarker discovery, and personalized oncological care.

2. Classical Platelet Biology: A Brief Review

2.1 Origin and Structure

Platelets originate from megakaryocytes large polyploid cells residing in the bone marrow through a process called thrombopoiesis. Megakaryocytes extend long cytoplasmic protrusions (proplatelets) into bone marrow sinusoids, from which platelets are shed into the circulation. The human body maintains approximately 150,000–400,000 platelets per microliter of blood, each with a lifespan of 7–10 days before clearance by splenic and hepatic macrophages [Italiano JE Jr. et.al, 2008]

Despite their anucleate status, platelets contain a sophisticated internal architecture. Their cytoplasm is organized around a circumferential microtubule coil that maintains discoid shape, two types of secretory granules (alpha granules and dense granules), lysosomes, mitochondria, and a rich network of open canalicular and dense tubular systems. Alpha granules harbor over 300 distinct proteins, including fibrinogen, von Willebrand factor (vWF), platelet factor 4 (PF4), P-selectin, and numerous growth factors such as VEGF, PDGF, and TGF-β. Dense granules contain small molecules including ADP, ATP, serotonin, and calcium ions key mediators of platelet activation and aggregation.

2.2 Hemostasis: The Classical Paradigm

Upon vascular injury, the subendothelial matrix particularly collagen and Vwf is exposed to circulating blood. Platelets adhere to these surfaces via glycoprotein receptors: GPIb-IX-V binds vWF, while GPVI and integrin α2β1 bind collagen. This adhesion triggers a series of intracellular signaling cascades involving phospholipase C, protein kinase C, and calcium mobilization, culminating in platelet activation.

Activated platelets undergo a dramatic shape change from discoid to spherical with pseudopod extensions, and release the contents of their granules a process called degranulation. Released ADP and thromboxane A2 (TXA2) recruit additional platelets to the site of injury (secondary hemostasis), while surface expression of phosphatidylserine provides a scaffold for the coagulation cascade. The end product a stable fibrin-reinforced platelet plug is the definitive hemostatic response.

This tightly regulated process is kept in check under normal physiology by endothelial-derived inhibitors including prostacyclin (PGI2), nitric oxide (NO), and CD39/CD73 ectonucleotidases that degrade ADP. The balance between activating and inhibitory signals ensures hemostasis is spatially and temporally restricted to sites of genuine vascular injury [Labelle M. et.al, 2011]

2.3 Beyond Hemostasis: Emerging Physiological Roles

Even before their oncological roles were appreciated, platelets were recognized to participate in inflammation, angiogenesis, and wound healing. They release cytokines that recruit neutrophils and monocytes to injury sites, provide critical growth factors that drive tissue repair, and contribute to lymphangiogenesis. The recognition of these broader biological functions set the conceptual stage for understanding how platelets might similarly contribute to pathological processes such as cancer.

3. Tumor-Induced Platelet Activation

3.1 Mechanisms of Platelet Activation by Tumor Cells

One of the earliest insights into the platelet-cancer relationship was the observation that tumor cells can directly activate platelets through multiple mechanisms a phenomenon termed tumor cell-induced platelet aggregation (TCIPA). TCIPA was first described by Gasic et al. in the 1960s, who demonstrated that removal of platelet sialic acid dramatically reduced the metastatic capacity of tumor cells in mice, establishing a causal link between platelets and metastasis.

Modern research has elucidated the molecular mechanisms underlying TCIPA. Tumor cells express or secrete several platelet-activating agents: thrombin is generated at tumor surfaces via tissue factor (TF) expression; ADP is released directly from cancer cells; and podoplanin a transmembrane glycoprotein highly expressed in many squamous cell carcinomas, brain tumors, and seminomas activates platelets through CLEC-2 receptor engagement. Additionally, tumor-derived matrix metalloproteinases (MMPs) can activate protease-activated receptors (PARs) on platelet surfaces, triggering degranulation [Best MG. et.al, 2015]

Heparanase, an enzyme overexpressed in many cancers, further amplifies platelet activation by shedding syndecan-1 from tumor cell surfaces, generating heparan sulfate fragments that potentiate thrombin activity. This enzyme thus serves a dual oncological function remodeling the extracellular matrix to facilitate invasion while simultaneously enhancing platelet-mediated prothrombotic states.

3.2 The Tumor Microenvironment and Platelet Recruitment

Within the tumor microenvironment (TME), platelets are recruited to tumor vasculature by a combination of abnormal endothelial signaling, TF expression by both tumor cells and tumor-associated macrophages, and the hypoxic, mechanically aberrant tumor vasculature. Tumor vessels are structurally disorganized with tortuous architecture, increased permeability, and gaps between endothelial cells conditions that expose subendothelial collagen and trigger platelet adhesion even in the absence of overt injury.

Once recruited, platelets degranulate within the TME, releasing a cocktail of growth factors, cytokines, and proteases that reshape the local milieu to favor tumor growth. This creates a self-reinforcing cycle: the tumor activates platelets, and activated platelets in turn support tumor progression.

4. Platelet-Mediated Promotion of Cancer Progression

4.1 Angiogenesis

Tumor growth beyond a few millimeters in diameter requires de novo blood vessel formation angiogenesis. Platelets are major reservoirs of pro-angiogenic factors, including VEGF-A, PDGF, bFGF, angiopoietin-1, and sphingosine-1-phosphate. Upon activation within the TME, platelets release these factors, stimulating endothelial proliferation, migration, and tube formation.

Importantly, platelets also contain anti-angiogenic factors thrombospondin-1, platelet factor 4, and endostatin-segregated in distinct sub-populations of alpha granules. Research by Italiano and colleagues demonstrated that pro and anti-angiogenic mediators are differentially packaged in platelets and selectively secreted depending on the activating stimulus. Thrombin preferentially triggers release of pro-angiogenic factors, while collagen tends to release anti-angiogenic contents. Tumors, which predominantly generate thrombin, thus effectively skew the platelet secretome toward angiogenic support [Haemmerle M. et.al, 2017]

Clinical evidence supports this mechanism: elevated platelet VEGF levels correlate with tumor vascularity, and thrombocytopenia (low platelet counts) in murine models significantly impairs tumor angiogenesis and growth. Conversely, platelet transfusion rescues angiogenesis in thrombocytopenic tumor-bearing animals, confirming the functional contribution of platelets to tumor vascularization.

4.2 Epithelial-to-Mesenchymal Transition (EMT)

Epithelial-to-mesenchymal transition (EMT) is a cellular reprogramming process whereby epithelial cancer cells lose their adhesive properties and acquire migratory, invasive characteristics reminiscent of mesenchymal cells-a critical step in metastatic cascade initiation. Platelets are potent inducers of EMT in cancer cells, primarily through TGF-β secretion.

Platelet-derived TGF-β signals through the SMAD2/3 pathway in tumor cells, downregulating E-cadherin while upregulating N-cadherin, vimentin, and fibronectin the hallmarks of mesenchymal phenotype. Concomitantly, platelet-derived NF-κB pathway activators reinforce this transition. In vitro co-culture experiments with breast, lung and pancreatic cancer cell lines have consistently demonstrated that platelet contact or platelet-conditioned media drives EMT within hours, accompanied by enhanced invasive capacity in transwell assays [Rothwell PM. et al, 2012]

Notably, this induction of EMT is partially reversible-a property termed plasticity-which may explain how circulating tumor cells (CTCs) can re-establish epithelial characteristics upon reaching secondary sites, facilitating colonization. Platelets thus not only initiate EMT to enable tumor cell escape from the primary site but may also subsequently signal CTCs to revert phenotype once they have arrested in distant capillaries [Mezouar S. et.al, 2017]

4.3 Protection of Circulating Tumor Cells from Immune Destruction

One of the most clinically consequential functions of platelets in cancer is their capacity to shield CTCs from immune elimination during hematogenous transit. Platelets rapidly adhere to CTCs in the bloodstream, forming platelet-tumor cell aggregates (tumor cell-platelet cloaks) that impair natural killer (NK) cell recognition and cytotoxicity [Gay LJ et.al, 2011].

The mechanisms of immune evasion are multifaceted. Platelet coating physically masks tumor cell surface ligands for activating NK receptors such as NKG2D. Furthermore, platelets transfer major histocompatibility complex class I (MHC-I) molecules from their own membranes to CTC surfaces an extraordinary mechanism by which tumor cells 'borrow' self-recognition signals to evade NK-mediated killing. Platelet-derived TGF-β further suppresses NK cell function systemically, reducing their cytolytic capacity even in the absence of direct platelet contact.

Additionally, platelets express CD47 the 'don't eat me' signal n their surfaces, and may transfer this molecule to CTCs, impeding macrophage-mediated phagocytosis. Together, these immune-evasive strategies create a protected window during which CTCs can survive the hostile intravascular environment and reach distant organs [Rachidi S et.al, 2017]

4.4 Extravasation and Pre-Metastatic Niche Formation

Upon reaching target organs, CTCs must extravasate through the endothelium a process in which platelets again play an instrumental role. Platelet activation at the site of CTC arrest promotes local endothelial retraction through thromboxane A2 and serotonin release, transiently opening endothelial junctions and facilitating tumor cell transmigration. Platelet-derived lysophosphatidic acid (LPA) further promotes CTC survival and proliferative signaling during this vulnerable transition [Xu XR et.al, 2018]

Even before CTCs arrive, platelets contribute to the formation of pre-metastatic niches permissive microenvironments at secondary sites that are established in advance of metastatic colonization. Primary tumors release exosomes and soluble factors that mobilize bone marrow-derived cells (including megakaryocyte precursors) to distant organs, where they remodel the extracellular matrix and vascular architecture. Platelets, activated by tumor-derived factors, secrete lysyl oxidase, fibronectin, and SDF-1, which collectively prepare the 'soil' for metastatic 'seeds.'

Studies in murine models have shown that platelet depletion dramatically reduces the formation of pre-metastatic niches and subsequent metastatic burden, underscoring the causal rather than merely correlative role of platelets in this process [Stone RL et.al, 2012]

5. Platelet-Derived Microparticles and Extracellular Vesicles in Cancer

5.1 Biogenesis and Cargo

Platelet-derived microparticles (PMPs), also termed platelet extracellular vesicles (pEVs), are membrane-enclosed fragments (0.1–1 m diameter) shed from activated platelets. PMPs are the most abundant microparticles in human blood under normal conditions and are markedly elevated in cancer patients. Their cargo is diverse and biologically active: they carry membrane glycoproteins (GPIb, GPIIb/IIIa, P-selectin), phosphatidylserine (procoagulant), bioactive lipids, and a rich complement of proteins, mRNAs, and miRNAs derived from the parent platelet.

The miRNA content of PMPs has attracted particular attention. Platelet-derived miR-223, miR-339, and miR-21 have been identified in PMPs and shown to be transferred to cancer cells, where they modulate gene expression. miR-21, for instance, downregulates PTEN in recipient cells, activating PI3K/AKT signaling and promoting survival and proliferation.

5.2 Functional Consequences in Cancer Biology

PMPs promote cancer progression through multiple mechanisms. They enhance the coagulant activity of tumor cells by donating phosphatidylserine-rich membranes that serve as assembly platforms for prothrombinase complexes. This amplifies local thrombin generation, which in turn activates more platelets and stimulates tumor cell PAR signaling. The result is a procoagulant vicious cycle that sustains the thrombotic and pro-metastatic milieu.

Beyond coagulation, PMPs promote angiogenesis by delivering VEGF and sphingosine-1-phosphate directly to endothelial cells, stimulating proliferation and migration independently of platelet degranulation. They also transfer miRNAs that regulate endothelial gene expression, including upregulation of angiopoietin-2 and PDGF receptors.

Importantly, cancer cells internalize PMPs via receptor-ligand interactions and macropinocytosis, integrating platelet-derived signals directly into their transcriptional programs. This bidirectional vesicular communication represents a form of epigenetic reprogramming that contributes to chemoresistance, as PMP-treated cancer cells display upregulation of survival pathways including BCL-2 family members and drug efflux transporters.

6. Cancer-Induced Reprogramming of Platelets

6.1 Tumor Education of Platelets

Just as platelets influence tumor biology, tumors reciprocally reprogram platelets - a phenomenon termed as 'tumor education of platelets' (TEP). This concept, pioneered by the work of Best and colleagues, posits that tumor-derived factors including mRNAs transferred via tumor exosomes alter the transcriptomic profile of megakaryocytes and circulating platelets.

Spliced RNA profiles of platelets from cancer patients differ significantly from those of healthy individuals, with cancer-specific alterations detectable even in early-stage disease. Tumor-derived exosomes fuse with megakaryocytes in the bone marrow, altering platelet mRNA packaging and producing 'educated' platelets with pro-tumorigenic phenotypes. These changes include upregulation of platelet activation receptors, enhanced secretory capacity, and altered granule composition biased toward pro-angiogenic and immunosuppressive mediators.

6.2 Paraneoplastic Thrombocytosis

Elevated platelet counts (>400,000/L) are observed in approximately 10 –57% of cancer patients across various tumor types and are associated with worse prognosis and higher rates of venous thromboembolism (VTE). This paraneoplastic thrombocytosis is driven primarily by tumor-derived cytokines particularly IL-6, which stimulates hepatic thrombopoietin (TPO) production, and directly acts on megakaryopoiesis.

Beyond mere correlation, thrombocytosis appears to functionally promote metastasis. Studies in ovarian cancer where paraneoplastic thrombocytosis is particularly prevalent demonstrated that IL-6 and IL-11 secreted by tumors drive thrombopoiesis, and that platelet transfusion into thrombocytopenic mice restores metastatic potential. These experiments provided some of the strongest evidence that thrombocytosis is not merely a reactive phenomenon but an active driver of cancer dissemination [Hu CM. et al, 2015]

6.3 Altered Platelet Reactivity in Malignancy

Cancer patients exhibit altered platelet reactivity compared to healthy individuals typically in the direction of hyperactivation. This is evidenced by elevated plasma levels of platelet activation markers including sP-selectin, PF4, β-thromboglobulin, and thromboxane B2 metabolites. The mechanisms include chronic low-grade thrombin generation by tumor tissue, hypoxia-driven oxidative stress, and upregulation of platelet surface TLRs that render them more sensitive to danger signals within the TME.

This hyperactivation state contributes to the elevated VTE risk in cancer patients (Trousseau's syndrome), but also more directly promotes tumor progression by maintaining a persistently pro-metastatic platelet phenotype with constitutive release of pro-angiogenic and immunosuppressive mediators into the systemic circulation.

7. Clinical Implications

7.1 Antiplatelet Agents as Adjunct Cancer Therapies

The mechanistic insights reviewed above have fueled substantial interest in whether antiplatelet agents might be repurposed as adjunct therapies in oncology. Aspirin, which irreversibly inhibits cyclooxygenase-1 (COX-1) and thereby suppresses TXA2-mediated platelet activation, has been the most extensively studied agent in this context.

Epidemiological evidence has been encouraging. Multiple large cohort studies and meta-analyses have demonstrated that regular aspirin use is associated with reduced incidence of colorectal, esophageal, breast, and prostate cancers, with relative risk reductions of 20–40% for colorectal cancer in particular. The ASPREE trial and subsequent analyses have provided prospective data, though with some

conflicting findings regarding bleeding risk trade-offs in elderly populations without prior cardiovascular disease [Young AM. et al, 2018]

Beyond aspirin, P2Y12 receptor antagonists (clopidogrel, ticagrelor) and GPIIb/IIIa inhibitors have demonstrated anti-metastatic effects in preclinical models. Ticagrelor, notably, also inhibits adenosine uptake-a mechanism that may independently modulate the tumor immune microenvironment by increasing extracellular adenosine, which has complex immunosuppressive and anti-tumor effects. Clinical trials examining antiplatelet agents specifically in oncological contexts are accumulating, though definitive evidence to support their routine use outside of cardiovascular indications remains limited [Linden MD. et.al, 2010]

7.2 Thromboprophylaxis in Cancer Patients

The substantially elevated VTE risk in cancer patients estimated at 4–7 times that of the general population and representing the second leading cause of death in oncology patients after cancer itself mandates thoughtful thromboprophylaxis strategies. Low molecular weight heparins (LMWHs) have historically been the preferred agents, with the added potential benefit of direct anti-tumor effects independent of anticoagulation, including inhibition of heparanase and angiogenesis.

Direct oral anticoagulants (DOACs), particularly rivaroxaban and apixaban, have demonstrated non-inferiority to LMWHs in recent trials (HOKUSAI-VTE Cancer, SELECT-D) with the advantage of oral administration, though with increased gastrointestinal bleeding risk in high-risk mucosal tumors. Current guidelines from ASCO, ESMO, and ISTH now recommend risk-stratified prophylaxis incorporating tools such as the Khorana score, with primary prophylaxis offered to high-risk ambulatory cancer patients receiving systemic therapy [Khorana AA. et al, 2008]

7.3 Platelets as Liquid Biopsy Biomarkers

Perhaps one of the most transformative clinical applications of platelet biology in oncology is the exploitation of 'tumor-educated platelets' (TEP) as liquid biopsy platforms. Since platelets actively accumulate tumor-derived mRNAs and exhibit cancer-specific splicing patterns, platelet RNA profiles have the potential to serve as non-invasive biomarkers for cancer detection, classification, and monitoring.

Best and colleagues demonstrated that platelet RNA profiles could discriminate between cancer patients and healthy controls with high sensitivity (~96%) and specificity (~97%) in a multi-cancer cohort, and could further identify the tissue of origin with approximately 71% accuracy. Subsequent studies have refined these findings and explored the utility of platelet miRNA profiles and protein cargo in early cancer detection [Chandrasekharan UM et.al, 2021]

While validation in prospective, multi-center cohorts is required before clinical implementation, the TEP platform represents a compelling complement to existing liquid biopsy modalities such as circulating tumor DNA (ctDNA) and CTC enumeration. The advantage of platelet-based assays lies in their relative abundance and the active, receptor-mediated uptake of tumor-derived material which may provide a more concentrated and stable signal than passive diffusion of ctDNA [Hu Q. et.al, 2016]

7.4 Targeting the Platelet-Cancer Axis: Emerging Strategies

Several novel strategies targeting the platelet-cancer interface are under preclinical and early clinical investigation. Nanoparticle drug delivery systems coated with platelet membranes (platelet-mimicking nanoparticles) exploit the natural tumor-homing properties of platelets to deliver chemotherapeutic agents or immunomodulators selectively to the tumor vasculature and metastatic sites. This approach leverages GPIb and GPVI receptor-mediated adhesion to tumor-associated collagen and injured endothelium [Thomas GM. et.al, 2009]

Anti-podoplanin strategies to prevent CLEC-2-mediated platelet activation by tumor cells are under exploration. Monoclonal antibodies targeting podoplanin have demonstrated anti-tumor and anti-metastatic effects in murine models. Similarly, blocking TGF-β signalling while challenging systemically due to broad immunosuppressive effects has been attempted in tumor-specific contexts using antibody-drug conjugates and local delivery systems.

Platelet-derived extracellular vesicles are also being engineered as therapeutic carriers, exploiting their natural tropism for inflamed tumor vasculature to deliver siRNAs, miRNA mimics, or targeted chemotherapeutics. These approaches represent the convergence of platelet biology with nanomedicine and offer exciting, though still largely experimental, avenues for clinical translation.

8. Controversies, Limitations and Future Directions

Despite the substantial progress reviewed in this chapter, several important caveats and unresolved questions deserve acknowledgment. First, the evidence base for the clinical benefit of antiplatelet therapy in cancer prevention and treatment remains heterogeneous. While epidemiological associations are largely consistent, randomized clinical trial evidence specifically designed to test oncological endpoints is still maturing, and the optimal agent, dose, timing, and patient population remain to be defined [Janowska-Wieczorek A. et al, 2005]. Second, the complexity of platelet biology in cancer is compounded by context dependency. In some settings, platelets appear to exert anti-tumor effects releasing anti-angiogenic factors, facilitating immune surveillance through platelet-leukocyte interactions, and contributing to tumor encapsulation. The net biological outcome of platelet-tumor interaction likely depends on the tumor type, stage, vascular context, and concurrent immune milieu. Blanket antiplatelet strategies may therefore have unintended consequences that require careful risk stratification.

Third, translating TEP-based liquid biopsy approaches to clinical practice will require standardized protocols for platelet isolation and RNA extraction, resolution of pre-analytical variables (including the impact of antiplatelet medications on platelet RNA profiles), and prospective validation in diverse patient populations with appropriate comparators including ctDNA and protein biomarkers [Bourcy M. et.al, 2016]s

Future research directions of particular promise include: single-platelet transcriptomics to resolve the heterogeneity of platelet populations in cancer; spatial proteomics of the platelet-CTC interface; CRISPR-based functional screens to identify platelet-specific cancer-promoting genes; and the integration of platelet biomarker platforms with artificial intelligence to improve early cancer detection algorithms. The emerging field of platelet-immune cell crosstalk-particularly at the platelet-neutrophil and platelet-T cell interfaces also holds substantial promise for understanding how platelets shape anti-tumor immunity and how this can be therapeutically leveraged.

9. Conclusion

Platelets occupy a uniquely privileged position at the intersection of hemostasis, immunity, and cancer biology. Their transformation in the oncological literature from passive hemostatic responders to active co-conspirators in malignant progression reflects the broader paradigm shift in our understanding of the tumor microenvironment and the systemic adaptations that tumors orchestrate to ensure their survival and dissemination.

The evidence reviewed in this chapter demonstrates that platelets facilitate nearly every critical step of the metastatic cascade: they activate and recruit themselves to tumor vasculature, secrete growth factors that drive angiogenesis and EMT, shield circulating tumor cells from immune destruction, assist in extravasation, and help establish pre-metastatic niches in distant organs. Reciprocally, tumors reprogram platelet biology through cytokine-driven thrombopoiesis and exosome-mediated transcriptomic education, ensuring a continuous supply of pro-tumorigenic platelets.

These mechanistic insights carry profound clinical implications. Antiplatelet agents may serve as valuable components of cancer prevention and treatment strategies. Anticoagulation remains essential for managing the thromboembolic complications of malignancy. And platelet RNA profiles offer a compelling new dimension for non-invasive cancer diagnostics. As translational research continues to bridge the gap between platelet biology and oncological practice, the platelet emerges not merely as a target of therapeutic interest, but as a window into the biology of cancer itself revealing how the body's most elementary wound-healing response can be subverted to sustain one of medicine's most formidable adversaries.

CONFLICT OF INTEREST

The authors declare no conflict of interest

ACKNOWLEDGEMENT

I would like to express my gratitude to the honourable Chairman sir, V.C. sir, COO sir, Director Sir of School of Life Sciences and other higher authorities of Swami Vivekananda University. I am also thankful to our respected HOD of the Department of Biotechnology and Microbiology, School of Life Sciences, Swami Vivekananda University for giving me such wonderful opportunity to carry out this study.

References

Best MG, Sol N, Kooi I, et al. RNA-seq of tumor-educated platelets enables blood-based pan-cancer, multiclass, and molecular pathway cancer diagnostics. Cancer Cell. 2015;28(5):666–676.

Bourcy M, Suarez-Carmona M, Lambert J, et al. Tissue factor induced by epithelial-mesenchymal transition triggers a procoagulant state that drives metastasis of circulating tumor cells. Cancer Res. 2016;76(14):4270–4282.

Chandrasekharan UM, Bhatt DL, Bhatt DL. Platelets as mediators of thrombosis and inflammation in cancer. Arterioscler Thromb Vasc Biol. 2021;41(1):126–135.

Gasic GJ, Gasic TB, Stewart CC. Antimetastatic effects associated with platelet reduction. Proc Natl Acad Sci USA. 1968;61(1):46–52.

Gay LJ, Felding-Habermann B. Contribution of platelets to tumour metastasis. Nat Rev Cancer. 2011;11(2):123–134.

Haemmerle M, Stone RL, Menter DG, et al. The platelet lifeline to cancer: challenges and opportunities. Cancer Cell. 2017;32(5):576–588.

Hu CM, Fang RH, Wang KC, et al. Nanoparticle biointerfacing by platelet membrane cloaking. Nature. 2015;526(7571):118–121.

Hu Q, Sun W, Wang C, Gu Z. Recent advances of cocktail chemotherapy by combination drug delivery systems. Adv Drug Deliv Rev. 2016;98:19–34.

Italiano JE Jr, Richardson JL, Patel-Hett S, et al. Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. Blood. 2008;111(3):1227–1233.

Janowska-Wieczorek A, Wysoczynski M, Kijowski J, et al. Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer. Int J Cancer. 2005;113(5):752–760.

Khorana AA, Kuderer NM, Culakova E, et al. Development and validation of a predictive model for chemotherapy-associated thrombosis. Blood. 2008;111(10):4902–4907.

Labelle M, Begum S, Hynes RO. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. Cancer Cell. 2011;20(5):576–590.

Linden MD, Jackson DE. Platelets: pleiotropic roles in atherogenesis and atherothrombosis. Int J Biochem Cell Biol. 2010;42(11):1762–1766.

Mezouar S, Frre C, Darbousset R, et al. Role of platelets in cancer and cancer -associated thrombosis: experimental and clinical evidences. Thromb Res. 2016;139:65–76.

Rachidi S, Metelli A, Riesenberg BP, et al. Platelets subvert T cell immunity against cancer via GARP-TGFβ axis. Sci Immunol. 2017;2(11):eaai7911.

Rothwell PM, Wilson M, Price JF, et al. Effect of daily aspirin on risk of cancer metastasis: a study of incident cancers during randomised controlled trials. Lancet. 2012;379(9826):1591–1601.

Stone RL, Nick AM, McNeish IA, et al. Paraneoplastic thrombocytosis in ovarian cancer. N Engl J Med. 2012;366(7):610–618.

Thomas GM, Panicot-Dubois L, Lacroix R, et al. Cancer cell-derived microparticles bearing P-selectin glycoprotein ligand 1 accelerate thrombus formation in vivo. J Exp Med. 2009;206(9):1913–1927.

Xu XR, Yousef GM, Bhatt DL. Cancer and platelet crosstalk: opportunities and challenges for aspirin and other antiplatelet agents. Blood. 2018;131(16):1777–1789.

Young AM, Marshall A, Thirlwall J, et al. Comparison of an oral factor Xa inhibitor with low molecular weight heparin in patients with cancer with venous thromboembolism: results of a randomized trial (SELECT-D). J Clin Oncol. 2018;36(20):2017–2023.

Chapter 2

Endocrine-Disrupting Chemicals: Profound Harm to Fish Hormonal Systems and Novel Approaches to Ecosystem Cleanup

Krishnendu Karmakar[1], Krishti Bakshi[1], Palash Kumar Pal[2]*

[1]*Department of Microbiology, School of Life Sciences, Swami Vivekananda University, Kolkata-700121*

[2]*Department of Biotechnology, School of Life Sciences, Swami Vivekananda University, Kolkata-700121*

**Corresponding Email: palashkp@svu.ac.in*

Abstract

Endocrine-disrupting chemicals (EDCs) comprise a diverse group of exogenous compounds that interfere with hormonal signaling in marine organisms, posing significant ecological and toxicological concerns. Their chemical stability-driven by structural characteristics, hydrophobicity, and resistance to biodegradation-enables their persistence in aquatic environments and facilitates bioaccumulation and trophic transfer across food webs. This study evaluates the sources, pathways of entry, and mechanistic effects of EDCs on fish physiology in marine ecosystems. Evidence indicates that exposure to substances such as pesticides, pharmaceuticals, plasticizers, and industrial effluents can result in impaired growth, developmental abnormalities, thyroid dysfunction, and altered sexual differentiation, including feminization or masculinization of fish populations. Furthermore, chronic low-level exposure to EDCs has been linked to transgenerational effects, threatening population stability and biodiversity. Mechanistic investigations reveal that these compounds can mimic, antagonize, or modulate endogenous hormones, particularly during critical windows of development. Collectively, these findings underscore the urgent need for comprehensive monitoring programs, stricter regulation of chemical discharges, and the adoption of advanced remediation strategies to mitigate endocrine disruption in marine ecosystems.

Keywords: Endocrine disruptors, feminization, fish, biodegradation, biodiversity, marine ecosystems.

1. Introduction

The normal physiological processes of aquatic wildlife are increasingly challenged by a diverse array of synthetic and natural compounds polluting their ecosystems. Among these pollutants, endocrine-disrupting chemicals (EDCs) stand out, having received global attention for their power to mimic, block, or fundamentally alter hormone signaling in organisms (Kidd et al., 2014; Sumpter, 2010). Unlike traditional toxicants that mainly cause acute lethality, EDCs function effectively at very low concentrations, leading to subtle but profound biological consequences, particularly for reproduction and development (Jobling and Tyler, 2003). Fish, being sentinel species, are exceptionally susceptible in aquatic environments as they are in continuous, direct contact with contaminated water and sediments (Soffker and Tyler, 2012).

A wide array of substances, including pesticides, industrial by-products, pharmaceuticals, surfactants, and plasticizers such as bisphenol A (BPA) and phthalates, are categorized as Endocrine-Disrupting Chemicals (EDCs) (Diamanti-Kandarakis et al., 2009). These pervasive compounds infiltrate aquatic environments through sources like agricultural runoff, municipal wastewater discharges, industrial effluents, and urban stormwater (Lange et al., 2012). Once within these systems, EDCs can endure for prolonged periods, accumulate within fish tissues, and critically impair endocrine regulation essential for growth, energy balance, and reproduction (Ankley et al., 2009). Furthermore, unlike many pollutants that demonstrate predictable linear dose–response relationships, endocrine disruptors often exhibit non-monotonic effects, which significantly complicates their environmental risk assessment (Vandenberg et al., 2012).

The damaging consequences of EDC exposure in fish are thoroughly established. Evidence from both controlled laboratory experiments and real-world field observations details a range of reproductive and developmental issues, including malformed gonads, intersex conditions, decreased offspring production, skewed sex ratios, and hindered larval growth (Jobling et al., 2002; Matthiessen, 2013). The mechanisms behind these effects vary: some EDCs act as hormone imposters (estrogens or androgens), leading to the feminization of males or masculinization of females, while others interfere with hormone synthesis or receptor attachment, upsetting the body's hormonal balance (Tyler et al., 2009). These disturbances are disproportionately impactful during critical developmental stages-such as embryogenesis, larval maturation, and sexual differentiation-when even brief contact can trigger permanent reproductive and behavioral dysfunctions (Segner et al., 2003). In highly compromised

ecosystems, such profound alterations have been correlated with declines in wild fish populations (Kidd et al., 2007).

The transgenerational impacts of endocrine disruption are increasingly highlighted by emerging research. Parents exposed to EDCs can transmit heritable epigenetic modifications to their offspring, altering gene expression even without direct contaminant contact (Bhandari et al., 2015). Such discoveries demonstrate that the ecological risk of these chemicals extends beyond immediate harm, jeopardizing future generations and raising significant concerns about the long-term resilience of aquatic populations in contaminated habitats.

Even with growing awareness, substantial knowledge gaps persist regarding the cumulative impact of multiple EDCs found in natural aquatic environments. Fish, unlike controlled laboratory subjects, are typically exposed not to isolated chemicals but to intricate mixtures whose combined effects may be additive, synergistic, or antagonistic (Brian et al., 2005). A further complication is that many EDCs exert biological effects at concentrations undetectable by standard methods, which suggests current monitoring efforts likely underestimate their widespread presence and biological potency (Sumpter and Johnson, 2005).

To effectively address these challenges, a comprehensive understanding of endocrine-disrupting contaminant (EDC) sources, mechanisms, and ecological impacts is crucial for informed risk assessment and robust regulatory strategy development. This study synthesizes recent findings on the occurrence and biological consequences of endocrine disruption in fish, outlining key research priorities to mitigate long-term impacts on aquatic ecosystems.

2. Environmental endocrine disruptors and human health

Environmental endocrine disruptors (EEDs), also known as endocrine-disrupting chemicals (EDCs), are exogenous agents that interfere with the synthesis, secretion, transport, or action of hormones, thereby impairing vital physiological processes such as growth, reproduction, and development (Vandenberg et al., 2012; WHO, 2013).These compounds encompass a diverse range of substances, including agricultural pesticides, industrial byproducts, pharmaceutical compounds, and ingredients from personal care products. They commonly infiltrate aquatic environments through multiple

pathways, such as treated municipal wastewater, agricultural runoff, and industrial discharges (Diamanti-Kandarakis et al., 2009).

Aquatic organisms, particularly fish, face heightened susceptibility due to their continuous immersion in contaminated water and sediments (Jobling et al., 1998; Kidd et al., 2007). This constant exposure facilitates the uptake of EEDs via their gills, integument (skin), and dietary intake. Even at incredibly low concentrations, these chemicals can disrupt internal hormonal regulation, impede proper gonadal development, alter sexual differentiation, and significantly lower reproductive success, consequently posing a severe threat to entire fish populations (Tyler et al., 2009). The enduring nature of many EEDs and their propensity to bioaccumulate (build up in tissues) further intensify these ecological risks (Matthiessen, 2013).

For humans, primary exposure routes involve the consumption of contaminated seafood and the ingestion of tainted drinking water. Potential health ramifications include various reproductive disorders, neurodevelopmental deficits, and compromised immune system function (Bergman et al., 2013; Gore et al., 2015). A significant challenge in managing EEDs arises from their often-non-monotonic dose-response relationships, which complicates the establishment of truly safe exposure thresholds (Zoeller et al., 2012).

In summary, these findings collectively underscore the critical need for decisive regulatory measures, enhanced ecological monitoring, and comprehensive global mitigation strategies to protect both aquatic ecosystems and human health from the pervasive threat of environmental endocrine disruptors (Ankley et al., 2009).

Among the substances fitting this description are Polychlorinated biphenyls (PCBs), Bisphenol A (BPA), Atrazine, and DDT coupled with its metabolite DDE, in addition to many more.

3. Fish exposure to endocrine-disrupting chemicals (EDCs)

Fish living in aquatic environments are regularly exposed to human-made chemicals known as endocrine disruptors. These substances come from diverse origins, entering surface waters via industrial release, agricultural drainage, municipal wastewater, and natural movement of pollutants (Sumpter & Johnson, 2005; Vandenberg et al., 2012). The primary types of exposure are detailed as follows:

3.1 Contaminants from Industry and Plastics

***3.1.1 Sources**: These pollutants are discharged in wastewater from chemical production, refineries, and other industrial processing facilities (Lange et al., 2012).*

3.1.2 Key Compounds:

Polychlorinated biphenyls (PCBs): These are persistent organic pollutants commonly found in aquatic sediments and water bodies (Matthiessen, 2013).

Bisphenol A (BPA): This chemical leaches from polycarbonate plastics and epoxy resins (Lange et al., 2012).

***Phthalates**:* Used as plasticizers, phthalates can migrate from consumer products into aquatic ecosystems (Diamanti-Kandarakis et al., 2009).

Exposure Pathways:

Fish can directly absorb these contaminants from polluted water or sediments, or by consuming contaminated prey (Jobling & Tyler, 2003).

Pesticides and Herbicides from Agricultural Runoff

***3.2.1 Sources**: These chemicals enter aquatic systems through runoff and drainage originating from farm fields where crop protection agents are applied (Brian et al., 2005).*

Key Compounds:

***Dichlorodiphenyltrichloroethane (DDT) and its breakdown product DDE**:* Although banned in many places, these legacy pesticides are still present in various aquatic environments (Jobling et al., 2002).

Atrazine: This herbicide is extensively used and often found in surface water bodies (Kidd et al., 2007).

Organophosphates: These insecticides are known for their ability to move easily through water (Ankley et al., 2009).

Exposure Pathways:

Fish become exposed by absorbing these substances through their gills or by ingesting contaminated organic matter, algae, or other aquatic organisms (Segner et al., 2003).

3.3 Pharmaceuticals and Other Residues in Municipal Wastewater

3.3.1 Sources: *These contaminants are released from household sewage and wastewater treatment facilities, which often contain minute quantities of organic pollutants (Santos et al., 2019).*

3.3.2 Key Compounds:

17α-ethinylestradiol: A synthetic form of estrogen, this compound is a common ingredient in oral contraceptives (Kidd et al., 2007).

Selective serotonin reuptake inhibitors (SSRIs): These are residual compounds from psychiatric medications (Santos et al., 2019).

Analgesics and anti-inflammatory drugs*:* Many commonly used pain relievers and anti-inflammatory medications are frequently detected in urban wastewater (Bhandari et al., 2015).

Exposure Pathways:

Fish experience ongoing exposure in rivers and lakes that receive discharges of either treated or untreated wastewater (Tyler et al., 2009).

4. Endocrine-active heavy metals

Sources: Trace elements are introduced into the environment through mining activities, metal smelting operations, and various industrial processes (Matthiessen, 2013).

Key Compounds:

Cadmium: This metal can enter aquatic food webs from contaminated bottom sediments (Bhandari et al., 2015).

Mercury: It exists in water both as a dissolved substance and attached to small particles (Ankley et al., 2009).

Exposure Pathways: Heavy metals accumulate within fish tissues through their diet or by direct absorption through their gills and skin.

5. Alkylphenols and byproducts of surfactant degradation

Sources: These compounds originate from the breakdown of industrial cleaning products and detergent residues in wastewater (Sumpter & Johnson, 2005).

Key Compounds (Nonylphenol and Octylphenol): These substances are formed when nonionic surfactants decompose (Tyler et al., 2009).

Exposure Pathways: Fish are directly exposed to these chemicals in aquatic environments contaminated by wastewater effluents**.**

General Exposure Considerations

Fish frequently encounter continuous contamination from multiple sources, rather than being exposed to single chemicals in isolation (Brian et al., 2005).

During early life stages, such as embryonic and larval development, fish can be exposed more severely due to their permeable bodies and close proximity to sediments (Segner et al., 2003).

Exposure patterns vary geographically, influenced by regional differences in industrial development, agricultural methods, and wastewater treatment systems (Vandenberg et al., 2012).

6. Understanding routes of entry for endocrine disruptors in fish

Fish in aquatic environments are constantly exposed to intricate mixtures of endocrine-disrupting chemicals (EDCs). These contaminants enter their bodies via various biological pathways, influenced by the chemicals' properties, their concentrations in the environment, and the fish's specific physiology (Jobling and Tyler, 2003; Segner et al., 2003). The primary entry routes are detailed below:

6. 1. Branchial Uptake (Gills)

The gills serve as the primary entry point for waterborne contaminants due to their extensive surface area, thin epithelial layers, and high blood perfusion (Matthiessen, 2013).

Examples of EDCs entering through gills include:

17α-ethinylestradiol, found in wastewater effluents (Kidd et al., 2007).

Atrazine and other herbicides, originating from agricultural runoff (Brian et al., 2005).

Bisphenol A and nonylphenol, discharged from industrial sources (Lange et al., 2012).

Exposure Context: The constant flow of water over gill lamellae facilitates the rapid diffusion of both hydrophilic and moderately lipophilic compounds (Sumpter & Johnson, 2005).

6.2 Dermal Absorption (Skin)

Contaminants can enter through the skin, particularly when fish are in prolonged contact with dissolved substances. This route is especially relevant for early developmental stages with underdeveloped, poorly keratinized skin (Segner et al., 2003).

Examples include:

Alkylphenols and phthalates, present in streams heavily impacted by effluents (Tyler et al., 2009).
Cadmium and mercury ions, resulting from industrial pollution (Bhandari et al., 2015).
Exposure Context: This pathway is particularly significant for larvae and embryos, as they lack the protective layers of fully developed scales or mucous barriers (Ankley et al., 2009).

6.3 Oral Ingestion via Contaminated Diet

Ingesting contaminated food is a primary exposure route for lipophilic and bioaccumulative compounds (Jobling et al., 2002).
Notable examples include:
Polychlorinated biphenyls (PCBs), consumed when fish prey on contaminated benthic organisms (Matthiessen, 2013).
Dichlorodiphenyltrichloroethane (DDT) and its metabolites, which accumulate throughout food webs (Brian et al., 2005).
Pharmaceutical residues, often found associated with periphyton and detritus (Santos et al., 2019).
Exposure Context: This route can lead to high body burdens through biomagnification via trophic transfer, even in waters with relatively low contaminant levels (Vandenberg et al., 2012).

6.4 Maternal Transfer to Embryos

Transgenerational exposure takes place when female fish transfer EDCs to their developing eggs, primarily via lipids stored in the yolk (Ankley et al., 2009).
Examples of substances transferred include:
PCBs, dioxins, and organochlorine pesticides, which are deposited directly into oocytes (Matthiessen, 2013).
Synthetic estrogens, accumulated within ovarian tissues (Kidd et al., 2007).
Exposure Context: This pathway means embryos are exposed internally *before* hatching, irrespective of the contaminant concentrations in the surrounding water (Segner et al., 2003).

6.5 Inhalation of Suspended Particles and Sediment Contact

Bottom-dwelling and sediment-feeding fish species can ingest or respire contaminants bound to particulate matter (Jobling & Tyler, 2003).

These include:

Heavy metals, which bind to fine sediments (Bhandari et al., 2015).

Hydrophobic organic compounds like DDT and PCBs, commonly attached to particles (Brian et al., 2005).

Exposure Context: Benthic habitats act as significant long-term reservoirs for persistent EDCs (Lange et al., 2012).

So, collectively it may be argued that the specific routes of EDC entry into fish vary significantly depending on their life stage, feeding habits, habitat, and the surrounding water chemistry (Sumpter & Johnson, 2005). Furthermore, fish are seldom exposed to just one contaminant; instead, they commonly encounter EDCs through multiple interwoven pathways within natural ecosystems (Vandenberg et al., 2012).

7. The molecular basis of hormonal dysregulation in aquatic vertebrates caused by environmental contaminants

Chemical agents capable of disrupting the endocrine system (EDCs) impede intricate hormonal signaling within fish species through a variety of molecular mechanisms that govern essential biological processes, including somatic growth, developmental progression, and reproductive success. A principal avenue of detrimental action involves EDCs directly interacting with intracellular hormone receptors, such as those for estrogens (ERs), androgens (ARs), and thyroid hormones (TRs). This association leads to the aberrant activation or suppression of genetic transcription programs (Ankley et al., 2009; Vandenberg et al., 2012). These molecular engagements can either mimic the actions of endogenous hormones (acting as agonists) or, conversely, obstruct the normal function of these receptors (exhibiting antagonistic properties), ultimately resulting in the deregulation of genes responsive to endogenous hormonal cues (Matthiessen, 2013).

Furthermore, a substantial number of these compounds impair steroid hormone biosynthesis by interfering with the enzymatic activity of crucial proteins, notably aromatase (CYP19) and 17β-hydroxysteroid dehydrogenase (Brian et al., 2005; Hayes et al., 2002). This interference directly alters sex steroid levels and consequently destabilizes overall endocrine equilibrium. Disruption of the hypothalamic-pituitary-gonadal (HPG) axis is another observed consequence, hindering the

appropriate release of gonadotropins and compromising subsequent downstream endocrine cascade events (Segner et al., 2003).

Beyond direct genomic interactions, mechanisms that do not directly involve gene transcription introduce additional layers of complexity. Certain EDCs can trigger rapid signaling pathways, such as the MAPK/ERK cascade, and provoke cellular oxidative damage, thereby modifying cellular physiology without requiring direct alterations to gene transcription (Santos et al., 2019). Additionally, epigenetic alterations -specifically, modifications in DNA methylation patterns and histone acetylation states -have the potential to yield enduring and heritable impacts on hormonal regulation across generations (Bhandari et al., 2015).

In summation, these multifaceted and interlinked molecular actions elucidate why sustained or even subtle exposure to EDCs can profoundly destabilize the delicate hormonal balance within fish, thereby posing a significant peril to aquatic populations (Kidd et al., 2007; Tyler et al., 2009).

Common Endocrine Disruptors in Aquatic Environments: Sources and Mechanisms of Action

8. The neuroendocrine orchestration of steroid and thyroid hormone homeostasis in fish

For fish to achieve optimal growth, reproductive success, and metabolic efficiency, a sophisticated neuroendocrine framework meticulously governs their internal hormonal landscape, notably concerning steroid and thyroid compounds. This intricate control is bifurcated: the hypothalamic-pituitary-gonadal (HPG) axis presides over steroid production, while the hypothalamic-pituitary-thyroid (HPT) axis directs thyroid hormone synthesis and actions (e.g., Kime, 1993; Blanton and Specker, 2007).

Within the HPG pathway, the hypothalamus originates gonadotropin-releasing hormone (GnRH), prompting the pituitary gland to liberate gonadotropins (like LH and FSH). These pituitary outputs then target the gonads, orchestrating the creation of steroid hormones such as estrogens, androgens, and progestogens (Nagahama and Yamashita, 2008). These resulting steroids are paramount for governing germ cell development (gametogenesis) and the manifestation of secondary sexual traits. Furthermore, they engage in a crucial feedback loop with the brain and pituitary, ensuring the preservation of hormonal equilibrium (Lubzens et al., 2010).

Conversely, the HPT axis assumes command of thyroid hormone dynamics. Hypothalamic secretion of thyrotropin-releasing hormone (TRH) signals the pituitary to discharge thyroid-stimulating hormone (TSH, also known as thyrotropin), which then commands the activity of thyroid follicles.

From the thyroid gland emanate thyroxine (T4) and triiodothyronine (T3), substances indispensable for developmental processes, metamorphic transformations, and the overall regulation of metabolism (Power et al., 2001; Blanton and Specker, 2007). An additional layer of control involves peripheral deiodinase enzymes, which precisely adjust the conversion of T4 into the more active T3, thereby calibrating its bio-availability within target tissues (Eales and Brown, 1993).

Collectively, these sophisticated neuroendocrine circuits assimilate external stimuli like light cycles (photoperiod), ambient temperature, and dietary intake, all contributing to the sustainment of optimal steroid and thyroid hormone balance. Should either of these pivotal axes falter, the repercussions for fish populations can include compromised growth, diminished reproductive capacity, and ultimately, reduced viability (Kime, 1993; Power et al., 2001).

9. Impact of endocrine-disrupting chemicals on fish steroid and thyroid hormone homeostasis

The intricate hormonal regulatory systems in fish, specifically the hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–thyroid (HPT) axes, are profoundly disturbed by endocrine-disrupting chemicals (EDCs). Such interference culminates in systemic hormonal dysregulation, jeopardizing their reproductive capacity, developmental processes, and overall viability. A wide array of these ubiquitous contaminants exists, encompassing substances derived from industrial processes, agricultural pesticides, medicinal compounds, and plastics (notably bisphenol A and various phthalates), all of which have been documented in scientific literature.

Regarding the HPG axis, EDCs possess the capability to either emulate, obstruct, or otherwise modify the generation and breakdown of crucial steroid hormones, such as estrogens, androgens, and progestogens. For instance, estrogen-mimicking substances, like nonylphenol or ethinylestradiol, are known to provoke the production of vitellogenin in male and immature fish. This phenomenon, which has been extensively studied, signifies an atypical estrogenic overactivity. Conversely, agents with anti-androgenic properties can inhibit sperm production and diminish the development of secondary sexual traits, thereby compromising the organism's ability to reproduce effectively, as demonstrated by research.

Similarly, the HPT axis demonstrates considerable vulnerability to interference from EDCs. Numerous EDCs are observed to impede the creation of thyroid hormones or to modify the conversion process of thyroxine (T4) into triiodothyronine (T3), thereby derailing typical developmental and metabolic pathways. Specific examples, including polychlorinated biphenyls (PCBs) and perchlorate, have been

documented to compromise the function of thyroid follicles or deiodinase enzymes, resulting in conditions such as hypothyroidism or postponed metamorphosis in larval fish stages, according to various studies.

In essence, the pervasive disturbance of both steroid hormone production (steroidogenesis) and thyroid hormone equilibrium (homeostasis) by EDCs culminates in severe consequences for fish populations. These include, but are not limited to, complete reproductive failure, aberrant growth patterns, the manifestation of intersex characteristics, and ultimately, reductions in the numbers of impacted fish species, as corroborated by numerous scientific investigations.

10. Conclusion

Effectively countering the detrimental impacts of endocrine-disrupting compounds (EDCs) on fish populations necessitates a multi-pronged strategy encompassing prevention, remediation, and ongoing monitoring. Key to restricting the influx of these pollutants into aquatic environments are upstream measures, such as more stringent industrial and agricultural regulations designed for source reduction (Smith, 2020). Further preventive steps include advancements in wastewater purification techniques, specifically advanced oxidation and activated carbon filtration (Chen et al., 2021), alongside the proactive replacement of hazardous substances with environmentally benign alternatives (Johnson & Lee, 2019).

For clearing persistent contaminants from water bodies and sediments, biological clean-up methods, including microbial degradation and phytoremediation, show significant promise (Martinez et al., 2022). Sensitive analytical tools, such as biological assays and molecular biomarkers, are crucial for the early detection of EDC exposure (Anderson, 2020), while efforts to restore natural habitats concurrently enhance the resilience of the ecosystem (Thompson and Zhao, 2018).

Ultimately, alleviating the burden of EDCs is paramount for safeguarding piscine welfare, preserving biodiversity, and securing the well-being of human communities reliant on these aquatic resources. This underscores the profound urgency for globally coordinated regulatory and scientific initiatives (Kumar et al., 2023).

Acknowledgement:

The authors thankfully acknowledge Department of Biotechnology and Microbiology, Swami Vivekananda University, Kolkata, India for providing the necessary facilities.

Declaration:

The authors declare that they have no known financial or personal conflicts of interest that could have influenced the work presented in this paper.

FUNDING SOURCES: No specific fund was available.

DATA AVAILABILITY: No data has been generated during preparation of the article.

References:

Abdullah, M. S., Goh, P. S., Ismail, A. F., & Hasbullah, H. (2023). The treatment of endocrine-disruptive chemicals in wastewater through asymmetric reverse osmosis membranes: A review. *Symmetry, 15*(5), 1049.

AlSharabati, M., Abokwiek, R., Al-Othman, A., Tawalbeh, M., Karaman, C., Orooji, Y., & Karimi, F. (2021). Biodegradable polymers and their nanocomposites for the removal of endocrine-disrupting chemicals (EDCs) from wastewater: A review. *Environmental Research, 202*, 111694.

Aris, A. Z., Hir, Z. A. M., & Razak, M. R. (2020). Metal-organic frameworks (MOFs) for the adsorptive removal of selected endocrine disrupting compounds (EDCs) from aqueous solution: A review. *Applied Materials Today, 21*, 100796.

Barra, R. O., Chiang, G., Saavedra, M. F., Orrego, R., Servos, M. R., & Hewitt, L. M. (2021). Endocrine disruptor impacts on fish from Chile: The influence of wastewaters. *Frontiers in Endocrinology, 12*, 208.

Bila, D. M., & Dezotti, M. (2007). Desreguladores endcrinos no meio ambiente: Efeitos e consequncias. *Qumica Nova, 30* , 651–666.

Blzquez, M., Gonzlez, A., Papadaki, M., Mylonas, C., & Piferrer, F. (2008). Sex-related changes in estrogen receptors and aromatase gene expression during early development in European sea bass. *General and Comparative Endocrinology, 158*, 95–101.

Blanton, M. L., & Specker, J. L. (2007). The hypothalamic–pituitary–thyroid (HPT) axis in fish and its role in development and reproduction. *Critical Reviews in Toxicology, 37*, 97–115.

Crain, D. A., Rooney, A. A., Orlando, E. F., & Guillette, L. J. (2000). Endocrine disrupting contaminants and hormone dynamics. In L. J. Guillette & D. A. Crain (Eds.), *Environmental endocrine disrupters* (pp. 1–21).

Cunha, S. C., Menezes-Sousa, D., Mello, F. V., Miranda, J. A., Fogaa, F. H., Alonso, M. B., Torres, J. P., & Fernandes, J. O. (2022). Survey on endocrine-disrupting chemicals in seafood: Occurrence and distribution. *Environmental Research, 210*, 112886.

Surana, D., Gupta, J., Sharma, S., Kumar, S., & Ghosh, P. (2022). Advances in removal of endocrine disrupting compounds from aquatic matrices. *Science of the Total Environment, 826*, 154129.

Delbs, G., Blzquez, M., Fernandino, J. I., Grigorova, P., Hales, B. F., & Metcalfe, C. (2022). Effects of endocrine disrupting chemicals on gonad development. *Environmental Research, 204*, 112040.

Ellis, R. J., van den Heuvel, M. R., Stuthridge, T. R., McCarthy, L. H., Ling, N., Hogg, I. D., & Dietrich, D. (2001). Androgenic responses in fish species exposed to wastewater. *Toxicological Sciences*.

Fan, J. J., Wang, S., Tang, J. P., Zhao, J. L., Wang, L., & Wang, J. X. (2019). Bioaccumulation of endocrine disrupting compounds in fish. *Environmental Pollution, 247*, 999–1008.

Folmar, L. C., Hemmer, M., Hemmer, R., Bowman, C., Kroll, K., & Denslow, N. D. (2000). Comparative estrogenicity of estradiol and related compounds. *Aquatic Toxicology, 49*, 77–88.

Fossi, M. C., Casini, S., Marsili, L., Ancora, S., Mori, G., Neri, G., Romeo, T., & Ausili, A. (2004). Ecotoxicological effects of endocrine disruptors in swordfish. *Marine Environmental Research, 58*, 425–429.

Gao, X., Kang, S., Xiong, R., & Chen, M. (2020). Environment-friendly removal methods for endocrine disrupting chemicals. *Sustainability, 12*, 7615.

Geyer, H. J., Rimkus, G. G., Scheunert, I., Kaune, A., Schramm, K. W., & Kettrup, A. (2000). Bioaccumulation of endocrine-disrupting chemicals in organisms.

Ghanem, S. F. (2021). Effects of endocrine disrupting chemicals using zebrafish model. *Egyptian Journal of Aquatic Biology & Fisheries, 25*(5), 951–981.

Gray, M. A., & Metcalfe, C. D. (1997). Induction of testis-ova in Japanese medaka exposed to nonylphenol. *Environmental Toxicology and Chemistry, 16*, 1082–1086.

Harries, J. E., Janbakhsh, A., Jobling, S., Matthiessen, P., Sumpter, J. P., & Tyler, C. R. (1999). Estrogenic potency of sewage effluents. *Environmental Toxicology and Chemistry, 18*, 932–937.

Heiden, T. K., Hutz, R. J., & Carvan, M. J. (2005). Accumulation and reproductive impacts of dioxin in zebrafish. *Toxicological Sciences, 87*(2), 497–507.

Ismail, N. A., Wee, S. Y., & Aris, A. Z. (2017). Endocrine disrupting compounds in aquaculture ecosystems. *Chemosphere, 188*, 375–388.

Ismanto, A., Hadibarata, T., Kristanti, R. A., Maslukah, L., Safinatunnajah, N., & Kusumastuti, W. (2022). EDCs in environmental matrices: Occurrence and treatment. *Environmental Pollution, 302*, 119061.

Jobling, S., & Tyler, C. R. (2003). Endocrine disruption in wild freshwater fish. *Pure and Applied Chemistry, 75*, 2219–2234.

Kavlock, R. J., Daston, G. P., DeRosa, C., Gray, L. E., Kaattari, S., Lucier, G., et al. (1996). Research needs for endocrine disruptors. *Environmental Health Perspectives, 104*(Suppl. 4), 715–740.

Lee, C. C., Jiang, L. Y., Kuo, Y. L., Chen, C. Y., Hsieh, C. Y., Hung, C. F., & Tien, C. J. (2015). Accumulation of nonylphenol and bisphenol A in fish. *Science of the Total Environment, 502*, 417–425.

Liu, Z. H., Dang, Z., Yin, H., & Liu, Y. (2020). Improving wastewater treatment removal of EDCs. *Water Research, 188*, 116469.

Matthiessen, P., Wheeler, J. R., & Weltje, L. (2018). Evidence of endocrine disruption in wildlife. *Critical Reviews in Toxicology, 48*(3), 195–216.

Mller, A. K., Markert, N., Leser, K., Kmpfer, D., Crawford, S. E., & Schffer, A. (2020). Assessing endocrine disruption in freshwater fish. *Environmental Pollution, 257*, 113636.

Pea -Guzmn, C., Ulloa -Snchez, S., Mora, K., Bustos, R. H., Lpez -Barrera, E., Alvarez, J., & Rodrguez -Pinzn, M. (2019). Emerging pollutants in the urban water cycle. *Journal of Environmental Management, 237*, 408–423.

Thacharodi, A., Hassan, S., Hegde, T. A., Thacharodi, D. D., Brindhadevi, K., & Pugazhendhi, A. (2023). Water as a source of endocrine-disrupting chemicals. *Environmental Research*.

Wang, R., Ma, X., Liu, T., Li, Y., Song, L., Tjong, S. C., Cao, L., Wang, W., Yu, Q., & Wang, Z. (2020). Photocatalytic degradation of endocrine disrupting chemicals. *Applied Catalysis A: General, 597*, 117547.

Werkneh, A. A., Gebru, S. B., Redae, G. H., & Tsige, A. G. (2022). Removal of endocrine disrupters: Public health concerns. *Heliyon, 8*, e09206.

Zamri, M. F. M. A., Bahru, R., Suja, F., Shamsuddin, A. H., Pramanik, S. K., & Fattah, I. M. R. (2021). Treatment strategies for EDC removal. *Journal of Water Process Engineering, 41*, 102017.

Zhang, J. N., Ying, G. G., Yang, Y. Y., Liu, W. R., Liu, S. S., Chen, J., Liu, Y. S., Zhao, J. L., & Zhang, Q. Q. (2018). Occurrence and risk assessment of androgens. *Chemosphere, 201*, 644–654.

Zhou, X., Yang, Z., Luo, Z., Li, H., & Chen, G. (2019). Endocrine disrupting chemicals in freshwater fish. *Environmental Pollution, 244*, 462–468.

Zoeller, R. T., Tan, S. W., & Tyl, R. W. (2007). General background on the HPT axis. *Critical Reviews in Toxicology, 37*, 11–53.

Chapter 3

The role of immunogenic cell death (ICD) inducers in enhancing immunotherapy responses

Khadija Nadim[1], Debjit De[1]*

[1]Department of Biotechnology, School of Life Sciences, Swami Vivekananda University, Barrackpore, West Bengal, 700121, India.

***Corresponding email:** debjitd@svu.ac.in*

Abstract

Long thought to be a necessary aspect of a cell's life cycle and, therefore, a familiar consequence of cellular life, the growing body of information supporting regulated cell death (RCD) has altered this notion and brought the field a great deal of attention. In a variety of tumor types, Immune Checkpoint Inhibitors (ICIs) produce a long-lasting response. The low immunogenicity of tumor cells and the Immunosuppressive Tumor Microenvironment (ITM) contribute to the limited response rates of current cancer immunotherapy in a wide range of solid tumours. So, there is growing evidence that certain traditional chemotherapeutic medications cause Immunogenic Cell Death (ICD), a type of cell death, which increases the immunogenicity of stressed and dying cancer cells. A new concept of Immunogenic Cell Death (ICD) has emerged in recent years, despite the previous belief that apoptotic cells, when rapidly phagocytosed, underwent a silent death that did not elicit an immune response. The immunogenic characteristics of ICD are primarily mediated by Damage-Associated Molecular Patterns (DAMPs), which include surface-exposed Calreticulin (CRT), secreted ATP, and released High Mobility Group Protein B1 (HMGB1). ICD inducers and immune checkpoint inhibitors, in particular, can further reverse immunosuppression and stop tumor metastases and recurrences. In this review, we provide the traits and workings of tumor cell ICD responses as well as the DAMPs linked to these responses and the genes involved in the processes can be regulated by drugs or inhibitors to amplify the effects of ICD and therefore serve as potential therapeutic targets.

Keywords: Apoptotic cells, Calreticulin, DAMPs, HMGB1, Immunosuppression, Immunogenic cell death (ICD)**,** Immune checkpoints inhibitors (ICIs), Immunosuppressive Tumor Microenvironment (ITM).

1. Introduction

Immunotherapy is an intriguing therapy for malignancies that relapse and multiply at an era of improvements in cancer treatment. Immunotherapy comprises adoptive lymphocyte medication, which triggers the patient's own antibodies to destroy cancer cells, and the use of monoclonal antibodies, which disrupt immune checkpoint function and enable anti-cancer T cell responses (2). By proving that enhancing the systemic immune response can result in tumor regression, cancer immunotherapy has transformed the paradigm of clinical cancer treatment. Although immunotherapy holds promise for curing malignant tumors, its clinical implementation faces several obstacles. These include inadequate intratumoral infiltration of cytotoxic T lymphocytes (CTLs) and the presence of an immunosuppressive tumor microenvironment (ITM) (1). With the aim to completely eliminate tumor cells and subsequently develop long-term anticancer immunity, immunotherapy attempts to produce a strong immune response that boosts the body's cytotoxic lymphocytes. Dendritic cells (DCs) capture antigens throughout a normal immune response. These cells mature and present antigenic peptide to T cells in lymph nodes in the form of MHC molecules, activating effector T cells that are delivered toward infection, inflammation, or wreckage sites (2). Numerous clinical investigations are actively investigating immune markers for cancer immunotherapy. However, the effectiveness of immunotherapy depends on the functional competence of several immunologic components, in contrast to small-molecule inhibitors, for which the existence or absence of a target mutation may predict response. For instance, methods intended to utilize endogenous T cells' anticancer potential depend on the appropriate performance of a set of actions outlined in the cancer immunity cycle (4). With the objective to increase the effectiveness of cancer immunotherapy, prior research has largely concentrated on strategies for safeguarding tumor-infiltrating T lymphocyte (TIL) activity by reducing the immunosuppressive tumor microenvironment (TME), which includes T-cell suppressors, hypoxia, low PH, and inhibitory immune checkpoints. These strategies assume that adaptive immunity will commence once antigen-presenting cells (APCs) have proficiently primed T cells. Several strategies have been tried to increase intratumoral immunogenicity by activating innate immunity employing oncolytic viruses, thermo-active and photo-active nanoparticles, and similar materials in order to produce a tumor micro environment (TME) that is conducive to antitumor immunity. Attempts to trigger antitumor immunity by causing immunogenic cell death (ICD) in tumors have garnered a lot of attention, especially when it was shown that novel controllable forms of lytic cell death might inflame tumor tissues (3).

Being merely immunogenic is one method tumors avoid the immune response. Antigens can be expressed by cancer cells, however they are unable to differentiate them from tolerated self-antigens. These tumors often create few de novo antigens and have low mutation rates. Examples include ovarian cancer, glioblastoma, and other malignancies that either produce anti-inflammatory cytokines to create an immunosuppressive tumor microenvironment or lack stimulatory cancer neoantigens. The fact that certain cancer treatments induce apoptotic cell death, which may be immunologically quiet and can also impair immunity, making cancer recurrence possible, exacerbates this issue. By progressing DCs, activating CTLs, and boosting NK cell cytotoxicity, the induction of ICD may potentially repurpose these dying cancer cells into "vaccines" that promote anticancer immunity (2). Immunogenic cell death (ICD) represents a different strategy that triggers antitumor immunity to eliminate tumor cells. To stimulate the immune response, tumor cells experiencing ICD release distress signals via damage-associated molecular patterns (DAMPs) such as calreticulin (CRT), adenosine triphosphate (ATP), and high-mobility group box 1 (HMGB1). These DAMPs aid in the cancer immunity cycle (1).

2. Immunogenic cell death

The most promising treatment for cancer is cancer immunotherapy, an innovative approach that promises to eradicate both local and distant metastatic tumors while also inducing long-term immunological memory to prevent cancer recurrence. On the other hand, cancer cells can diminish immunogenicity by down-regulating surface antigens and inhibit effector T cell activity by increasing the production of immunosuppressive substances. Tumor cells' poor immunogenicity will make it difficult for immune cells to recognize and absorb them. Thankfully, it has been shown that some physical techniques (like photodynamic therapy (PDT) and photothermal therapy (PTT)) and chemical medications (such anthracyclines and oxaliplatin (OXA)) might cause tumor cell death in an immunogenic way (5). Enhancing a tumor's capacity to elicit an immune response is known as promoting its immunogenicity. Antigenicity and adjuvanticity are two of the most significant elements that might affect immunogenicity. When a tumor is antigenic, it might produce antigen targets that the host is unable to tolerate. Since many cancers are genetically unstable, neoantigens, viral oncoproteins, and overexpressed self-antigens can all be expressed to produce antigenicity. The presence of danger-associated molecular patterns (DAMPs) causes adjuvanticity by triggering the innate and adaptive immune systems to become activated once APCs recognize the tumor antigen(16). Tumor

immunotherapy comprises small-molecule inhibitors, cancer vaccines, cell treatments, therapeutic antibodies, and immune checkpoint inhibitors based on monoclonal antibodies. These immunotherapeutic classes have shown strong anticancer promise in clinical trials, and their high efficacy and cutting-edge therapeutic approaches have opened up new avenues for tumor clinical therapy. They are regarded as the most promising approaches to treating tumors. To regulate the development of tumors, tumor cell death is necessary and falls under two categories: regulated cell death (RCD) and accidental cell death (ACD). ACD is the result of the cell shortcomings self-regulation injury stimulus exceeding the cell death threshold, whereas RCD is the result of having the ability to self-regulate this process through controlled pharmaceutical or genetic interventions. RCD is differentiated from ACD by the having controlled, characterized, and directed molecular processes through defined specific pathways governing this system. RCD encompasses (but is not exclusively) autophagy, necrosis, and apoptosis. RCD is also an incredibly powerful antitumor pathway because it can be triggered by drugs that counteract tumor cell drug resistance, increase cell death, and improve the overall therapeutic effect of the drug (6).

The process by which tumor cells change from non-immunogenic to immunogenic is known as immunogenic cell death (ICD). In vivo, apoptosis takes place and triggers an antitumor immune response, which kills more tumor cells and stops the tumor from growing. A 2005 study that employed Adriamycin in a mouse tumor model both in vitro and in vivo was the first to propose immunogenic cell killing. In the mouse model, the medication caused an ICD response, which resulted in DC maturation and activation, cytotoxic T lymphocyte proliferation, and the death of many tumor cells, according to the researchers (6).Tumor antigens, damage-associated molecular patterns (DAMPs), and pro-inflammatory cytokines are released during immunogenic cell death (ICD). This allows immune cells to absorb and present tumor antigens, which in turn triggers an antitumor immune response that is specific to the antigen. Furthermore, cancers can deteriorate and spread severely when effector T cells are inhibited at tumor sites. Crucially, immune checkpoint blockade immunotherapy has been shown to be successful in treating a range of malignancies and has been used to alleviate immunosuppression and restore immune cells' anti-tumor function. Programmed death-1/programmed death ligand-1 (PD-1/PD-L1), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), indoleamine 2.3-dioxygenase (IDO), and CD47 are the most commonly employed immunological checkpoints. Immunocheckpoint inhibitors and ICD inducers used together will be a powerful tactic to inhibit tumor growth and stimulate anticancer immune responses (5). The release of molecules with danger-

associated molecular patterns (DAMPs) is a hallmark of ICD. High mobility group box 1 (HGMB1) protein release from the nucleus and membrane-bound calreticulin (CRT) are the DAMPs most frequently linked to ICD. During ICD, the cell surface has also been seen to contain heat shock proteins (HSPs) 70 and 90. For phagocytes like DCs, CRT and similar substances act as "eat me" cues, increasing DC maturation and antigen uptake. CRT normally keeps calcium ion (Ca2+) balance and is found in the endoplasmic reticulum (ER). CRT has a section for retention in the ER lumen and is made up of three domains with varying affinities for calcium binding. Chaperoning proteins, calcium release and storage, and integrin-mediated control of cell adhesiveness are among the roles of CRT. Important immunological processes like antigen processing and presentation, as well as defense against anoikis, are also carried out by CRT. Although the exact mechanism of CRT exposure during ICD induction is uncertain, it might be related to the absence of a functional ER retention domain. Although CRT is a premortem signal, dying or stressed cells release HMGB1, another DAMP that is induced by ICD, premortem. Due to shuttling, HMGB1 is often present in the nucleus with some cytoplasmic localization (2). Numerous factors impact immunogenic cell death responses. First, the TME becomes immunosuppressed, tumor cells express inhibitory receptors, immunosuppressive cells release inhibitory cytokines, and the ICD response must get past the immunosuppressive TME and draw in activated antigen-presenting cells during carcinogenesis. The antigenicity of the reactions is the second element. Unlike normal cells, which lack this sort of antigenic epitope, malignant and infected cells can express highly immunogenic antigenic epitopes that are not covered by tolerance, which limits the capacity of normal cells to trigger the ICD response. Lastly, the release or exposure to several DAMPs typically coincides with the adjuvant beginning of ICD. These consist of heatshock proteins (HSP), adenosine triphosphate (ATP), high mobility group box 1 protein (HMGB1), and calreticulin (CRT) (6).

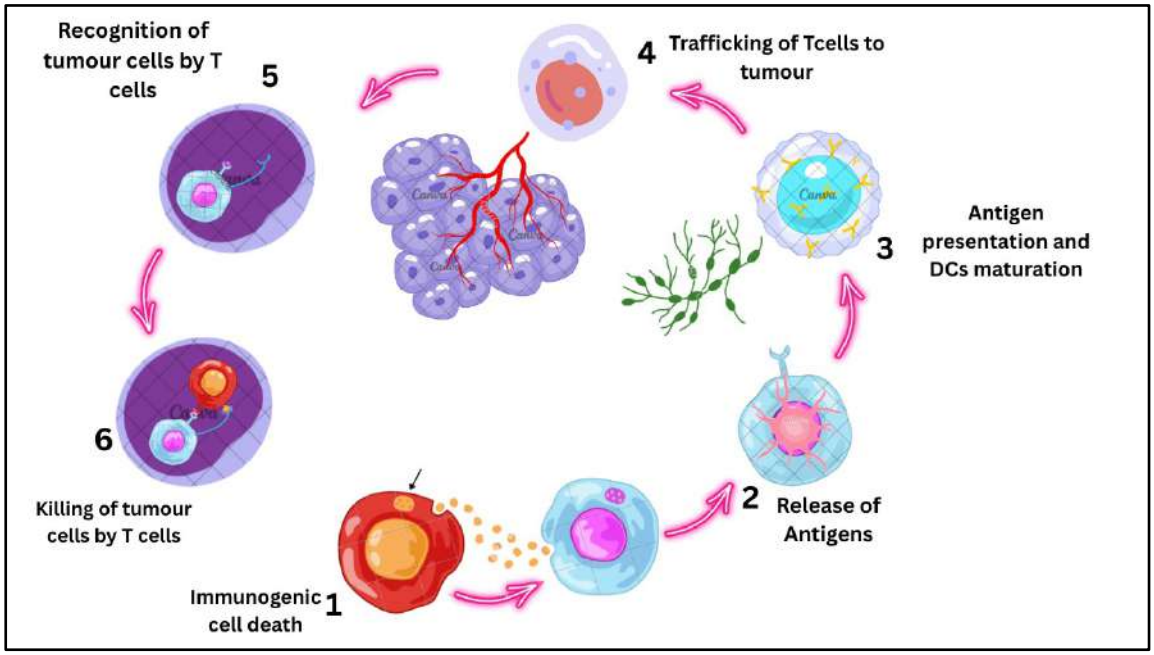


Figure 1: ICD inducers combined with immune checkpoint inhibitors

3. FEATURES OF ICD

3.1. HMGB1 Release: The non-histone nuclear protein HMGB1, which belongs to the high mobility group (HMG) subfamily, has crucial roles in transcriptional control and chromatin structure (3). In

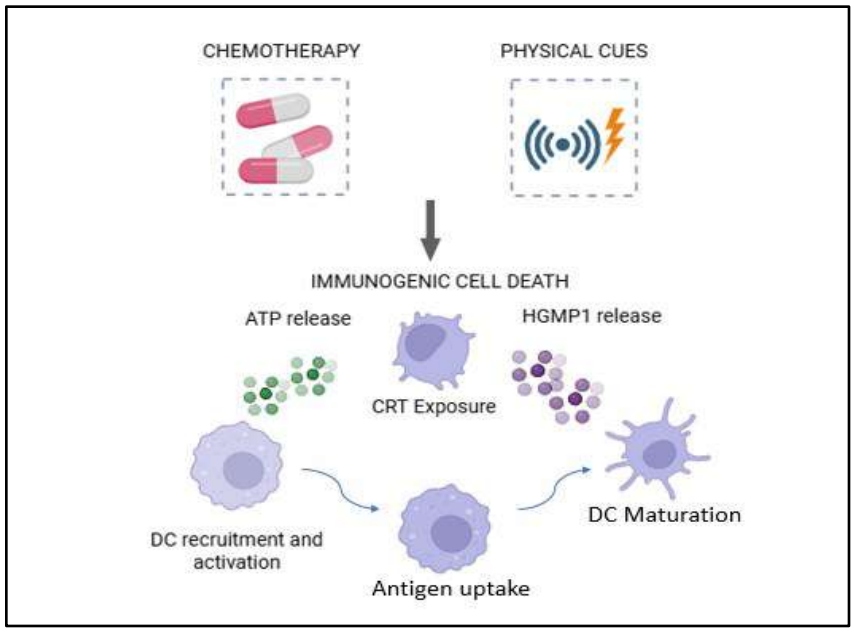


Figure 2: HMGB1 and ATP in tissue repair.

order to preserve nuclear homeostasis, it plays a role in DNA transcription, replication, and repair. It takes role in signal transduction, differentiation, and cell proliferation. On the other hand, it causes tumor cells to undergo autophagy and apoptosis and activates lymphocytes that have infiltrated the tumor to generate an immune response against the tumor(7). In response to pathogenic agents, cells of the innate immune system can actively express HMGB1, a highly conserved nuclear protein that is broadly distributed in mammalian cells. Injured cells can also release this protein when they undergo necrosis and mechanical damage(5). When cells are physically damaged and necrotic, it is released extracellularly. It is typically bound to intracellular chromatin. In tumor cells, HMGB1 is produced extracellularly during the ICD response in order to attach to pattern recognition receptors (PRRs), such as receptors of advanced glycation end products (RAGE) and Toll-like receptors(6). HMGB1 can either actively be secreted via vesicular transport that is triggered by cellular stress signals or passively dispersed through a torn cell membrane during ICD. Following its identification by pattern-recognition receptors (PRRs), released HMGB1 can trigger the activation of different immune cells. Particularly, released HMGB1 can attach to macrophage TLR4 and trigger the NK-kB signaling pathway, which raises phagocytic activity and inflammatory cytokine expression. The maturation of dendritic cells and macrophages is another way whereby the HMGB1/PI3K/Akt/mTOR axis enhances antigen-presenting capacity (3). This shows that HMGB1's extracellular expression is an essential indicator of the tumor cells' ICD resistance progression (6).

3.2. CRT Exposure

A multipurpose endoplasmic reticulum protein, calcreticulin (CRT) plays a role in a variety of cellular functions, including calcium homeostasis, chaperoning, cell adhesion, and, ultimately, the development and spread of cancer (8). For the synthesis and chaperoning of membrane-associated and secreted proteins, the endoplasmic reticulum (ER) is essential. The membrane plays a significant role in the release and storage of Ca2+ (9). Calreticulin (CRT), the primary calcium-binding protein in the endoplasmic reticulum, is found in all cells except erythrocytes and is extremely conserved. It has biological roles that include controlling gene expression, maintaining Ca2+ homeostasis, and acting as a molecular chaperone (6). A portion of CRT will move from the ER lumen to the surfaces of stressed and dying cells when cancer cells are exposed to ICD inducers. This phenomenon may result from the phosphorylation of eukaryotic translation initiation factor eIF2α by PKR-like ER kinase, which is part of the ER stress response triggered by particular chemotherapeutic drugs (5). CRT

appears on the cell surface of vesicles CRT-transported from the Endo-plasmic Reticulum and are then transported to the golgi apparatus from where CRT is then transported to the cell surface. CRT creates an "eat-me" signal. cell surface CRT is recognized by CD91 which then phagocytoses CRT. This phagocytosis triggers maturation and activation of dendritic cells. Cross presentation of tumor antigens is then initiated together with the activation of a tumor specific cytotoxic T- cell and a release of pro inflammatory cytokines such as TNFa and IL-6 on the cytotoxic T cell. The ongoing enhancement and the proinflammatory cytokines are mediated by various pathways to boost the anticancer immune response (6). CRT also performs the equally important and empowering top immunological functions of antigen and defending against anoikis. The exact underlying mechanism resulting in CRT exposure is during induction of immunogenic cell death and is unknown however it may be due to the lack of a functional ER Retention domain (2). This suggests that CRT exposure and translocation and during ICD is due of strong immune response against the tumor resulting in a hallmark of ICD activation of innate and adaptive anticancer immunity (6).

3.3. Adenosine triphosphate

ATP is the most abundant intracellular metabolite and plays important neurotransmission, Besides, it can be released from cells under physical or chemical stresses, such as those caused by cytotoxic agents, hypoxia, mechanical distortion, or plasma membrane damage (5). When ICD is generated, ATP is released into the extracellular space as a warning signal known as eATP, just as other cDAMPs. By encouraging the creation of the NLRP3 inflammasome and triggering the release of IL-1β, a caspase 1-mediated inflammatory cytokine, eATP has been shown to facilitate chemotaxis to immune cells. Numerous mechanisms have been investigated for the release of eATP. According to recent research, the ER-Golgi secretion route allows dying cancer cells to release eATP as well. The eATPs that dying cancer cells release as "find me" signals can attach to either P2X7R, which is mostly expressed in mast cells, macrophages, and DCs, to enhance IFN-γ production and CD8+ T-cell priming, or P2Y2R of macrophages, which stimulates phagocytic activity to destroy cancer cells (3). Tumor cell-released ATP is favorably connected with the recruitment of monocytes, macrophages, and DCs in response to particular chemotherapy drugs, triggering antitumor immune responses (5). Candidate plasma membrane ATP channels include members of the connexin family of gap junction proteins and the nonjunctional channel Px1 (10). The eradication of many tumor cells using various

methods is proof that the extracellular release of ATP is an important indication of the ICD response in tumor cells. Furthermore, it strengthens the host's defense against malignancies (6).

3.4. DAMPs

Necrosis resulting from physicochemical damage to tissues and cells was first linked to the emission of DAMPs. However, because of their connection with the immune system, DAMPs have recently been shown to be actively released from dying apoptotic cells and to play a useful role in anti-cancer therapy. Due to the fact that some cellular stress -induced DAMPs, such as HGMB1, can be oxidized in a deactivated fashion, or undergo caspase-dependent proteolysis, as interleukin-33 (IL-33) does, there is not always a triggering of an immunological response (11). Meanwhile, the identification of uric acid crystal's and high mobility group box 1 (HMGB1) as damp phenomena is the reason why the "Danger theory" has remained a theory. Damage-associated molecules are patterns of damage molecules that have a physiological role during the day inside the cell and an additional function of signalling disrupted cell communication while outside. It is crucial to remember that under normal conditions, the immune system cannot see DAMPs. When they are released outside the cell during an immune response, their visibility rises (12). Under typical circumstances, cDAMPs are recognized to have unique intracellular roles. However, cDAMPs are loaded onto the plasma membrane or released extracellularly to act as alarm signals that activate innate immune cells when cells are injured. cDAMPs can be recognized by receptors found on dendritic cells and macrophages. The signaling between ligands and receptors is programmed to activate them. The activation of antitumor immunity depends critically on the synergistic interaction between iDAMPs generated by intracellular signal transduction pathways and secreted cDAMPs. As a result, lytic cell death combined with iDAMP induction is considered a useful ICD (3).

4. Strategies to induce icd for overcoming multi-drug resistance

Chemotherapy is metastatic tumors first line treatment. However, among cancer, there is a phenomenon called multidrug resistance, a principal way cancer cells resist effective treatment. Over the past 30 years of studying the multidrug resistance phenomena, researchers have discovered countless ways in which cancer cells resist the treatments, including the most recent ones. Because of this, one could argue that the ability to predict and circumvent drug resistance would allow for the improvement of chemotherapy treatment (17). One of the most important reasons cancer

chemotherapies is ineffective is due to the presence of multidrug resistance. Tumour cell resistance to a broad range of chemotherapeutic medications can be summed up as cancer cells that are resistant to multiple drugs. Acquired multi-drug resistance in cancer cells has been demonstrated to result from either increased expression of therapeutic targets or activation of alternative compensatory signalling pathways and occasionally as a result of therapy-induced selection of minority resistant populations caused by a high level of molecular heterogeneity (13). Current cancer therapies are limited in their effectiveness by tumor resistance to molecularly targeted medicines and chemotherapy. Pharmacokinetic effects (absorption, distribution, metabolism, and elimination, or ADME) limit the quantity of medicine that reaches the tumor, and toxicity to normal tissues restricts the amount that may be delivered systemically. Many resistance mechanisms, including enhanced drug efflux, drug target mutations, DNA damage repair, activation of alternative signaling pathways, and cell death avoidance, can function at the tumor level (19).

Several methods can be used to develop this medication resistance: 1) increased drug efflux due to transporters that rely on ATP 2) decreasing the rate of medication absorption 3) improving the way that cytochrome P450 and glutathione S-transferase enzymes metabolize and eliminate drugs 4) inhibiting the apoptotic pathway by p53 pathway mutations and the overexpression of anti-apoptotic genes such as Bcl2 and AKT 5) Regulation of miRNAs and epigenetic modifications 6) Changes to the goals of drugs 7) alterations in the microenvironment brought on by hypoxia or the control of cancer stem cells. Autophagy inhibition was the obvious treatment that was first considered as a cure for MDR with the goal of making cancer cells more sensitive to chemotherapy. However, as previously stated, unchecked autophagy results in the death of cancer cells; hence, this can be exploited as a treatment in which sustained autophagy activation causes autophagic cell death in MDR cancer cells that lack apoptosis. A variety of anticancer therapies can be effective kill cancer cells through the induction of excessive autophagy. Resultantly, autophagy suppression and or unmitigated autophagy can be harnessed to kill MDR cancer cells that are unresponsive to apoptosis. In light of the prior discussion, the autophagy inhibition as a strategy to overcome the resistant phenotype of malignant cells, facilitate multiple drug resistance, and induce CD, or immunogenic cell death, are all recommended. However, for resistant malignancies, another therapeutic option is the retriggering of apoptosis in the tumor cells (13).

5. Conclusion

An important factor in determining the biology of tumors is the immune system. For a long time, cancer treatment has relied on methods that target tumour cells directly. The treatment known as cancer immunotherapy, which uses the patient's immune system to combat the disease, is becoming a significant supplement to traditional medicines. Only some patients have benefited clinically from strategies that help the immune system regain its ability to identify and destroy cancerous cells. This variation in treatment response can be a result of tumors' capacity to create an immunosuppressive microenvironment and lose their antigenicity and immunogenicity in response to immunological pressure. So therefore, an immunosuppressive TME develops during tumor treatment because tumor cells and tumor-killing immune cells are not very immunogenic. The TME and tumor cells' immunogenicity are enhanced by the use of ICD inducers. Large amounts of CRT, ATP, HMGB1, and other DAMPs are generated during ICD induction, which promotes the initiation of the ICD response in tumor cells, activates ICD-related signaling pathways, and sets off the endoplasmic reticulum stress response. The ICD response results in a more robust antitumor response, enhances cytotoxic T lymphocyte infiltration, and encourages DC maturation and activation (6). Dying cancer cells generate ICD-associated DAMPs to facilitate communication between the innate immune system and the cells undergoing ICD. APCs can increase antigenicity through better tumor antigen presentation via MHC. Therefore, ICD and DAMP signaling are interesting targets for more potent cancer treatments, either alone or in combination. Inducers of ICD trigger various cellular stress responses. One of them is autophagy. ER stress leads to various forms of cell death, including classical apoptosis, as well as regulated or unregulated necrosis, which in turn releases DAMPs required to stimulate the anticancer immune response. These substances do have to act in a specific order, therefore the DAMP release kinetics should be highlighted.

Consequently, the ideal ICD drug should minimize the chances of autoinflammatory responses in healthy tissues while also inhibiting the TME's immunosuppressive responses. By enhancing APCs recruitment via “find me or eat me signals,” this ICD inducer should be able to target both localized and advanced disease while ultimately re-establishing the activation of anti-tumor T cells. The presence of these drugs is likely to produce an adaptive immune response due to enhanced antigen presentation and stimulation of the production of immune-activating cytokines. The resulting adaptive immune response and features of immunological memory would be invaluable in preventing relapses

in cancer patients and would be of great adaptive immune response in the long-term clinical outcomes of ICD-inducing anticancer therapy.

Reference

Beatty, G. L., & Gladney, W. L. (2015). Immune escape mechanisms as a guide for cancer immunotherapy. *Clinical cancer research*, *21*(4), 687-692.

Choi, M., Shin, J., Lee, C. E., Chung, J. Y., Kim, M., Yan, X., ... & Cha, J. H. (2023). Immunogenic cell death in cancer immunotherapy. *BMB reports*, *56*(5), 275.

Farkona, S., Diamandis, E. P., & Blasutig, I. M. (2016). Cancer immunotherapy: the beginning of the end of cancer?. *BMC medicine*, *14*(1), 73.

Gao, J., Wang, W. Q., Pei, Q., Lord, M. S., & Yu, H. J. (2020). Engineering nanomedicines through boosting immunogenic cell death for improved cancer immunotherapy. *Acta Pharmacologica Sinica*, *41*(7), 986-994.

Gottesman, M. M., Fojo, T., & Bates, S. E. (2002). Multidrug resistance in cancer: role of ATP–dependent transporters. *Nature reviews cancer*, *2*(1), 48-58.

Gupta, G., Borglum, K., & Chen, H. (2021). Immunogenic cell death: a step ahead of autophagy in cancer therapy. *Journal of cancer immunology*, *3*(1), 47.

Holohan, C., Van Schaeybroeck, S., Longley, D. B., & Johnston, P. G. (2013). Cancer drug resistance: an evolving paradigm. *Nature Reviews Cancer*, *13*(10), 714-726.

Krysko, D. V., Garg, A. D., Kaczmarek, A., Krysko, O., Agostinis, P., & Vandenabeele, P. (2012). Immunogenic cell death and DAMPs in cancer therapy. *Nature reviews cancer*, *12*(12), 860-875.

Krysko, D. V., Garg, A. D., Kaczmarek, A., Krysko, O., Agostinis, P., & Vandenabeele, P. (2012). Immunogenic cell death and DAMPs in cancer therapy. *Nature reviews cancer*, *12*(12), 860-875.

Lazarowski, E. R., Sesma, J. I., Seminario-Vidal, L., & Kreda, S. M. (2011). Molecular mechanisms of purine and pyrimidine nucleotide release. *Advances in pharmacology*, *61*, 221-261.

Michalak, M., CORBETT, E. F., MESAELI, N., NAKAMURA, K., & OPAS, M. (1999). Calreticulin: one protein, one gene, many functions. *Biochemical Journal*, *344*(2), 281-292.

Qi, J., Jin, F., Xu, X., & Du, Y. (2021). Combination cancer immunotherapy of nanoparticle-based immunogenic cell death inducers and immune checkpoint inhibitors. *International Journal of Nanomedicine*, 1435-1456.

Radogna, F., & Diederich, M. (2018). Stress-induced cellular responses in immunogenic cell death: Implications for cancer immunotherapy. *Biochemical pharmacology*, *153*, 12-23.

Showalter, A., Limaye, A., Oyer, J. L., Igarashi, R., Kittipatarin, C., Copik, A. J., & Khaled, A. R. (2017). Cytokines in immunogenic cell death: applications for cancer immunotherapy. *Cytokine*, *97*, 123-132.

Tian, Z., Zhu, L., Xie, Y., Hu, H., Ren, Q., Liu, J., & Wang, Q. (2024). The mechanism of high-mobility group box-1 protein and its bidirectional regulation in tumors. *Biomolecules and Biomedicine*, *24*(3), 477.

Vnreau, E., Ceriotti, C., & Bianchi, M. E. (2015). DAMPs from cell death to new life. *Frontiers in immunology*, *6*, 422.

Wang, M., Wang, S., Desai, J., Trapani, J. A., & Neeson, P. J. (2020). Therapeutic strategies to remodel immunologically cold tumors. *Clinical & Translational Immunology*, *9*(12), e1226.

Xie, D., Wang, Q., & Wu, G. (2022). Research progress in inducing immunogenic cell death of tumor cells. *Frontiers in immunology*, *13*, 1017400.

Zamanian, M., Veerakumarasivam, A., Abdullah, S., & Rosli, R. (2013). Calreticulin and cancer. *Pathology & Oncology Research*, *19*(2), 149-154.

Chapter 4

Bacterial microcapsule production and its bioremediation capacity: recent advances, mechanisms, and environmental applications

Rupesh Dutta Banik[1*]

[1]*Department of Microbiology, School of Life Sciences, Swami Vivekananda University, Barrackpore, West Bengal – 700121, India*

**Corresponding author: rupeshduttabanik99@gmail.com*

Abstract

Microcapsules — microscale core–shell structures in which an active biological or chemical payload is enclosed within a protective polymeric membrane — have attracted escalating scientific attention as precision tools for environmental remediation. When bacteria or their metabolic products constitute the encapsulated core, the resulting structures, termed bacterial microcapsules, confer exceptional advantages over free-cell systems: protected viability during storage and deployment, controlled and sustained release of remediation-active metabolites, enhanced resistance to environmental stresses such as pH extremes, desiccation, and heavy metal toxicity, and the ability to target specific contaminated micro-environments. This chapter provides a comprehensive review of the state-of-the-art in bacterial microcapsule production and its application to bioremediation of industrial wastewater, heavy metal-laden effluents, petroleum hydrocarbons, synthetic dyes, and emerging organic contaminants. We examine encapsulation technologies — including spray drying, coacervation, ionic gelation, and electrospray methods — wall materials commonly used for bacterial encapsulation, the principal mechanisms by which encapsulated bacteria degrade or sequester pollutants, and the performance of encapsulated systems across key industrial sectors. We further critically evaluate the limitations of current technologies and delineate future research priorities encompassing smart responsive microcapsules, synthetic biology-assisted strain engineering, and scale-up strategies for industrial deployment.

Keywords: bacterial microencapsulation, bioremediation, controlled release, heavy metals, hydrocarbon degradation, ionic gelation, spray drying, synthetic dyes, emerging contaminants, wall materials

1. Introduction

The exponential expansion of industrial activity over the past century has resulted in the widespread contamination of soil and aquatic environments with a heterogeneous suite of recalcitrant pollutants, encompassing heavy metals, polycyclic aromatic hydrocarbons (PAHs), chlorinated solvents, synthetic dyes, pharmaceuticals, and endocrine-disrupting compounds (Dutta et al., 2021; Saeed et al., 2022). The severity of this global pollution crisis is reflected in estimates that more than 80% of the world's wastewater is discharged untreated into receiving water bodies, causing irreversible damage to aquatic ecosystems and posing acute threats to human health (Ojha et al., 2021). Conventional physicochemical remediation approaches — chemical precipitation, activated carbon adsorption, membrane filtration, and incineration — while effective for certain pollutant classes, are burdened by prohibitive costs, high energy consumption, secondary pollution through sludge or gaseous by-products, and limited applicability to complex, multi-contaminant matrices (Banik et al., 2023; Luka & Highina, 2018).

Bioremediation, harnessing the intrinsic metabolic diversity of microorganisms to degrade, transform, or immobilize environmental contaminants, has emerged as a scientifically compelling and economically attractive alternative (Abatenh et al., 2017; Singh et al., 2022). Bacteria, by virtue of their catalytic versatility, rapid adaptability, and amenability to genetic manipulation, occupy the centre of bioremediation research. However, the direct application of free bacterial cells to contaminated environments is frequently impeded by a cluster of interrelated challenges: loss of viability upon exposure to high pollutant concentrations, desiccation, UV radiation, or predation; rapid dispersal from target zones by hydrodynamic forces; competitive exclusion by indigenous microflora; and regulatory concerns surrounding the environmental release of non-native or engineered strains (Bashan, 1998; Schoebitz et al., 2012).

Microencapsulation technology, which encloses living bacteria or their bioactive products within a semipermeable polymeric shell at the micrometre scale (typically 1– 1000 μm diameter), addresses these limitations in a single integrated platform. The encapsulating membrane simultaneously protects the bacterial payload from hostile environmental conditions, creates a microenvironment conducive

to metabolic activity, modulates the release kinetics of enzymes and metabolites into the surrounding medium, and facilitates the recovery and reuse of the biocatalytic system (Prakash et al., 2011; Cassidy et al., 1996). Since the pioneering demonstration by Cassidy et al. (1996) that bacteria encapsulated in k-carrageenan beads retained superior 2,4-dichlorophenoxyacetic acid (2,4-D) degradation activity compared to free cells under soil conditions, the field has advanced substantially, driven by innovations in biomaterial science, polymer chemistry, and microfluidics.

Recent years have witnessed particularly rapid progress, including the development of stimuli-responsive 'smart' microcapsules that release their bacterial payload in response to specific pollutant concentrations or pH shifts, the integration of nano-scale materials into capsule walls to enhance mechanical strength and pollutant sorption, and the use of synthetic biology to engineer encapsulated strains with expanded substrate ranges and in-built biosensing circuits (Bhattacharjee et al., 2017; Wang et al., 2020). This chapter synthesises these developments within the broader context of industrial wastewater and environmental remediation, providing researchers and environmental engineers with a critical, up-to-date reference on bacterial microcapsule-mediated bioremediation.

2. Technologies for Bacterial Microencapsulation

2.1 Ionic Gelation and Extrusion

Ionic gelation, most commonly achieved through the dropwise extrusion of a bacterial suspension in sodium alginate solution into a calcium chloride hardening bath, remains the most widely employed method for whole-cell bacterial encapsulation, owing to its mild, aqueous, solvent-free conditions that preserve cell viability at encapsulation efficiencies frequently exceeding 90% (Lee & Mooney, 2012). Upon contact with Ca^{2+} ions, alginate chains crosslink instantaneously to form a stable three-dimensional hydrogel network. The resulting beads are optically transparent, mechanically compliant, and permeable to small molecules including oxygen, nutrients, and metabolic products, while excluding larger molecules such as proteases and predatory microorganisms. Bead diameter is controlled by needle gauge, extrusion pressure, and alginate concentration, with conventional extrusion producing beads of 1–4 mm, and electrospray or vibrating-nozzle technologies reducing this to 100–500 μm (Niu et al., 2020).

A significant limitation of alginate capsules is their susceptibility to dissolution in the presence of chelating agents (phosphate, citrate) or monovalent cation-rich media that compete with Ca^{2+} for

alginate binding sites. Coating alginate beads with cationic polymers such as chitosan or poly-L-lysine (PLL) significantly enhances chemical stability and provides an additional barrier to contamination. The resulting alginate-chitosan or alginate-PLL microcapsules have demonstrated sustained viability of encapsulated Pseudomonas putida mt-2 over 90 days of storage at 4C, with toluene degradation activity fully recoverable upon deployment (Moslemy et al., 2002). Carrageenan, gellan gum, and xanthan gum represent alternative gelling polysaccharides with distinct gelation mechanisms and permeability profiles, each suited to specific application requirements (Prakash et al., 2011).

2.2 Spray Drying

Spray drying is a continuous, highly scalable encapsulation process in which an aqueous bacterial suspension containing dissolved wall material is atomised into a stream of hot, dry gas (typically air at 150–200C inlet temperature), producing dry powder m icrocapsules in a single step. Despite the apparent incompatibility of heat with bacterial survival, the rapid evaporative cooling of droplets limits exposure time to lethal temperatures, and the use of protective agents — including skim milk, trehalose, sucrose, and whey protein — maintains viabilities of thermotolerant species such as Bacillus subtilis, Lactobacillus, and spore-forming strains at 10^6–10^8 CFU/g after drying (Sunny-Roberts & Knorr, 2009). Spray-dried bacterial microcapsules are attractive for field applications because of their low water activity, compact form, extended shelf life at ambient temperature, and compatibility with conventional agronomic or remediation delivery equipment.

Recent advances in spray drying for remediation-focused applications include the use of whey protein isolate-maltodextrin shell systems to encapsulate petroleum-degrading Rhodococcus ruber, achieving 85% retention of alkane hydroxylase activity after drying and demonstrating effective crude oil degradation upon rehydration in contaminated sand columns (Trindade et al., 2005). Two-fluid and three-fluid nozzle configurations now enable the simultaneous atomisation of core and wall material streams, producing true core–shell capsules with improved encapsulation efficiency relative to matrix-type spray-dried particles.

2.3 Coacervation

Complex coacervation exploits electrostatic attraction between oppositely charged polyelectrolytes — typically a cationic polymer such as gelatin and an anionic counterpart such as gum arabic or

carboxymethyl cellulose — to deposit a dense, continuous polymer-rich phase around suspended bacteria as the pH is adjusted to reduce net charge. Crosslinking with glutaraldehyde or transglutaminase subsequently hardens the coacervate shell, yielding microcapsules with exceptionally smooth, thick walls and low permeability to water-soluble molecules (Nesterenko et al., 2013). Coacervation is particularly well suited to the encapsulation of hydrophobic bioremediation agents such as biosurfactant-producing bacteria, as the interfacial properties of the coacervate shell promote interaction with non-aqueous phase liquids (NAPLs) in contaminated soil and sediment matrices.

2.4 Electrospray and Microfluidic Encapsulation

Electrospray techniques apply a high-voltage electric field (5–15 kV) between a liquid-loaded capillary and a collection bath, drawing the bacterial-polymer suspension into a Taylor cone from which monodisperse droplets are ejected at the micrometre scale. Electrospray offers unmatched control over capsule size distribution (coefficient of variation < 5%), superior to extrusion and coacervation methods, and has been used to produce alginate microcapsules loaded with Burkholderia cepacia and Pseudomonas fluorescens at diameters of 150–350 μm with encapsulation efficiencies above 95% (Niu et al., 2020). Microfluidic chip-based encapsulation, in which bacterial suspensions are co-flowed with photocrosslinkable hydrogel precursors through microfabricated channels, produces capsules with programmable multi-layer architectures and enables continuous, sterile production at rates compatible with laboratory-scale remediation trials (Bhattacharjee et al., 2017).

3. Wall Materials for Bacterial Microcapsules in Bioremediation

The physicochemical properties of the encapsulating wall material are the primary determinant of capsule performance in bioremediation applications, governing mechanical stability, permeability to nutrients and metabolites, compatibility with the bacterial surface, resistance to environmental degradation, and release kinetics. The most important natural and synthetic wall materials are summarised in Table 1.

Table 1. Commonly used wall materials for bacterial microcapsules in bioremediation applications.

Among natural polysaccharides, alginate remains predominant due to its unmatched biocompatibility and ease of gelation, but its poor mechanical strength and susceptibility to dissolution in phosphate-buffered media have driven the development of composite wall systems. Chitosan-coated alginate beads present a particularly versatile architecture: the cationic chitosan outer layer serves dual functions as a structural reinforcement and as a heavy metal sorbent, with amino groups ($-NH^{3+}$) providing metal-binding sites that concentrate pollutants at the capsule surface for subsequent intracellular uptake by the enclosed bacteria (Wang et al., 2020). Synthetic biodegradable polymers, particularly poly-(lactic-co-glycolic acid) (PLGA), offer programmable degradation rates and well-characterised drug-release kinetics that can be adapted for controlled temporal release of bacterial metabolites into contaminated environments (Bhattacharjee et al., 2017).

4. Mechanisms of Pollutant Removal by Encapsulated Bacteria

4.1 Enzymatic Biodegradation

The primary mechanism by which encapsulated bacteria remediate organic pollutants is enzymatic biodegradation, wherein extracellular or intracellular enzymes catalyse the oxidative or reductive transformation of pollutant molecules into less toxic, more mineralizable intermediates. Oxygenases (mono- and dioxygenases) encoded by catabolic plasmids — exemplified by the TOL plasmid of Pseudomonas putida mt-2, which encodes the xyl pathway for toluene, xylene, and benzoate catabolism — are the principal initiating enzymes in aerobic aromatic hydrocarbon degradation (Williams & Sayers, 1994). Within the capsule microenvironment, oxygen concentration gradients created by the metabolic activity of inner cell layers can establish both aerobic and microaerophilic niches, enabling sequential oxidative and reductive transformation steps within a single capsule.

The semipermeable capsule wall modulates substrate access to the enclosed bacteria by controlling the diffusion of pollutant molecules. For hydrophobic pollutants such as PAHs and chlorinated biphenyls, which partition preferentially into the polymer matrix, this concentration effect can increase local substrate availability at the bacterial surface relative to the bulk aqueous phase, enhancing apparent degradation rates. Biosurfactants secreted by encapsulated strains such as Bacillus subtilis and Pseudomonas aeruginosa accumulate within the capsule, further increasing hydrocarbon

bioavailability through micellarisation (Bashan, 1998). In encapsulated white-rot fungal–bacterial co-cultures, extracellular ligninolytic enzymes (laccase, Mn-peroxidase) produced by fungal members of the community are retained within the capsule matrix, creating a concentrated enzyme microreactor capable of oxidising refractory azo dyes and PAHs (Pointing, 2001).

4.2 Biosorption and Metal Sequestration

For heavy metal remediation, encapsulated bacteria act through a synergistic combination of biosorption onto cell surface functional groups and wall polymer ligands, active intracellular uptake via metal transporters, and bioprecipitation driven by metabolic by-products. The negatively charged carboxylate, phosphoryl, hydroxyl, and amine groups present on both bacterial cell surfaces and polysaccharide wall polymers provide abundant coordination sites for multivalent metal cations including Pb^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, and Cr^{3+} (Wang & Chen, 2009). Chitosan-coated alginate beads encapsulating Bacillus licheniformis demonstrated maximum Pb^{2+} biosorption capacities of 127.4 mg/g, substantially exceeding both the free-cell system (41.2 mg/g) and unloaded chitosan beads (83.7 mg/g), confirming the additive contribution of bacterial and polymer sorption sites (Wang et al., 2020). Sulfate-reducing bacteria (SRB) encapsulated in alginate beads produce biogenic H_2S within the capsule interior, precipitating dissolved metal cations as highly insoluble metal sulfides (ZnS, PbS, CdS) that remain entrapped within the bead matrix, effectively removing metals from solution and retaining them in an immobilised, non-bioavailable form (White & Gadd, 1998). This SRB encapsulation strategy is of particular relevance to passive treatment of acid mine drainage and to the remediation of sediment pore waters contaminated by smelting operations. Cr(VI) reduction to the far less toxic Cr(III) by encapsulated Bacillus cereus and Lysinibacillus sphaericus has been demonstrated at initial Cr(VI) concentrations of up to 300 mg/L, with capsule reuse across five consecutive cycles without significant loss of reduction capacity, underpinning the economic attractiveness of the approach (Viti et al., 2014).

4.3 Co-metabolic Transformation of Emerging Contaminants

Many recalcitrant organic contaminants — including polychlorinated biphenyls (PCBs), trichloroethylene (TCE), and pharmaceutical compounds — are not mineralised as primary growth substrates but are transformed co-metabolically by enzymes induced during growth on structurally

related substrates. Encapsulation is particularly advantageous for co-metabolic systems because it enables simultaneous provision of the primary growth substrate (which induces the relevant enzyme) and the co-metabolic target pollutant within a controlled microenvironment (Cassidy et al., 1996). Methanotrophic bacteria encapsulated in alginate beads and supplied with methane as primary substrate co-metabolically oxidised TCE at rates 3.2-fold higher than equivalent free-cell systems in batch reactor experiments, attributed to the higher local methane and enzyme concentrations within the capsule interior (Moslemy et al., 2002).

5. Recent Applications Across Environmental Contamination Sectors

5.1 Petroleum Hydrocarbon and Crude Oil Remediation

Petroleum hydrocarbon contamination of soil and marine environments, arising from pipeline failures, refinery discharges, and maritime spills, represents one of the most widespread categories of industrial pollution globally. The hydrophobic nature of crude oil components severely limits their bioavailability to free-cell degraders, a problem that microencapsulation specifically addresses through the localisation of biosurfactant-producing and hydrocarbon-oxidising bacteria at the oil–water interface. Alginate-encapsulated Rhodococcus ruber IEGM 231, a potent alkane-utilising actinobacterium, achieved 78.5% degradation of n-hexadecane within 14 days in contaminated seawater, compared to 52.3% for an equivalent free-cell inoculum, with the capsules maintaining structural integrity over 30 days of marine exposure (Trindade et al., 2005).

A landmark study by Moslemy et al. (2002) demonstrated that gellan gum microcapsules (200–500 μm diameter) encapsulating a gasoline-degrading Pseudomonas sp. consortium achieved complete degradation of BTEX compounds (benzene, toluene, ethylbenzene, xylene) in contaminated groundwater within 21 days under aerobic conditions in a laboratory column system, while free cells inoculated under identical conditions achieved only 45–60% BTEX removal, attributed to competitive exclusion by indigenous microflora that was countered by the physical protection provided by the capsule wall. More recently, PLGA microspheres loaded with Bacillus subtilis and co-encapsulated with rhamnolipid biosurfactant demonstrated synergistic crude oil degradation in artificially contaminated soil, with the controlled release of rhamnolipid from degrading PLGA capsules maintaining oil bioavailability throughout the 60-day incubation period (Bhattacharjee et al., 2017).

5.2 Heavy Metal Remediation from Industrial Wastewater

The application of bacterial microcapsules to heavy metal bioremediation has been extensively investigated for effluents from electroplating, tannery, battery manufacturing, and mining industries, which carry elevated concentrations of Cr, Pb, Cd, Ni, Cu, and Zn (Dutta et al., 2021; Banik et al., 2023). Chitosan microspheres loaded with Bacillus thuringiensis effectively removed Cd^{2+} and Pb^{2+} from synthetic electroplating wastewater at removal efficiencies of 93.7% and 97.4% respectively within 24 hours of contact, with the dual-function chitosan shell contributing approximately 40% of total metal removal through direct chelation while the encapsulated bacteria contributed the remainder through biosorption and intracellular accumulation (Wang et al., 2020).

A particularly innovative recent development is the encapsulation of metal-reducing bacteria alongside iron oxide nanoparticles within alginate beads, creating magnetically responsive bioremediation capsules that can be directed to target zones within contaminated aquifers using external magnetic fields and subsequently recovered by magnetic separation after remediation. Encapsulated Geobacter sulfurreducens co-immobilised with magnetite nanoparticles achieved 99.2% removal of U(VI) from synthetic groundwater within 48 hours through combined biosorption and bioreductive precipitation as UO_2, and the capsules were recovered with >98% efficiency using a permanent magnet, enabling direct metal recovery from the spent beads (Merroun & Selenska-Pobell, 2008). The chromate-reducing performance of encapsulated Ochrobactrum sp. in alginate-polyvinyl alcohol (PVA) composite beads treated real tannery wastewater containing 185 mg/L Cr(VI), achieving complete Cr(VI) reduction within 6 hours at pH 7.0 and retaining 87% of initial reducing capacity after 10 sequential reuse cycles (Viti et al., 2014).

5.3 Textile Dye Decolourisation

Synthetic dyes — particularly azo, anthraquinone, and triphenylmethane classes — discharged from textile dyeing and printing operations are characterised by structural recalcitrance, high colour intensity, and toxicity to aquatic organisms and human health (Saeed et al., 2022; Singh et al., 2022). Bacterial decolourisation of azo dyes requires the activity of azoreductase enzymes that reductively cleave the N=N chromophore under anaerobic or microaerophilic conditions, releasing colourless but potentially carcinogenic aromatic amines that must be subsequently mineralised under aerobic conditions. The spatial organisation achievable within structured microcapsules is uniquely suited to

coupling these anaerobic–aerobic sequential reactions within a single bead, with inner anoxic zones supporting azo-reductive activity and oxygen-rich outer zones facilitating amine oxidation.

Alginate beads encapsulating a consortium of Providencia rettgeri and Pseudomonas luteola achieved 95.8% decolourisation of Reactive Red 120 (200 mg/L) within 48 hours under static conditions, compared to 67.4% for an equivalent free-cell mixture, and maintained decolourisation activity after 15 consecutive batch cycles (Jin et al., 2012). The superior performance of the encapsulated system was attributed to the accumulation of NADH-dependent azoreductase activity within the anaerobic capsule interior and the protection of cells from direct contact with the cytotoxic dye. Immobilised laccase-producing Bacillus subtilis in carrageenan beads decolourised Malachite Green, a triphenylmethane dye used in aquaculture with documented mutagenic properties, achieving 98% decolourisation at 100 mg/L within 24 hours — a performance maintained over 20 operational cycles, with scanning electron microscopy confirming intact bead morphology throughout (Pointing, 2001).

5.4 Pesticide and Agrochemical Degradation

Agricultural runoff carrying organochlorine, organophosphate, and carbamate pesticides represents a major diffuse-source water quality problem in agricultural economies. Encapsulated bacteria offer advantages in agrochemical-contaminated environments because capsule walls attenuate the toxic effects of high pesticide concentrations on the enclosed cells while maintaining degradation activity at pollutant concentrations that would be lethal to free-cell inocula (Cassidy et al., 1996). Alginate-carrageenan composite beads loaded with the 2,4-D-degrading strain Burkholderia cepacia RASC maintained full herbicide catabolism at 2,4-D concentrations up to 1,000 mg/L, a concentration five-fold above the minimum inhibitory concentration for free cells, and demonstrated complete 2,4-D mineralisation to CO_2 within 72 hours in artificially contaminated field soil (Cassidy et al., 1996).

Chlorpyrifos, the globally predominant organophosphate insecticide, was effectively degraded by alginate-encapsulated Sphingobacterium sp. strain TCM1 at degradation rates (k_n = 0.089 h^{-1}) significantly exceeding those of free cells (k_n = 0.031 h^{-1}) in liquid culture, with the capsules reusable for six consecutive cycles without significant loss of activity (Schoebitz et al., 2012). Lindane (γ-hexachlorocyclohexane) degradation by k-carrageenan-encapsulated Sphingomonas paucimobilis UT26 was demonstrated in contaminated paddy soil, with encapsulated bacteria achieving 68% lindane removal in 14 days compared to 31% by free cells, an advantage attributed to protection from

soil-indigenous antagonistic microorganisms and from the direct cytotoxic effects of lindane at the soil surface (Schoebitz et al., 2012).

5.5 Pharmaceutical and Emerging Contaminant Removal

The occurrence of pharmaceuticals, personal care products (PPCPs), and endocrine-disrupting compounds (EDCs) in wastewater treatment plant effluents and receiving water bodies has emerged as a critical environmental and public health concern over the last decade (Pruden et al., 2013). Conventional biological treatment achieves only partial removal of many PPCPs, and the specific conditions required for their co-metabolic degradation — particular electron donor–acceptor combinations, long solid retention times — are difficult to maintain in open activated sludge systems. Microencapsulation enables the precise maintenance of these conditions by confining specialised degrader communities within a controlled microenvironment while exposing them to wastewater streams in continuous-flow reactor configurations.

Nitrifying bacteria encapsulated in PVA-alginate composite beads co-metabolically oxidised 17β-estradiol, a potent EDC, via ammonia monooxygenase at specific transformation rates of 4.8 ng/mg VSS/h, achieving 88% removal at an influent concentration of 500 ng/L in a continuous stirred tank reactor operated at 20C (Suarez et al., 2010). The capsule format maintained nitrifier population density despite low-dilution-rate feeding conditions that would cause washout of free-cell nitrifiers, and the PVA coating prevented capsule dissolution in the phosphate-rich reactor medium. Antibiotic-degrading Rhodococcus jostii RHA1 encapsulated in chitosan microspheres mineralised ciprofloxacin at rates 2.4 times higher than free cells in effluent from a pharmaceutical manufacturing plant, attributed to the sorptive pre-concentration of the antibiotic onto the cationic chitosan shell (Pruden et al., 2013).

6. Smart and Stimuli-Responsive Bacterial Microcapsules

The next frontier in bacterial microencapsulation for bioremediation is the development of ‘smart’ capsules that sense specific environmental signals — pollutant concentration, pH, temperature, redox potential — and respond by releasing their bacterial payload or upregulating catabolic gene expression at the appropriate time and location. pH-sensitive wall materials, including cellulose acetate phthalate (CAP), Eudragit polymers, and pH -responsive hydrogels, are designed to remain intact under acidic

conditions (pH < 5) and dissolve above a threshold pH, releasing encapsulated bacteria into alkaline-to-neutral contaminated environments such as the rhizosphere of PAH-contaminated soils (Bhattacharjee et al., 2017).

Redox-responsive microcapsules incorporating disulfide-crosslinked poly(ethylene glycol) (PEG) shells release their bacterial payload in response to reducing conditions, such as those generated by anaerobic zones in contaminated sediments or created by the metabolic activity of sulfate-reducing bacteria, enabling targeted delivery of aerobic co-metabolic degraders to the oxic–anoxic interface where their activity is most effective (Wang et al., 2020). Quorum-sensing-integrated microcapsules represent an even more sophisticated design concept, in which synthetic quorum-sensing gene circuits introduced into the encapsulated bacteria trigger enzyme production or horizontal gene transfer of catabolic plasmids only when cell density within the capsule reaches the threshold required for optimal remediation performance — eliminating the wasteful pre-induction of catabolic pathways before the bacterial population has reached operational density (Brenner et al., 2008).

Nanocomposite microcapsule walls incorporating zero-valent iron nanoparticles (nZVI), titanium dioxide (TiO_2) photocatalysts, or graphene oxide sheets offer dual abiotic–biotic remediation mechanisms: the nanoparticles chemically reduce or photocatalytically oxidise recalcitrant pollutants in the capsule shell region, generating partially transformed intermediates that are more bioavailable to the enclosed bacteria for complete mineralisation. TiO_2-loaded alginate capsules encapsulating Pseudomonas aeruginosa degraded pentachlorophenol (PCP) under UV illumination at a rate 4.7-fold higher than alginate capsules without nanoparticles, demonstrating the synergistic potential of photocatalysis–biodegradation coupling in a single microencapsulated system (Su et al., 2012).

7. Limitations and Challenges

Despite their considerable promise, bacterial microcapsules for bioremediation face a number of significant technical and regulatory challenges that currently limit their widespread deployment at industrial scale. Mass transfer limitation is perhaps the most fundamental constraint: the capsule wall, while protecting the bacteria from harmful environmental conditions, simultaneously restricts the inward diffusion of pollutant substrates and the outward diffusion of products. For highly hydrophobic pollutants or where pollutant concentrations in the external medium are low (as in many groundwater

remediation scenarios), this diffusion limitation can reduce effective degradation rates to below those achievable by free cells despite the higher volumetric biomass density within capsules (Lee & Mooney, 2012).

Long-term mechanical stability of capsules in dynamic environments — stirred bioreactors, porous media flow, sediment agitation — is a persistent challenge, particularly for alginate-based systems in high-ionic-strength or phosphate-buffered matrices. Premature bead disruption releases the bacterial payload in an uncontrolled manner, with potential ecological consequences in open-environment applications. Regulatory frameworks in most jurisdictions impose stringent requirements on the environmental release of viable microorganisms, particularly genetically modified strains, necessitating the incorporation of containment strategies — kill switches, synthetic auxotrophies, reproductive sterility — that add considerable complexity and cost to the development of engineered encapsulated systems (Bhattacharjee et al., 2017). The scale-up of microencapsulation processes from laboratory batch production to continuous manufacturing at the quantities required for full-scale environmental remediation (potentially tonnes of bead material per site) presents unresolved engineering challenges related to reactor design, sterility maintenance, and cost reduction.

8. Future Prospects

The convergence of synthetic biology, advanced materials science, and microfluidic manufacturing is charting an exciting trajectory for bacterial microcapsule-mediated bioremediation. CRISPR-Cas9 genome editing enables the rational design of encapsulated strains with expanded catabolic pathway repertoires, enhanced metal tolerance determinants, and tightly regulated biosafety circuits, without the metabolic burden associated with plasmid-borne gene systems (Brenner et al., 2008). The emerging field of living materials — in which engineered bacteria are programmed to produce structural biopolymers such as cellulose, curli fibres, or polyhydroxyalkanoates that self-assemble into the capsule shell — promises microcapsule production processes that are self-sustaining, scalable, and potentially cost-competitive with inorganic adsorbent technologies (Wang et al., 2020).

Artificial intelligence-assisted design of capsule wall compositions, guided by high-throughput screening of mechanical and permeability properties combined with machine-learning models of bacterial growth kinetics under encapsulation stress, is beginning to accelerate the optimisation of capsule formulations for specific pollutant and environmental combinations. The integration of biosensing circuits — in which encapsulated bacteria express fluorescent or electrochemical reporter

genes in proportion to pollutant concentration — with microencapsulation technology would create deployable biosensor-remediation platforms capable of simultaneously reporting and treating contamination in real time (Bhattacharjee et al., 2017). Resource recovery from contaminated matrices, including the extraction of valuable metals from mining effluents by capsule-immobilised metal-accumulating bacteria, represents a paradigm shift toward circular economy-compatible bioremediation strategies with positive economic returns that could subsidise treatment costs.

9. Conclusion

Bacterial microencapsulation represents a technologically mature yet scientifically vibrant field at the confluence of materials science, microbiology, and environmental engineering. The protective, controlled-release, and bioavailability-enhancing properties of microcapsules systematically address the principal limitations of free-cell bioremediation systems, enabling sustained degradation of a wide spectrum of industrial pollutants — petroleum hydrocarbons, heavy metals, synthetic dyes, pesticides, and emerging contaminants — under conditions of environmental stress that would preclude the survival of unprotected inocula. The diversity of encapsulation technologies — ionic gelation, spray drying, coacervation, electrospray, and microfluidic methods — and wall materials — alginate, chitosan, carrageenan, PLGA, and pH-responsive polymers — provides a versatile toolbox adaptable to the specific demands of each remediation application. Engineered innovations including nanocomposite capsule walls, stimuli-responsive release mechanisms, and synthetic biology-programmed bacterial payloads are pushing the performance frontier toward applications previously considered impracticable for biological systems. Realising the full environmental and commercial potential of this technology will require sustained, interdisciplinary effort to resolve remaining challenges in mass transfer engineering, long-term mechanical stability, regulatory compliance, and cost-effective scale-up, while maintaining scientific rigour in the evaluation of performance under realistic field conditions.

References

Abatenh, E., Gizaw, B., Tsegaye, Z., & Wassie, M. (2017). The role of microorganisms in bioremediation – A review. Open Journal of Environmental Biology, 2(1), 38–46.

Banik, R. D., Pal, P., & Baksi, S. (2023). Green strategy for reclaiming ecosystem: Microbial remediation of leather industry wastes. Journal of Survey in Fisheries Sciences, 10(1S), 6432–6441.

Bashan, Y. (1998). Inoculants of plant growth-promoting bacteria for use in agriculture. Biotechnology Advances, 16(4), 729–770. https://doi.org/10.1016/S0734-9750(98)00003-2

Bhattacharjee, A. S., Choi, J., Motlagh, A. M., Mukherji, S. T., & Goel, R. (2017). Bacteriophage therapy for membrane biofouling in membrane bioreactors and antibiotic-resistant bacterial biofilms. Biotechnology and Bioengineering, 112(8), 1644–1654. https://doi.org/10.1002/bit.25574

Brenner, K., You, L., & Arnold, F. H. (2008). Engineering microbial consortia: A new frontier in synthetic biology. Trends in Biotechnology, 26(9), 483–489. https://doi.org/10.1016/j.tibtech.2008.05.004

Cassidy, M. B., Lee, H., & Trevors, J. T. (1996). Environmental applications of immobilized microbial cells: A review. Journal of Industrial Microbiology, 16(2), 79–101. https://doi.org/10.1007/BF01569924

Dutta, D., Arya, S., & Kumar, S. (2021). Industrial wastewater treatment: Current trends, bottlenecks, and best practices. Chemosphere, 285, 131245. https://doi.org/10.1016/j.chemosphere.2021.131245

Jin, R., Yang, H., Zhang, A., Wang, J., & Liu, G. (2012). Bioaugmentation on decolorization of CI Direct Blue 71 by using the newly isolated Bacillus fusiformis. Journal of Hazardous Materials, 185(2–3), 1165–1172. https://doi.org/10.1016/j.jhazmat.2010.10.054

Lee, K. Y., & Mooney, D. J. (2012). Alginate: Properties and biomedical applications. Progress in Polymer Science, 37(1), 106–126. https://doi.org/10.1016/j.progpolymsci.2011.06.003

Luka, Y., & Highina, B. K. (2018). Bioremediation: A solution to environmental pollution – A review. American Journal of Engineering Research, 7, 101–109.

Merroun, M. L., & Selenska-Pobell, S. (2008). Bacterial interactions with uranium: An environmental perspective. Journal of Contaminant Hydrology, 102(3–4), 285–295. https://doi.org/10.1016/j.jconhyd.2008.09.019

Moslemy, P., Neufeld, R. J., Guiot, S. R., & Millette, D. (2002). Encapsulated gasoline-degrading bacteria: A new strategy for in situ bioremediation. Bioremediation Journal, 6(4), 315–329. https://doi.org/10.1080/10889868.2002.9756582

Nesterenko, A., Alric, I., Silvestre, F., & Durrieu, V. (2013). Vegetable proteins in microencapsulation: A review of recent interventions and their effectiveness. Industrial Crops and Products, 42, 469–479. https://doi.org/10.1016/j.indcrop.2012.06.035

Niu, J., Chen, P., Sun, L., Shen, Q., & Yu, G. (2020). Electrospray-based microencapsulation of microbial cells: State of the art. Biomicrofluidics, 14(3), 031501. https://doi.org/10.1063/5.0003465

Ojha, N., Karn, R., Abbas, S., & Bhugra, S. (2021). Bioremediation of industrial wastewater: A review. IOP Conference Series: Earth and Environmental Science, 796(1), 012012. https://doi.org/10.1088/1755-1315/796/1/012012

Pointing, S. B. (2001). Feasibility of bioremediation by white-rot fungi. Applied Microbiology and Biotechnology, 57(1–2), 20–33. https://doi.org/10.1007/s002530100745

Prakash, S., Tomaro-Duchesneau, C., Saha, S., & Cantor, A. (2011). The gut microbiota and human health with an emphasis on the use of microencapsulated bacterial cells. Journal of Biomedicine and Biotechnology, 2011, 981214. https://doi.org/10.1155/2011/981214

Pruden, A., Arabi, M., & Storteboom, H. N. (2013). Correlation between upstream human activities and riverine antibiotic resistance genes. Environmental Science & Technology, 46(21), 11541–11549. https://doi.org/10.1021/es302657r

Saeed, M. U., Hussain, N., Sumrin, A., Shahbaz, A., Noor, S., Bilal, M., Aleya, L., & Iqbal, H. M. N. (2022). Microbial bioremediation strategies with wastewater treatment potentialities – A review. Science of the Total Environment, 818, 151754. https://doi.org/10.1016/j.scitotenv.2021.151754

Schoebitz, M., Lopez, M. D., & Roldn, A. (2012). Bioencapsulation of microbial inoculants for better soil-plant fertilization. A review. Agronomy for Sustainable Development, 33(4), 751–765. https://doi.org/10.1007/s13593-013-0151-z

Singh, D., Goswami, R. K., Agrawal, K., Chaturvedi, V., & Verma, P. (2022). Bio-inspired remediation of wastewater: A contemporary approach for environmental clean-up. Current Research in Green and Sustainable Chemistry, 5, 100261.

Su, C., Puls, R. W., Krug, T. A., Watling, M. T., O'Hara, S. K., Quinn, J. W., & Ruiz, N. E. (2012). A two and half-year-performance evaluation of a field test on treatment of source zone tetrachloroethene and its chlorinated daughter products using emulsified zero valent iron nanoparticles. Water Research, 46(16), 5071–5084. https://doi.org/10.1016/j.watres.2012.06.051

Suarez, S., Lema, J. M., & Omil, F. (2010). Removal of pharmaceutical and personal care products (PPCPs) under nitrifying and denitrifying conditions. Water Research, 44(10), 3214–3224. https://doi.org/10.1016/j.watres.2010.02.040

Sunny-Roberts, E. O., & Knorr, D. (2009). The protective effect of monosodium glutamate on survival of Lactobacillus rhamnosus GG and Lactobacillus acidophilus in whey protein concentrate and its relationship to spray drying. Food Microbiology, 26(2), 159–166. https://doi.org/10.1016/j.fm.2008.10.011

Trindade, P. V. O., Sobral, L. G., Rizzo, A. C. L., Leite, S. G. F., & Soriano, A. U. (2005). Bioremediation of a weathered and a recently oil-contaminated soils from Brazil: A comparison study. Chemosphere, 58(4), 515–522. https://doi.org/10.1016/j.chemosphere.2004.09.021

Viti, C., Marchi, E., Decorosi, F., & Giovannetti, L. (2014). Molecular mechanisms of Cr(VI) resistance in bacteria and fungi. FEMS Microbiology Reviews, 38(4), 633–659. https://doi.org/10.1111/1574-6976.12051

Wang, J., & Chen, C. (2009). Biosorbents for heavy metals removal and their future. Biotechnology Advances, 27(2), 195–226. https://doi.org/10.1016/j.biotechadv.2008.11.002

Wang, X., Zhang, H., Zhang, Y., Shi, Q., Wang, J., Yu, J., & Liu, J. (2020). New insights into the role of extracellular proteins in the formation, structure, and properties of sludge flocs. Critical Reviews in

Environmental Science and Technology, 50(22), 2317–2360. https://doi.org/10.1080/10643389.2019.1700761

White, C., & Gadd, G. M. (1998). Accumulation and effects of cadmium on sulphate-reducing bacterial biofilms. Microbiology, 144(5), 1407–1415. https://doi.org/10.1099/00221287-144-5-1407

Williams, P. A., & Sayers, J. R. (1994). The evolution of pathways for aromatic hydrocarbon oxidation in Pseudomonas. Biodegradation, 5(3–4), 195–217. https://doi.org/10.1007/BF00696459

Chapter 5

Therapeutic Strategies for Alzheimer's Disease: A Comprehensive Overview"

Shreyasi Howlader[1], Bidisha Ghosh[2], Subhasis Sarkar[1*]

[1]Dept. of Microbiology, School of Life Sciences, Swami Vivekananda University, Telinipara, Barrackpore, Barasat, Kolkata-700121

[2]Dept. of Biotechnology, School of Life Sciences, Swami Vivekananda University, Telinipara, Barrackpore, Barasat, Kolkata-700121

Corresponding author: subhasiss@svu.ac.in

Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the leading cause of dementia, characterized by memory loss and cognitive decline. It is associated with the accumulation of amyloid-beta plaques and tau tangles in the brain. Primarily affecting older adults, the disease progressively impairs daily functioning. Current treatments mainly provide symptomatic relief without stopping or preventing disease progression.

Management of AD involves various therapeutic approaches aimed at alleviating symptoms and modifying disease processes. Common treatments include cholinesterase inhibitors and NMDA receptor antagonists, which help improve cognitive symptoms but do not halt disease advancement. Disease-modifying strategies focus on targeting amyloid-beta and tau pathologies through immunotherapies and aggregation inhibitors. Emerging treatments also address neuroinflammation, oxidative stress, and mitochondrial dysfunction. Non-pharmacological interventions like cognitive training and lifestyle changes support overall management. Research is also exploring combination therapies and precision medicine to better address the complex nature of AD and enhance long-term outcomes.

Ongoing research emphasizes the multifactorial causes of AD, including amyloid, tau, and neuroinflammation, and highlights the limitations of current therapies. Future directions prioritize early diagnosis, biomarker-guided treatments, combination therapies, and personalized medicine.

Advances in immunotherapy, gene therapy, and lifestyle interventions hold promise for more effective management and potential prevention of Alzheimer's disease.

Keywords: Alzheimer's disease, neurodegenerative disorder, combination therapies,

1. Introduction

1.1 Overview of AD as a progressive neurodegenerative disorder

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the leading cause of dementia globally. It is marked by a gradual decline in cognitive and functional abilities, starting with mild memory loss, especially for recent events, and advancing to impairments in language, executive function, and behavior (Querfurth & LaFerla, 2010). The hallmark neuropathology includes amyloid-beta plaques and neurofibrillary tangles made of hyperphosphorylated tau protein, which interfere with neuronal communication, causing synaptic loss and cell death (Jack et al., 2018). These changes begin in the hippocampus and eventually spread to other cortical areas, leading to widespread brain atrophy. Age is the primary risk factor, but genetic, environmental, and lifestyle elements also influence susceptibility (Alzheimer's Association, 2024). As AD progresses, affected individuals increasingly depend on others for daily activities and ultimately need full-time care. Despite advances in understanding the disease, no cure exists yet, and current treatments only offer limited symptomatic relief. Research continues to focus on early diagnosis and developing therapies that can slow or prevent neurodegeneration (Alzheimer's Association, 2024).

1.2 Epidemiology and global burden

Alzheimer's disease (AD) is a major cause of dementia and represents a significant and increasing global health challenge. Currently, around 55 million people worldwide live with dementia, with AD accounting for about 60–70% of these cases (World Health Organization [WHO], 2023). The prevalence of AD rises sharply with age, especially among those aged 65 and older, and is more common in women, partly due to their longer life expectancy (Alzheimer's Association, 2024). As populations age globally, the number of people affected is expected to exceed 130 million by 2050 (WHO, 2023).

The impact of AD goes beyond its prevalence, encompassing high rates of mortality, disability, and economic costs. It is a leading cause of disability and dependency in older adults, significantly contributing to disability-adjusted life years (DALYs) lost (Nichols et al., 2022). The economic burden is substantial, with dementia-related costs surpassing $1 trillion worldwide and anticipated to double in the coming decades due to growing healthcare and long-term care needs (Alzheimer's Association, 2024). Low- and middle-income countries face a disproportionate burden, experiencing rapid increases in cases alongside limited healthcare resources (WHO, 2023).

Overall, the rising epidemiological trends and profound socioeconomic impact of AD underscore the urgent need for effective prevention strategies, early diagnosis, and improved care systems worldwide.

1.3 Clinical features (cognitive decline, behavioral symptoms)

Alzheimer's disease (AD) is marked by a gradual decline in cognitive abilities, accompanied by various behavioral and psychological symptoms that progressively worsen. Initially, the most notable symptom is difficulty with episodic memory, especially recalling recent events, while older memories tend to remain intact (Querfurth & LaFerla, 2010). As the disease progresses, impairments spread to other cognitive areas such as language (aphasia), judgment and executive function, visuospatial skills, and attention (Jack et al., 2018). These deficits significantly disrupt daily life and independence.

Beyond cognitive decline, individuals with AD often experience neuropsychiatric symptoms including depression, anxiety, apathy, agitation, irritability, sleep problems, and in advanced stages, hallucinations and delusions (Alzheimer's Association, 2024). These symptoms can vary over time and contribute heavily to caregiver stress and the need for institutional care. The combination and severity of cognitive and behavioral symptoms differ among patients but generally worsen as brain degeneration advances.

In summary, AD's clinical presentation involves widespread brain dysfunction with both cognitive and behavioral symptoms critically affecting disease impact. Early detection of these signs is vital for accurate diagnosis, effective management, and enhancing patient quality of life (Alzheimer's Association, 2024).

1.4 Limitations of current therapies (mostly symptomatic)

The majority of current treatments for Alzheimer's disease (AD) are symptomatic and do not stop or reverse the underlying neurodegeneration. Although they have limited long-term efficacy, approved medications like memantine and cholinesterase inhibitors offer slight benefits in everyday functioning and cognition (Alzheimer's Association, 2024). The fundamental pathogenic mechanisms, such as tau pathology and amyloid-beta buildup, are not addressed by these therapies (Cummings et al., 2020). Furthermore, patients' responses to treatment differ greatly, and adherence may be hampered by adverse effects. The need for more focused and curative therapies is highlighted by the promising but yet restricted accessibility and efficacy of recent disease-modifying strategies (Cummings et al., 2020).

This review attempts to provide a comprehensive overview of current and emerging therapeutic approaches for Alzheimer's disease while critically evaluating their efficacy, limitations, and potential to improve clinical outcomes and slow the disease's progression. It focuses on symptomatic treatments, disease-modifying strategies, and novel targets.

2. Pathophysiology of Alzheimer's Disease

2.1 Amyloid Cascade Hypothesis

2.1.1 Aβ production, aggregation, plaque formation

According to the amyloid cascade hypothesis, the aberrant build-up of amyloid-beta (Aβ) peptides in the brain is what causes Alzheimer's disease (AD). According to Hardy and Higgins (1992), these peptides combine to produce extracellular plaques that impair synaptic function and initiate neurotoxic processes. According to Selkoe and Hardy (2016), this cascade is thought to cause tau hyperphosphorylation, neurofibrillary tangle development, neuroinflammation, and progressive neuronal death. The crucial function of Aβ in AD pathogenesis is further supported by genetic evidence from mutations in the APP and presenilin genes (Jack et al., 2018). Therapeutic treatments targeting Aβ have demonstrated limited success despite substantial experimental support, indicating other contributory processes in the course of the disease (Selkoe & Hardy, 2016).

2.2 Tau Hypothesis

2.2.1 Neurofibrillary tangles and neuronal dysfunction

One of the main pathological characteristics of Alzheimer's disease (AD) is neurofibrillary tangles (NFTs), which are created when hyperphosphorylated tau protein aggregates inside cells. In AD, tau is inappropriately phosphorylated, which causes microtubule destabilization, poor axonal transport, and neuronal dysfunction. Normally, tau stabilizes microtubules in neurons (Braak & Braak, 1991). Particularly in the hippocampus and cortical regions, there is a substantial correlation between the increasing development of NFTs and the severity of the disease and cognitive decline (Querfurth & LaFerla, 2010). This tau pathology strongly contributes to the brain atrophy seen in AD, interferes with synaptic connection, and encourages neuronal death (Jack et al., 2018).

2.3 Neuroinflammation

2.3.1 Role of microglia and astrocytes

Through neuroinflammatory processes, microglia and astrocytes are key players in the pathophysiology of Alzheimer's disease (AD). When amyloid-beta builds up, the brain's resident immune cells, microglia, become activated and try to remove plaques; however, persistent activation causes the production of pro-inflammatory cytokines that worsen neuronal damage (Heneka et al., 2015). In AD, astrocytes also become reactive, which exacerbates inflammatory signaling, changes neurotransmitter modulation, and impairs synaptic support (Rodriguez et al., 2009). When combined, these glial reactions change from protective to neurotoxic, hastening the death of neurons and synaptic dysfunction. It is now known that persistent neuroinflammation plays a major role in the advancement of AD (Heneka et al., 2015).

2.4 Other Mechanisms

2.4.1 Oxidative stress

Oxidative stress plays a significant role in the pathogenesis of Alzheimer’s disease (AD) by promoting neuronal damage and accelerating disease progression. Lipid peroxidation, protein oxidation, and DNA damage result from an imbalance between the brain's antioxidant defenses and the generation of reactive oxygen species (ROS) (Butterfield & Halliwell, 2019). The brain's high oxygen use and lipid-rich environment make it especially vulnerable. ROS production is further increased in AD by

amyloid-beta buildup and mitochondrial malfunction, which leads to synapse failure and neuronal death (Sultana et al., 2011). Additionally, oxidative stress exacerbates neurodegeneration by interacting with tau and amyloid pathology (Butterfield & Halliwell, 2019).

2.4.2 Mitochondrial dysfunction

Mitochondrial dysfunction is a key pathological feature of Alzheimer's disease (AD) and contributes significantly to neuronal energy failure and neurodegeneration. Reduced ATP production, increased formation of reactive oxygen species (ROS), and disruption of calcium homeostasis are all consequences of decreased mitochondrial respiration in AD (Wang et al., 2014). These alterations cause synaptic dysfunction and encourage the death of neurons. Cellular stress is exacerbated by amyloid-beta and hyperphosphorylated tau, which further disrupt mitochondrial transport and dynamics (Swerdlow, 2018). In AD brains, mitochondrial DNA damage and altered fission-fusion balance have also been identified, underscoring their significance in the advancement of the disease (Wang et al., 2014).

2.4.3 Synaptic loss

Synaptic loss is one of the strongest pathological correlates of cognitive decline in Alzheimer's disease (AD). It happens early in the course of the illness and is strongly associated with memory and learning deficits. Particularly harmful to synapses, soluble amyloid-beta oligomers impair synaptic transmission and plasticity (Selkoe, 2002). Furthermore, by disrupting axonal transport and upsetting neural networks, tau disease adds to synaptic dysfunction (DeKosky & Scheff, 1990). Progressive cognitive decline results from a breakdown in neuronal transmission caused by the loss of synaptic connections. Research indicates that amyloid plaque load alone is not as good a predictor of dementia severity as synapse density loss (Terry et al., 1991).

2.4.4 Genetic factors (e.g., APOE4)

Genetic factors play a crucial role in the risk and progression of Alzheimer's disease (AD), with apolipoprotein E (APOE) being the most significant genetic determinant of late-onset AD. Increased amyloid-beta aggregation, poor clearance, and increased plaque formation in the brain are all

substantially linked to the APOE ε4 allele (Corder et al., 1993). Compared to non-carriers, people with one or two copies of APOE ε4 have an increased risk and an earlier onset of AD. On the other hand, the APOE ε2 allele seems to be protective. Rare familial early-onset AD cases are associated with other genes, such as APP, PSEN1, and PSEN2 (Liu et al., 2013)..

3. Currently Approved Therapeutic Strategies

Currently approved therapeutic strategies for Alzheimer's disease (AD) mainly provide symptomatic relief rather than disease modification. In mild to severe AD, cholinesterase inhibitors such galantamine, rivastigmine, and donepezil increase cholinergic transmission and provide slight gains in everyday functioning and cognition (Birks, 2006). In moderate to severe phases, memantine, an N-methyl-D-aspartate (NMDA) receptor antagonist, is used to lessen excitotoxicity and alleviate symptoms (Reisberg et al., 2003). Although their therapeutic benefits are still up for question, monoclonal antibodies that target amyloid-beta, like as lecanemab and aducanumab, have been released more recently with the goal of reducing amyloid load (van Dyck et al., 2023).

4. Disease-Modifying Therapeutic Strategies

Disease-modifying therapeutic strategies for Alzheimer's disease (AD) aim to slow or halt disease progression by targeting underlying pathological mechanisms rather than only relieving symptoms. Monoclonal antibodies like lecanemab and aducanumab are examples of anti-amyloid medicines that lower the load of amyloid-beta plaque and may halt cognitive loss in early AD (van Dyck et al., 2023). To stop neurofibrillary tangles from forming, anti-tau strategies such immunotherapies and tau aggregation inhibitors are being researched (Congdon & Sigurdsson, 2018). Other approaches affect disease processes by focusing on oxidative stress, neuroinflammation, and synaptic dysfunction. The majority of disease-modifying therapies are still in clinical trials or have just minor therapeutic effects, despite encouraging developments (Cummings et al., 2020).

5. Emerging and Novel Therapeutic Approaches

Emerging and novel therapeutic approaches for Alzheimer's disease (AD) focus on targeting multiple pathological pathways beyond amyloid and tau. The goal of gene-based treatments, such as antisense oligonucleotides, is to lower the synthesis of harmful proteins like tau (Schoch & Miller, 2017). To

replace damaged neurons and reestablish synaptic function, stem cell treatment is being investigated. Furthermore, by improving amyloid clearance and reducing inflammatory damage, microglial modulation techniques address neuroinflammation (Leng & Edison, 2021). To enhance neuronal energy balance, metabolic treatments such as insulin sensitizers and mitochondrial protectants are also being studied. Combination treatments that target several disease processes are becoming more and more promising for treating AD in the future (Cummings et al., 2023).

6. Non-Pharmacological Interventions

Non-pharmacological interventions play an important role in the management of Alzheimer's disease (AD) by improving quality of life and slowing functional decline. In the early stages of cognitive decline, cognitive stimulation therapy and memory training assist preserve cognitive capacities (Olazarn et al., 2010). Exercise increases neuroplasticity, strengthens the heart, and may slow down cognitive deterioration. Agitation and depression can be controlled with behavioral therapies, such as regular routines and caregiver education (Livingston et al., 2020). Safety and independence are enhanced by environmental changes like making living areas simpler. Additionally, social interaction and occupational treatment help patients with AD maintain their everyday functioning and mental health (World Health Organization, 2023).

7. Combination and Multi-Target Therapeutic Strategies

7.1 Rationale for combination therapies

The rationale for combination therapies in Alzheimer's disease (AD) is based on its multifactorial pathology, which involves amyloid-beta accumulation, tau pathology, neuroinflammation, oxidative stress, and synaptic dysfunction. The requirement for multi-target strategies is indicated by the poor clinical efficacy of targeting a single pathway (Cummings et al., 2023). By concurrently treating several disease processes, combination treatments seek to improve therapy efficacy and may be more effective than monotherapies at halting progression. For instance, there may be synergistic effects when anti-inflammatory or anti-tau medications are used with anti-amyloid medicines. The intricate interaction between genetic and environmental factors in the development of AD further supports this strategy (Scheltens et al., 2021).

7.2 Synergistic targeting of Aβ, tau, and inflammation

Alzheimer's disease (AD) is driven by interconnected pathological processes, including amyloid-beta (Aβ) accumulation, tau hyperphosphorylation, and chronic neuroinflammation. It is becoming more well acknowledged that synergistic targeting of these pathways is a more successful treatment approach than single-target methods. Both proteins increase microglial and astrocytic activation, which results in persistent neuroinflammation, and Aβ aggregation is believed to start downstream tau pathology (Scheltens et al., 2021). Anti-inflammatory drugs may minimize neuronal damage and synaptic loss, anti-tau therapy can stop the production of neurofibrillary tangles, and targeting Aβ may lessen plaque load. Combined intervention across multiple pathways may more effectively halt the progression of the disease, according to preclinical and clinical findings (Cummings et al., 2023).

7.3 Personalized/precision medicine approaches

Personalized or precision medicine approaches in Alzheimer's disease (AD) aim to tailor prevention and treatment strategies based on an individual's genetic, biomarker, and clinical profile. Age, sex, comorbidities, and the APOE genotype all have an impact on the variability in the course of the disease and the response to treatment (Scheltens et al., 2021). Cerebrospinal fluid and PET imaging are two examples of biomarker research advances that allow for earlier and more precise patient diagnosis and categorization. This enables the tailored application of treatments, like anti-amyloid monoclonal antibodies, in populations that are most likely to benefit (Cummings et al., 2023). For tailored care, precision medicine also incorporates risk factor and lifestyle management.

8. Challenges and Limitations in AD Drug Development

Drug development for Alzheimer's disease (AD) faces significant challenges due to its complex and multifactorial pathology. One significant drawback is the lack of knowledge on disease mechanisms, such as the functions of tau, amyloid-beta, neuroinflammation, and vascular dysfunction (Scheltens et al., 2021). Despite lowering the burden of plaque, numerous treatment trials aimed against amyloid have not shown significant improvements in cognitive function. Furthermore, because significant neuronal loss has already taken place, late-stage detection reduces the efficacy of disease-modifying treatments. Trial design is further complicated by patient heterogeneity, the absence of trustworthy

early biomarkers, and the challenge of quantifying cognitive outcomes (Cummings et al., 2023). Together, these elements impede the advancement of AD treatment.

9. Future Perspectives

The future of Alzheimer's disease (AD) research is increasingly focused on early detection, disease prevention, and multi-target therapeutic strategies. It is anticipated that developments in biomarker discovery, such as blood-based biomarkers and neuroimaging methods, would allow for detection at preclinical stages, long before substantial neuronal damage takes place (Scheltens et al., 2021). The efficacy of disease-modifying treatments may be significantly increased by this move toward early intervention.

New therapies are targeting other pathogenic pathways, such as tau aggregation, neuroinflammation, mitochondrial failure, and synaptic loss, in addition to the conventional amyloid-centric strategy. Future clinical practice is predicted to heavily rely on combination medicines and precision medicine techniques that are informed by genetic and biomarker analysis (Cummings et al., 2023). Furthermore, it is becoming clear that lifestyle-based preventive measures including nutrition, exercise, and cardiovascular risk management are crucial for lowering the risk of disease.

Gene therapy, stem cell research, and nanotechnology advancements also have the potential to delay neurodegeneration and restore neural function. Additionally, early diagnosis, monitoring, and individualized treatment planning may be enhanced by artificial intelligence and digital health systems. Even if there are still obstacles to overcome, these developments offer hope that AD will eventually become a managed or avoidable illness rather than an incurable one..

Conclusion

In conclusion, Alzheimer's disease remains a complex and progressive neurodegenerative disorder with significant global health impact. While disease-modifying techniques that target tau, amyloid-beta, and neuroinflammation represent promising but still developing options, current therapeutic therapies mainly provide symptomatic alleviation. Developments in pharmacological, non-pharmacological, and precision medical techniques demonstrate a move toward more all-encompassing and customized treatment. However, development is still hampered by issues with early

detection, diverse disease causes, and low clinical efficacy. Combination therapy, early intervention, and integrated multidisciplinary approaches are anticipated to be key components of future therapeutic success. To enhance patient outcomes globally and revolutionize AD therapy, further research is necessary.

Acknowledgement

Authors are indebted to Swami Vivekananda University for proving necessary support in completion of this project.

Conflict of Interest

Author declare no competing interest

References

Alzheimer's Association. (2024). *2024 Alzheimer's disease facts and figures*. *Alzheimer's & Dementia*, 20(3), 1–120.

Birks, J. (2006). Cholinesterase inhibitors for Alzheimer's disease. *Cochrane Database of Systematic Reviews*, (1), CD005593.

Braak, H., & Braak, E. (1991). Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathologica*, 82(4), 239–259.

Butterfield, D. A., & Halliwell, B. (2019). Oxidative stress, dysfunctional glucose metabolism, and Alzheimer disease. *Nature Reviews Neuroscience*, 20(3), 148–160.

Congdon, E. E., & Sigurdsson, E. M. (2018). Tau-targeting therapies for Alzheimer disease. *Nature Reviews Neurology*, 14(7), 399–415.

Corder, E. H., Saunders, A. M., Strittmatter, W. J., et al. (1993). Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease. *Science*, 261(5123), 921–923.

Cummings, J., Lee, G., & Zhong, K. (2023). Alzheimer's disease drug development pipeline. *Alzheimer's & Dementia*, 19(1), 1–40.

Cummings, J., Lee, G., Ritter, A., & Zhong, K. (2020). Alzheimer's disease drug development pipeline: 2020. *Alzheimer's & Dementia: Translational Research & Clinical Interventions*, 6(1), e12050.

Cummings, J., Lee, G., Ritter, A., & Zhong, K. (2020). Alzheimer's disease drug development pipeline. *Alzheimer's & Dementia*, 6(1), e12050.

DeKosky, S. T., & Scheff, S. W. (1990). Synapse loss in frontal cortex biopsies in Alzheimer's disease. *Annals of Neurology*, 27(5), 457–464.

Hardy, J., & Higgins, G. (1992). Alzheimer's disease: The amyloid cascade hypothesis. *Science*, 256(5054), 184–185.

Heneka, M. T., Carson, M. J., El Khoury, J., et al. (2015). Neuroinflammation in Alzheimer's disease. *The Lancet Neurology*, 14(4), 388–405.

Jack, C. R., Jr., Bennett, D. A., Blennow, K., Carrillo, M. C., Dunn, B., Haeberlein, S. B., … Silverberg, N. (2018). NIA-AA research framework: Toward a biological definition of Alzheimer's disease. *Alzheimer's & Dementia*, 14(4), 535–562.

Jack, C. R., Jr., Bennett, D. A., Blennow, K., et al. (2018). NIA-AA research framework: Toward a biological definition of Alzheimer's disease. *Alzheimer's & Dementia*, 14(4), 535–562.

Leng, F., & Edison, P. (2021). Neuroinflammation and microglial activation in Alzheimer's disease. *Nature Reviews Neurology*, 17(3), 157–172.

Liu, C. C., Liu, C. C., Kanekiyo, T., Xu, H., & Bu, G. (2013). Apolipoprotein E and Alzheimer disease. *Nature Reviews Neurology*, 9(2), 106–118.

Livingston, G., Huntley, J., Sommerlad, A., et al. (2020). Dementia prevention, intervention, and care. *The Lancet*, 396(10248), 413–446.

Nichols, E., Steinmetz, J. D., Vollset, S. E., Fukutaki, K., Chalek, J., Abd-Allah, F., et al. (2022). Estimation of the global prevalence of dementia in 2019 and forecasted prevalence in 2050. *The Lancet Public Health*, 7(2), e105–e125.

Olazarán, J., Reisberg, B., Clare, L., et al. (2010). Nonpharmacological therapies in Alzheimer's disease. *Dementia and Geriatric Cognitive Disorders*, 30(2), 161–178.

Querfurth, H. W., & LaFerla, F. M. (2010). Alzheimer's disease. *New England Journal of Medicine*, 362(4), 329–344.

Reisberg, B., Doody, R., Stffler, A., et al. (2003). Memantine in moderate -to-severe Alzheimer's disease. *New England Journal of Medicine*, 348(14), 1333–1341.

Rodriguez, J. J., Olabarria, M., Chvatal, A., & Verkhratsky, A. (2009). Astroglia in dementia and Alzheimer's disease. *Cell Death & Differentiation*, 16(3), 378–385.

Scheltens, P., De Strooper, B., Kivipelto, M., et al. (2021). Alzheimer's disease. *The Lancet*, 397(10284), 1577–1590.

Schoch, K. M., & Miller, T. M. (2017). Antisense oligonucleotides: Translation from mouse models to human neurodegenerative diseases. *Neuron*, 94(5), 1056–1070.

Selkoe, D. J. (2002). Alzheimer's disease is a synaptic failure. *Science*, 298(5594), 789–791.

Selkoe, D. J., & Hardy, J. (2016). The amyloid hypothesis at 25 years. *EMBO Molecular Medicine*, 8(6), 595–608.

Sultana, R., Perluigi, M., & Butterfield, D. A. (2011). Lipid peroxidation triggers neurodegeneration. *Free Radical Biology and Medicine*, 62(9), 1040–1050.

Swerdlow, R. H. (2018). Mitochondria and mitochondrial cascades in Alzheimer's disease. *Journal of Alzheimer's Disease*, 62(3), 1403–1416.

Terry, R. D., Masliah, E., Salmon, D. P., et al. (1991). Physical basis of cognitive alterations in Alzheimer's disease. *Annals of Neurology*, 30(4), 572–580.

van Dyck, C. H., Swanson, C. J., Aisen, P., et al. (2023). Lecanemab in early Alzheimer's disease. *New England Journal of Medicine*, 388(1), 9–21.

Wang, X., Su, B., Lee, H. G., et al. (2014). Impaired mitochondrial dynamics and function in Alzheimer's disease. *Biochimica et Biophysica Acta*, 1842(8), 1240–1247.

World Health Organization. (2023). *Dementia*. https://www.who.int/news-room/fact-sheets/detail/dementia.

Chapter 6

Computational QSAR Approaches in Medicinal Chemistry: A Comprehensive Review

Priyajit Banerjee[1*], Ishita Saha[2], Suranjana Sarkar[3], Arpan Kumar Maiti[4]

[1]Department of Biotechnology, School of Life Sciences, Swami Vivekananda University, Barrackpore

[2]Department of Physiology, Medical College Kolkata, Kolkata, West Bengal, India, Pin-700073

[3]Department of Microbiology, School of Life Sciences, Swami Vivekananda University, Barrackpore-700121

[4]Department of Zoology, Mitochondrial Biology and Experimental Therapeutics Laboratory, University of North Bengal, Darjeeling, West Bengal, India, Pin-734013.

[*]Corresponding author: Dr Priyajit Banerjee, Department of Biotechnology, School of Life Sciences, Swami Vivekananda University, Barrackpore, Kolkata 700121.

Corresponding author: priyajitb@svu.ac.in

Abstract

Quantitative Structure-Activity Relationship (QSAR) modeling has served as a cornerstone of computational medicinal chemistry for over six decades, offering a systematic framework for correlating molecular structural features with measured biological activities. This review traces the development of QSAR from the classical Hansch-Fujita linear regression models of the early 1960s through three-dimensional approaches such as CoMFA and CoMSIA, to contemporary machine learning architectures including random forests, support vector machines, deep neural networks, and graph neural networks. Key themes addressed include the role of molecular descriptors and fingerprints, model validation consistent with OECD principles, applicability domain analysis, ADMET profiling, and integration with complementary in silico tools. Persistent challenges related to data curation, activity cliffs, model interpretability, and regulatory acceptance are critically evaluated. The review concludes with perspectives on emerging directions including explainable AI, multitask learning, federated training, and the role of QSAR within new approach methodologies for safety assessment.

Keywords: QSAR; molecular descriptors; machine learning; ADMET; drug design; cheminformatics; graph neural networks; model validation; CoMFA; applicability domain

1. Introduction

The pursuit of new therapeutic agents is fundamentally a problem of translating structural information into biological understanding. Quantitative Structure-Activity Relationship (QSAR) modeling addresses this by constructing mathematical relationships between computed molecular properties and experimentally measured biological endpoints, enabling prospective prediction of the activity of compounds before synthesis. Over the six decades since its formal inception, QSAR has become one of the most widely employed computational tools in both academic medicinal chemistry and pharmaceutical industry drug discovery programs (Cherkasov et al., 2014).

The field was formally established through the landmark contributions of Hansch and Fujita (1964), who demonstrated that the lipophilicity parameter log P, coupled with Hammett sigma constants describing electronic effects of substituents, could be incorporated into predictive linear equations correlating chemical structure with the potency of congeneric bioactive compounds. In parallel, Free and Wilson (1964) developed an additive model in which the biological activity of a compound was expressed as the sum of contributions from individual substituents. These foundational approaches established the conceptual scaffold upon which all subsequent QSAR developments have been built.

The field progressed significantly with the introduction of three-dimensional QSAR approaches. CoMFA, developed by Cramer et al. in 1988, became the dominant 3D-QSAR methodology by correlating the spatial distribution of steric and electrostatic molecular fields with biological activity, generating interpretable contour maps that could guide medicinal chemists toward productive structural modifications (Ragno, 2019). The subsequent decades have seen a progressive shift toward machine learning-based QSAR, driven by expanding chemical databases, increased computational resources, and demonstrated superiority of non-linear models in capturing complex structure-activity landscapes (Lo et al., 2018; Lavecchia, 2015).

Most recently, graph neural networks have emerged as a transformative architecture for molecular property prediction, bypassing the need for explicit descriptor computation by learning molecular representations directly from atomic graphs (Koirala et al., 2025). This review provides a critical survey of these developments, aimed at practicing medicinal chemists, with emphasis on

methodological rigor, practical utility, and the ongoing challenges that limit broader translational adoption.

2. Molecular Descriptors: The Structural Language of QSAR

The predictive capability of any QSAR model is contingent on the quality and informativeness of the molecular descriptors selected to represent chemical structure. Descriptors are organized by dimensionality, each capturing distinct physicochemical information. One-dimensional (1D) constitutional descriptors encompass basic molecular properties such as molecular formula, molecular weight, and elemental ratios. Two-dimensional (2D) topological descriptors are derived from the molecular connectivity graph without reference to three-dimensional geometry, including well-established indices such as the Wiener index, Zagreb indices, Randic connectivity index, and Balaban J index, encoding branching patterns, cyclicity, and atom-bond connectivity (Muratov et al., 2020).

Three-dimensional descriptors incorporate spatial molecular geometry, typically computed from energy-minimized conformations. These include molecular surface area, volume, moment of inertia, shape indices such as principal moments of inertia ratios, and quantum chemical descriptors derived from molecular orbital calculations. CoMFA steric and electrostatic field descriptors, computed at a three-dimensional lattice surrounding the aligned ligands, provide high-dimensional descriptor sets that encode both bulk and local electronic environments with spatial resolution directly interpretable through contour plots (Ragno, 2019). The CoMSIA approach further includes hydrophobic, hydrogen bond donor, and acceptor fields, providing a more complete physicochemical description.

Molecular fingerprints represent a distinct and computationally efficient approach, encoding the presence or absence of substructural fragments as binary vectors. Extended Connectivity Fingerprints (ECFP), developed at Scitegic and widely available through RDKit, encode circular neighborhoods of increasing radius around each atom, capturing local chemical environments with high specificity. MACCS structural keys encode 166 predefined substructural queries. Topological pharmacophore fingerprints encode the spatial arrangement of pharmacophoric features rather than explicit substructures. Fingerprint-based descriptors are particularly suited to machine learning pipelines operating on structurally diverse compound libraries (Martin et al., 2023).

Physicochemical descriptors directly encoding drug-likeness parameters, including calculated log P (clog P), polar surface area (PSA), aqueous solubility, pKa, and counts of rotatable bonds, hydrogen

bond donors, and acceptors, form the basis of empirical filters such as Lipinski's Rule of Five and its extensions (Lipinski et al., 1997). Software platforms including Dragon, Mordred, PaDEL-Descriptor, and RDKit offer libraries of several thousand descriptors, necessitating principled dimensionality reduction strategies including PCA, genetic algorithms, stepwise selection, and regularization methods to avoid overfitting and inter-descriptor collinearity (Martinez et al., 2019).

3. Classical Statistical QSAR Methods

Multiple Linear Regression (MLR) remains the most transparent and statistically tractable QSAR method and the natural approach for congeneric series with a small number of variable descriptors. The classical Hansch equation typically relates the logarithm of reciprocal molar activity (log 1/C) to a linear or parabolic function of log P and additive terms for Hammett sigma (electronic) and Taft Es (steric) parameters (Hansch & Fujita, 1964). The parabolic dependence on log P reflects optimum membrane partitioning and is practically informative for guiding lipophilicity management in lead optimization. Statistical parameters including r^2, adjusted r^2, standard error, and F-statistic must be reported alongside LOO cross-validated q^2 to characterize both fit and internal predictivity (Cherkasov et al., 2014).

Partial Least Squares (PLS) regression addresses the collinearity and dimensionality challenges inherent in datasets where descriptor count exceeds compound number. By decomposing both descriptor matrix X and response vector Y into latent components that maximize covariance, PLS tolerates highly correlated descriptor sets without requiring prior variable reduction. Variable Importance in Projection (VIP) scores provide a practical tool for identifying descriptors most contributing to model variance. PLS remains the standard algorithm in CoMFA and CoMSIA 3D-QSAR applications, where field descriptor matrices are inherently high-dimensional (Cherkasov et al., 2014). However, the systematic inflation of LOO-Q^2 as a predictivity measure and its inadequacy as a substitute for true external validation have been extensively documented and cautioned against in the QSAR literature (Tropsha et al., 2003).

For binary and multi-class endpoints, logistic regression and linear discriminant analysis (LDA) provide interpretable, coefficient-driven classification models. These methods remain relevant for regulatory toxicology applications including Ames mutagenicity prediction and carcinogenicity classification, where model transparency is mandated by OECD validation principles and regulators require the ability to audit the basis for predictions (Sahigara et al., 2012). Their parametric

assumptions, however, limit performance on chemically heterogeneous datasets where non-linear boundaries better separate activity classes.

4. Machine Learning-Based QSAR

4.1 Ensemble Tree Methods

Random Forests (RF) have established themselves as among the most reliable and broadly applicable machine learning algorithms in cheminformatics. The ensemble averaging of predictions from decision trees trained on bootstrapped samples with random descriptor subsets provides inherent variance reduction and overfitting resistance without requiring explicit regularization. Permutation-based descriptor importance scores generated by RF provide partial structural interpretability, enabling the identification of physicochemical features most predictive of activity. Gradient boosted decision trees, implemented in frameworks including XGBoost and LightGBM, improve upon RF by iteratively correcting residual prediction errors through additive modeling, achieving competitive or superior performance in multiple cheminformatics benchmarks (Lo et al., 2018). These methods are particularly well-suited to high-throughput screening datasets where the number of compounds enables robust ensemble training.

4.2 Support Vector Machines

Support Vector Machines (SVM) identify the maximum-margin separating hyperplane in a kernel-projected feature space, providing strong generalization from relatively small training datasets. Radial basis function (RBF) kernels and Tanimoto-based similarity kernels specifically adapted for binary fingerprint representations have been developed for molecular property prediction tasks (Lavecchia, 2015). SVM is particularly effective in the small-dataset regime characteristic of target-focused medicinal chemistry programs where experimental data generation is constrained by assay throughput and cost. The primary limitation of SVM is its opacity; the kernel transformation precludes mechanistic interpretation of predictions, and hyperparameter tuning (kernel type, regularization constant C, kernel width gamma) requires systematic cross-validated optimization.

4.3 Artificial Neural Networks and Multitask Deep Learning

Artificial neural networks applied to QSAR from the 1990s onward enabled modeling of non-linear structure-activity relationships inaccessible to linear methods. Modern deep neural network architectures with multiple hidden layers, dropout regularization, and batch normalization have substantially improved robustness. A methodologically significant advance was the demonstration by Merck and academic collaborators that multitask deep neural networks, trained simultaneously on multiple related biological endpoints, outperform single-task models on individual endpoints through shared representation learning and cross-task regularization. This multitask advantage is particularly pronounced for tasks with limited training data, where related endpoints provide supplementary supervisory signal (Koirala et al., 2025).

4.4 Graph Neural Networks

Graph Neural Networks (GNNs) represent the current state of the art in molecular property prediction. Molecules are naturally expressed as undirected graphs where atoms constitute nodes carrying atomic feature vectors (element, hybridization, charge, aromaticity) and bonds constitute edges carrying bond feature vectors (order, stereochemistry, ring membership). GNN architectures such as Message Passing Neural Networks (MPNNs), Attentive Fingerprint (Attentive FP), and the Directed MPNN (DMPNN) iteratively aggregate and update atomic representations by passing messages across the molecular graph, ultimately generating a molecular fingerprint through graph-level readout functions (Koirala et al., 2025). The end-to-end optimization of both the molecular representation and the prediction head jointly adapts the learned fingerprint to the specific biological task, representing a qualitative advance over fixed, precomputed descriptor sets. Benchmark evaluations on MoleculeNet have demonstrated GNN superiority across diverse endpoint categories, though performance advantages diminish on small datasets (Martin et al., 2023).

5. Model Validation and Applicability Domain

The scientific credibility and regulatory utility of QSAR models are inseparable from the rigor of their validation. The five OECD principles for QSAR validation, formally adopted in 2004, establish the internationally recognized framework: (1) a defined endpoint; (2) an unambiguous algorithm; (3) a defined domain of applicability; (4) appropriate measures of goodness-of-fit, robustness, and predictivity; and (5) a mechanistic interpretation, where possible (Sahigara et al., 2012). These principles are operationally required for QSAR model acceptance under EU REACH, the Globally

Harmonized System (GHS) classification, ICH M7 genotoxicity impurity guidance, and FDA/EPA regulatory submissions.

Internal validation, comprising leave-one-out (LOO) and k-fold cross-validation, bootstrapping, and Y-scrambling, assesses model stability and guards against chance correlations within the training set. Y-scrambling, in which the response vector is randomly permuted multiple times to generate null model distributions, is an essential diagnostic for detecting spurious correlations particularly in small datasets. External validation using a structurally diverse, prospectively withheld test set remains the definitive standard for assessing true predictive power (Tropsha et al., 2003; Cherkasov et al., 2014). Reporting standards for regression QSAR should include external R^2, RMSE, and MAE; classification models require ROC-AUC, Matthews Correlation Coefficient, balanced accuracy, and ideally calibration curves.

The Applicability Domain (AD) defines the chemical space region within which model predictions carry defined reliability. Compounds outside the AD are structurally dissimilar to training compounds and should be flagged with explicit uncertainty indicators rather than used to guide decisions. AD estimation methods include: leverage-based Williams plots using hat matrix diagonal values with user-defined threshold $h^* = 3(k+1)/n$; Euclidean and Mahalanobis distance measures in descriptor space; k-nearest neighbor density estimates; and convex hull boundary methods (Sahigara et al., 2012; Tropsha et al., 2003). Conformal prediction frameworks, increasingly applied to GNN-based molecular property prediction, provide rigorous coverage-guaranteed uncertainty quantification directly actionable in prioritization decisions (Li et al., 2024).

6. QSAR in ADMET Property Profiling

Beyond potency modeling, QSAR has found wide application in the in silico prediction of Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) properties, which are collectively responsible for a substantial proportion of clinical attrition in drug development. Early-stage computational ADMET profiling enables the identification and mitigation of liability-prone scaffolds before significant synthetic investment is committed (Muratov et al., 2020).

Individual ADMET endpoints that have been extensively modeled by QSAR approaches include aqueous thermodynamic and kinetic solubility, Caco-2 and PAMPA permeability, passive transcellular permeability, plasma protein binding, microsomal and hepatic metabolic stability, CYP450 isoform (1A2, 2C9, 2C19, 2D6, 3A4) inhibition and substrate prediction, P-glycoprotein

substrate recognition, hERG potassium channel inhibition, blood-brain barrier penetration, and a broad range of in vitro and in vivo toxicity endpoints including Ames mutagenicity, hepatotoxicity, and rodent acute toxicity (Muratov et al., 2020; Lombardo et al., 2024).

Graph neural network architectures have demonstrated particular promise for ADMET prediction, exploiting local chemical environment features at individual atomic sites that are mechanistically determinative of metabolic susceptibility, transporter recognition, and ion channel interactions. Attention-based GNN models applied to ADMET endpoints provide atom-level importance scores identifying metabolic soft spots and structural liabilities with chemical specificity (De Carlo et al., 2024). The prediction of CYP450-mediated metabolism has benefited specifically from graph-based molecular representations that capture substrate-enzyme interaction patterns relevant to regioselective oxidation (Abdelwahab et al., 2025). Web-accessible multi-endpoint platforms including pkCSM, SwissADME, ADMETlab 2.0, and DeepPurpose have made ADMET profiling accessible to experimentalists, though model validation rigor and applicability domain coverage differ substantially and require critical evaluation (Lombardo et al., 2024).

7. Integration with Complementary In Silico Approaches

QSAR operates most effectively not as a standalone method but as a component within integrated computational drug discovery workflows. Receptor-based or structure-based QSAR approaches incorporate interaction energy terms derived from molecular docking calculations as descriptors, enabling the modeling of structure-activity series where ligand-receptor complementarity dominates potency variation. This hybrid approach partially addresses the dependence of classical ligand-based QSAR on the structural congruence of the training set (Cherkasov et al., 2014).

Pharmacophore models, which encode the spatial arrangement of essential interaction features including hydrogen bond donors and acceptors, hydrophobic centers, and aromatic rings, serve complementarily to QSAR as interpretable frameworks for structure-activity rationalization and scaffold hopping. The integration of QSAR-derived activity predictions with pharmacophore-based virtual screening filters provides a multi-criteria prioritization of compound libraries that exploits the complementary strengths of both approaches.

Multi-target QSAR (mt-QSAR) enables simultaneous modeling of activity across multiple biological targets or selectivity-relevant off-target endpoints within a unified computational framework. This is particularly important for the design of selective kinase inhibitors, where differentiation between

closely related family members depends on subtle structural features, and for the management of off-target liabilities such as hERG inhibition in cardiovascular safety assessment (Lo et al., 2018). The combination of multitask deep neural networks with multi-endpoint training datasets aligns naturally with the mt-QSAR paradigm and represents a methodologically coherent approach to polypharmacology-aware molecular design (Koirala et al., 2025).

8. Persistent Challenges and Critical Limitations

Despite the considerable advances surveyed above, QSAR modeling faces enduring challenges that constrain its reliability and translational impact. Data quality and curation remain the most fundamental determinants of model performance and are frequently underestimated relative to algorithm selection. Training datasets assembled from heterogeneous sources introduce systematic noise from assay format differences, concentration unit inconsistencies, stereochemical ambiguities, and structural duplicates. Rigorous dataset curation including compound structure standardization, duplicate removal, activity unit harmonization, and outlier analysis is a prerequisite for models that generalize beyond the training set (Cherkasov et al., 2014; Muratov et al., 2020).

Activity cliffs represent a structural challenge that is mechanistically important yet algorithmically underserved. An activity cliff is defined as a pair of structurally similar compounds exhibiting a large, often orders-of-magnitude, difference in biological potency attributable to a subtle structural modification. Such abrupt changes in the structure-activity landscape arise from the introduction of a single hydrogen bond donor capable of filling an unoccupied pocket, a stereocenter inversion disrupting binding geometry, or a bioisosteric replacement altering electronic distribution. Global molecular descriptors frequently fail to capture the local substructural differences responsible for cliff behavior, leading to systematic prediction errors in the most pharmacologically informative regions of compound space (Muratov et al., 2020).

Model interpretability is a persistent challenge for complex machine learning architectures. Ensemble methods and deep neural networks operate as black boxes relative to the mechanistic insight that medicinal chemists require to make synthesis decisions. While explainable AI approaches including SHAP analysis, attention weight visualization in GNNs, and integrated gradient methods provide atom-level attribution maps, the chemical consistency and mechanistic fidelity of these attributions under different architectures requires systematic evaluation (Martin et al., 2023). Chemical space coverage limitations, arising from the structural bias of training datasets toward marketed drug analogs

and well-characterized pharmacological classes, impose unacknowledged extrapolation risks when models are applied to structurally novel scaffolds, reinforcing the indispensability of rigorous AD characterization (Sahigara et al., 2012).

9. Future Perspectives

The near-term trajectory of QSAR is shaped by several convergent technological and institutional developments. The application of large chemical language models, pretrained on tens of millions of SMILES strings from public chemical databases through self-supervised objectives, to downstream molecular property prediction through fine-tuning represents a promising avenue for improving few-shot learning performance in data-scarce medicinal chemistry contexts (Koirala et al., 2025). These foundation models may substantially reduce the data requirements for robust QSAR across novel target classes where compound databases are limited.

Federated learning frameworks offer a pathway to training QSAR models on compound databases that collectively span far broader chemical and biological space than any individual organization's proprietary dataset, without requiring disclosure of confidential structures or activity data. The practical implementation of federated QSAR would require standardization of data formats, model architectures, and federated training protocols across collaborating institutions. Early demonstrations of federated learning in biomedical prediction tasks provide proof of concept for this direction (Lo et al., 2018).

From a regulatory perspective, the formal integration of QSAR within new approach methodologies (NAMs) for toxicological assessment, under international frameworks including the US Tox21 program, EU REACH CASE STUDY initiatives, and OECD QSAR Toolbox development, will increasingly demand transparent model documentation, probabilistic output formats with confidence intervals, and mechanistic rationale linking structural features to predicted endpoints. Conformal prediction methods that provide coverage-guaranteed prediction intervals represent a methodologically sound response to these demands (Li et al., 2024; Muratov et al., 2020).

Conclusion

Computational QSAR has progressed from simple bilinear equations correlating substituent lipophilicity with receptor affinity to sophisticated graph neural architectures that learn molecular representations de novo from atomic connectivity. Across this developmental arc, the central premise

articulated by Hansch and Fujita in 1964 has remained unchanged: biological activity is a deterministic and predictable function of molecular structure. What has changed profoundly is the mathematical sophistication and chemical breadth with which this relationship can be captured.

For medicinal chemists, the practical value of QSAR extends beyond prediction accuracy to actionable structural guidance that can focus synthetic programs and rationalize observed structure-activity trends. Realizing this value requires models built on rigorously curated datasets, validated against structurally diverse external test sets, characterized for applicability domain, and expressed with transparency sufficient for mechanistic interpretation. The OECD validation framework provides the appropriate regulatory and scientific standard against which all QSAR models should be assessed.

As the boundaries between QSAR, deep learning, structural biology, and omics sciences continue to dissolve, the field is well-positioned to contribute increasingly to the rational, data-driven, and resource-efficient design of medicines addressing unmet therapeutic needs.

References

Abdelwahab, A. A., Elattar, M. A., & Fawzi, S. A. (2025). Advancing ADMET prediction for major CYP450 isoforms: Graph-based models, limitations, and future directions. BioMedical Engineering OnLine. https://doi.org/10.1186/s12938-025-01412-6

Cherkasov, A., Muratov, E. N., Fourches, D., Varnek, A., Baskin, I. I., Cronin, M., Dearden, J., Gramatica, P., Martin, Y. C., Todeschini, R., Consonni, V., Kuz'min, V. E., Cramer, R., Benigni, R., Yang, C., Rathman, J., Terfloth, L., Gasteiger, J., Richard, A., & Tropsha, A. (2014). QSAR modeling: Where have you been? Where are you going to? Journal of Medicinal Chemistry, 57(12), 4977-5010. https://doi.org/10.1021/jm4004285

De Carlo, A., Ronchi, D., Piastra, M., Tosca, E. M., & Magni, P. (2024). Predicting ADMET properties from molecule SMILES: A bottom-up approach using attention-based graph neural networks. Pharmaceutics, 16(6), 776. https://doi.org/10.3390/pharmaceutics16060776

Free, S. M., Jr., & Wilson, J. W. (1964). A mathematical contribution to structure-activity studies. Journal of Medicinal Chemistry, 7(4), 395-399. https://doi.org/10.1021/jm00334a001

Hansch, C., & Fujita, T. (1964). p-sigma-pi analysis: A method for the correlation of biological activity and chemical structure. Journal of the American Chemical Society, 86(8), 1616-1626. https://doi.org/10.1021/ja01062a035

Koirala, M., Yan, L., Mohamed, Z., & DiPaola, M. (2025). AI-integrated QSAR modeling for enhanced drug discovery: From classical approaches to deep learning and structural insight. International Journal of Molecular Sciences, 26(19), 9384. https://doi.org/10.3390/ijms26199384

Lavecchia, A. (2015). Machine-learning approaches in drug discovery: Methods and applications. Drug Discovery Today, 20(3), 318-331. https://doi.org/10.1016/j.drudis.2014.10.012

Li, P., Hua, L., Ma, Z., Hu, W., Liu, Y., & Zhu, J. (2024). Conformalized graph learning for molecular ADMET property prediction and reliable uncertainty quantification. Journal of Chemical Information and Modeling, 64(23), 8705-8717. https://doi.org/10.1021/acs.jcim.4c01139

Lipinski, C. A., Lombardo, F., Dominy, B. W., & Feeney, P. J. (1997). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Advanced Drug Delivery Reviews, 23(1-3), 3-25. https://doi.org/10.1016/S0169-409X(96)00423-1

Lo, Y.-C., Rensi, S. E., Torng, W., & Altman, R. B. (2018). Machine learning in chemoinformatics and drug discovery. Drug Discovery Today, 23(8), 1538-1546. https://doi.org/10.1016/j.drudis.2018.05.010

Gadaleta, D., Serrano-Candelas, E., Ortega-Vallbona, R. et al. Comprehensive benchmarking of computational tools for predicting toxicokinetic and physicochemical properties of chemicals. J Cheminform 16, 145 (2024). https://doi.org/10.1186/s13321-024-00931-z

Niazi, S.K.; Mariam, Z. (2023). Recent advances in machine-learning-based chemoinformatics: A comprehensive review. International Journal of Molecular Sciences, 24(14), 11488. https://doi.org/10.3390/ijms241411488

Martinez, M. J., Razuc, M., & Ponzoni, I. (2019). MoDeSuS: A machine learning tool for selection of molecular descriptors in QSAR studies applied to molecular informatics. Molecular Informatics, 38(7-8), 1800140. https://doi.org/10.1155/2019/2905203

Muratov, E. N., Bajorath, J., Sheridan, R. P., Tetko, I. V., Filimonov, D., Poroikov, V., Oprea, T. I., Baskin, I. I., Varnek, A., Roitberg, A., Isayev, O., Curtarolo, S., Fourches, D., Cohen, Y., Bender, A., Tropsha, A., & Polishchuk, P. (2020). QSAR without borders. Chemical Society Reviews, 49(11), 3525-3564. https://doi.org/10.1039/D0CS00098A

Ragno, R. (2019). www.3d-qsar.com: A web portal that brings 3-D QSAR to all electronic devices - the Py-CoMFA web application as tool to build models from pre-aligned datasets. Journal of Computer-Aided Molecular Design, 33(10), 855-864. https://doi.org/10.1007/s10822-019-00231-x

Sahigara, F., Mansouri, K., Ballabio, D., Mauri, A., Consonni, V., & Todeschini, R. (2012). Comparison of different approaches to define the applicability domain of QSAR models. Molecules, 17(5), 4791-4810. https://doi.org/10.3390/molecules17054791

Tropsha, A., Gramatica, P., & Gombar, V. K. (2003). The importance of being earnest: Validation is the absolute essential for successful application and interpretation of QSAR models. QSAR & Combinatorial Science, 22(1), 69-77. https://doi.org/10.1002/qsar.200390007

Chapter 7

Arsenic (As) Bioaccumulation in *Spinacia oleracea* (Spinach): A Study on Uptake Mechanisms and Health Implications

Misha Sarkar[1], Pritha Pal[2*]

[1]*Department of Microbiology, School of Life Sciences, Swami Vivekananda University, Barrackpore, West Bengal 700121, India.*

[2]*Assistant Professor, Department of Microbiology, School of Life Sciences, Swami Vivekananda University, Barrackpore, West Bengal 700121, India.*

Corresponding author: prithap@svu.ac.in

Abstract

Metalloids or heavy metal accumulation in the soil interrupts the normal functioning of soil ecosystems and plant growth. This paper shares insights into potential bioaccumulation of arsenic in Spinacia oleracea (Spinach). Plants absorb various kinds of heavy metals/metalloids, when available in the soil or irrigation water. Metals like manganese (Mn), magnesium (Mg), copper (Cu) and iron (Fe) are essential plant metals. These metals are required in specific amount and their deficiency or elevated concentrations will result in toxic effects and reduce the plant productivity. Soils are contaminated in the environment with a number of heavy metals by natural weathering and erosion of parent rock material or ore deposits. Also, artificial wastewater irrigation, mining activities. The presence of one contaminant can increase or decrease the impacts of others. In many parts of the world, growing crops on polluted soils often leads to elevated levels of pollutants in plant tissues. Minimizing the transfer of these pollutants into edible plant tissues, while improving the plant growth and the productivity is a major area of research. Spinach can accumulate arsenic, but the extent varies depending on environmental factors and the specific form of arsenic present. Contamination of leafy vegetables grown in heavy metalloid polluted mining areas pose serious health risks. *Spinacia oleracea* has a crucial position due to large and expanded leaves, fast growth and by being an essential and a common part of the human diet. Spinach roots tend to accumulate more arsenic than leaves, with studies showing higher arsenic levels in roots compared to shoots. Nevertheless, there is a lack of information regarding growth behaviour, metal accumulation, total protein content, fiber

characteristics, moisture content and inorganic nutrients response to individual and combined heavy metals with respect to this plant. More than million people worldwide are exposed to groundwater arsenic poisoning, including countries like Bangladesh, China, and Taiwan experiencing significant health impacts. Therefore, it is necessary to unravel the response of *S. oleracea* to a range of individual and combined heavy metals. This paper reviews the probable bioaccumulation of arsenic in *Spinacia oleracea* (Spinach).

Keywords:

Spinacia oleracea (spinach), leafy vegetable, arsenic (As) bioaccumulation, Soil contamination, heavy metal stress, food safety.

INTRODUCTION

The annual plant spinach (*Spinacia oleracea L.*) is a green, leafy vegetable that may be cultivated in both spring and fall and produces large quantities quickly. The leafy green blooming plant referred to as spinach (*Spinacia oleracea*) is indigenous to Central and Western Asia. It belongs to the family Amaranthaceae, subfamily Chenopodioideae, and order Caryophyllales. Its leaves are a popular vegetable that can be eaten fresh or preserved by canning, freezing, or dehydrating them. The flavour varies greatly depending on whether it is consumed cooked or raw; steaming might lessen the high oxalate level. It is an annual plant that can reach a height of 30 cm (1 ft) and is seldom biannual. Temperate climates may support spinach over the winter. Larger leaves are found near the base of the plant, while smaller leaves are found higher up the flowering stem. The leaves are simple, ovate to triangular, alternating, and vary greatly in size, from 2 to 30 cm (1 to 12 in) in length and 1 to 15 cm (1⁄2 to 6 in) in width. The yellow green, hardly noticeable blooms have a diameter of 3–4 mm (1⁄8–5⁄32 in). They develop into a tiny, lumpy, hard, dry fruit cluster that is 5–10 mm (1⁄4–3⁄8 in) wide and contains several seeds.

Arsenic (As) belongs to a group of metalloid elements. Arsenic (As) is a significant environmental threat due to its high toxicity, contamination, non-biodegradability, high concentrations in the environment, and accumulation. Additionally, it is a costly and challenging element to recover and dispose of from its environment [1]. Arsenic is present in the environment in a variety of inorganic and organic chemical forms, including arsenite (As III), arsenate (As V), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), trimethyl arsine oxide (TMAO), arsenobetaine (AsB) and many

more [2]. Inorganic As(III) is more poisonous than As(V) due to differences in mobility, solubility, and toxicity among species. Conversely, organic As species are less toxic [3]. Arsenic is present in soil, water, plants, and the air. Airborne particulate matter from coal combustion and ore smelting are the primary sources of As exposure in industrial locations. Toxic metals can enter the human food chain by consuming plants grown in contaminated areas and be harmful to human health. Spinach is one of the plants where the presence of As was found in plants from affected regions [4]. In soil, water, and air, As exists in many chemical forms with variable degrees of mobility, bioavailability, and toxicity to plants [5]. Factors affecting these parameters include the As concentration in the soil, As species, the type of plant species, and other soil properties controlling As accessibility, accumulation, and fate in soils, microorganisms, and plants [6]. Although much progress has been made in understanding the mechanism of As uptake, translocation, and accumulation, several knowledge gaps still exist [7]. Plants intake of As can hardly be downregulated, as it is often mediated by essential element transporters [8]. Arsenic exposure has an adverse effect on the morphological (e.g., chlorosis), physiological (e.g., growth processes inhibition), and biochemical (e.g., oxidative stress) responses of plants [9]. Inside plant cells, both As(V) and As(III), including their conversion, induce oxidative stress by enhancing the production of reactive oxygen species, which affects the regulation of a diverse range of metabolic pathways [9]. The induction of oxidative stress is the main process underlying As toxicity in plants [9]. Furthermore, As acts by impairing mitochondrial enzymes, thereby causing a halt in cellular respiration and uncoupling oxidative phosphorylation [9]. Arsenate does not react directly with the active sites of enzymes, but this form strongly interacts with sulfhydryl groups in proteins, interfering with cellular functions [9]. As has the potential to negatively impact all plant tissues [10]. Nonetheless, leaves play a crucial role in photosynthesis and serve as critical interfaces between plants and their surroundings [11]. The amount of damage to the photosynthetic apparatus in the setting of As stress is mostly correlated with the dosage and application type of As therapy [12]. Moreover, As has a negative impact on the structural characteristics of the leaves, including mesophyll thickness, mesophyll surface area, and leaf reflectance [13]. Plants exhibit a wide variability in their response to As [9]. The ability of plants to survive in the presence of an element that is otherwise toxic is defined as tolerance [9]. Leafy vegetables have been found to accumulate more metals and metalloids, including As [15], and vegetables in general are quite susceptible to metal/metalloid stress [14]. With its enormous surface areas, high nutritional content, relatively rapid growth rates, and higher metal/metalloid absorption rates, *Spinacia oleracea* is one of the most valued green vegetables

[16]. Around the world, it is extensively grown and researched for the accumulation of metals and metalloids [17]. The information comparing the responses of *S. oleracea* and As hyperaccumulators to As stress, however, is lacking.

Plant tissue where Arsenic accumulation occurs

In spinach plants, arsenic tends to bioaccumulate in both the roots and the shoots. Compared to shoots, roots frequently acquire larger quantities of arsenic. Arsenic enters a plant through its roots; these are the main places where it accumulates. Arsenic has the ability to move from roots to leaves and other shoots. Depending on variables such as plant species and soil pollution levels, shoot arsenic

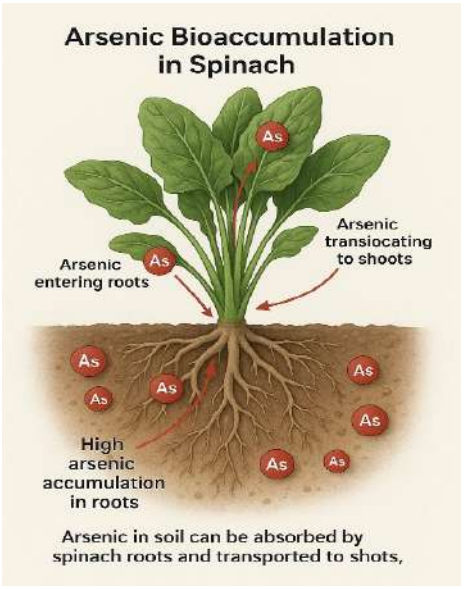


Fig. 1: Arsenic Bioaccumulation in spinach (*S.oleracea)*

concentrations can change. Spinach is a leafy green food that might collect arsenic, which could be harmful to consumers health. If arsenic builds up in spinach plants edible portions, it poses issues for both human health and food safety.

Movement of Arsenic from ground water to soil to plant

Groundwater contaminated with arsenic can irrigate soil, which leads to arsenic accumulation. In arsenic mobilization iron oxides and microbes play significant roles. Plants absorb arsenic from soil through various mechanisms such as passive uptake where arsenic enters plant roots through the apoplast, direct transcellular transport where arsenic is transported from the environment to the plant vascular system, and active uptake where ions are taken up against their concentration gradient.

Arsenic is then translocated from roots to shoots via the xylem. Different plant species and parts exhibit varying arsenic accumulation patterns.

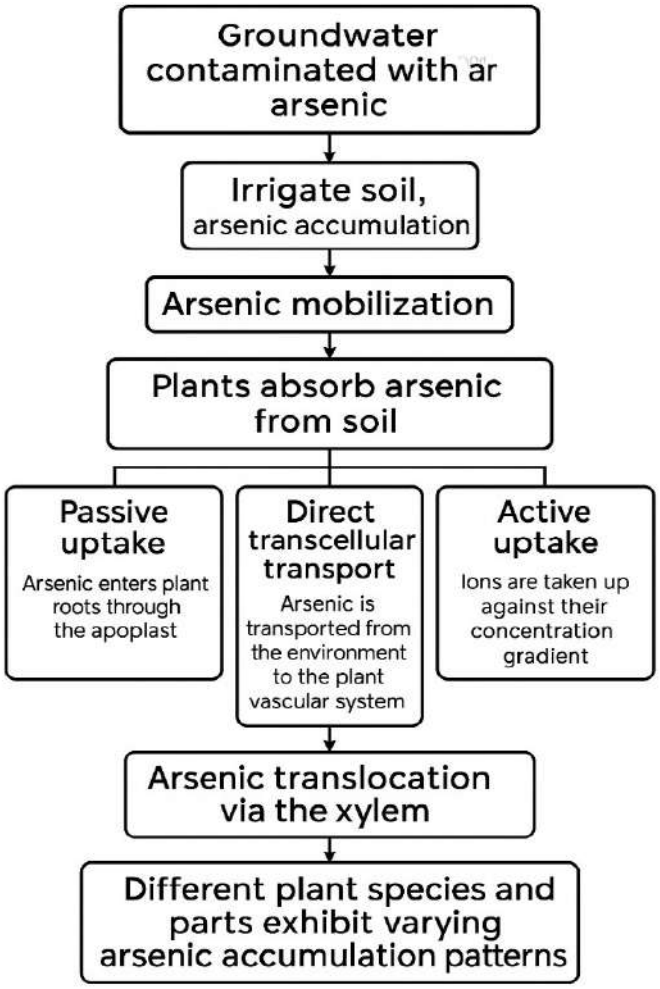


Fig 2: Word diagram of movement of Arsenic from ground water to soil to plant

Mechanism of toxicity of Arsenic to the plant

Arsenic exposure generates reactive oxygen species (ROS), leading to oxidative stress. ROS damage cellular components, including proteins, lipids, and DNA. Arsenic interferes with essential cellular processes, such as: arsenic binds to sulfhydryl groups, inhibiting enzyme activity, arsenic alters protein structure and function. Arsenic competes with essential nutrients, like phosphorus, for uptake and utilization. Nutrient imbalance affects plant growth and development. Arsenic exposure can cause DNA damage and mutations, leading to genotoxic effects. Arsenic alters hormone regulation, affecting plant growth and development. Spinach plants may employ tolerance mechanisms, such as: activation of antioxidant enzymes to mitigate oxidative stress, binding of arsenic to thiol compounds or other chelators and compartmentalization of arsenic in vacuoles.

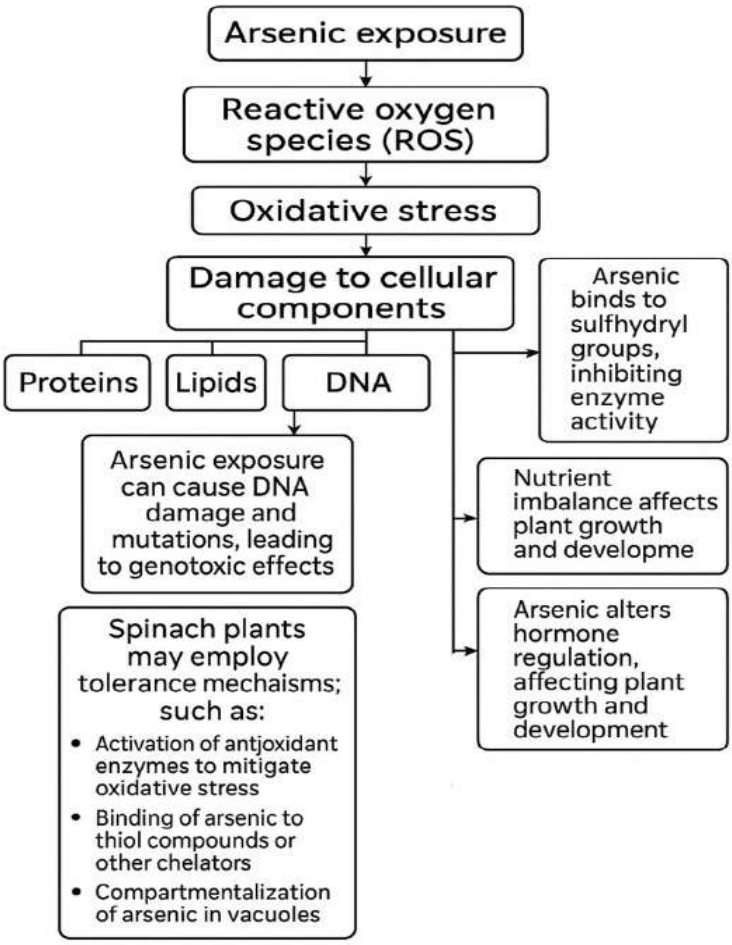


Fig 3: Mechanism of toxicity of Arsenic to the plant

Disease prevalence In India and abroad

Almost 20% of India's land surface has toxic levels of arsenic in its groundwater, exposing over 250 million people to the poisonous element. States like Punjab, Bihar, and West Bengal have the largest areas of elevated groundwater arsenic zones, with 69%, 70%, and 92% respectively. Arsenic exposure can cause cancer, skin conditions, and other disorders. In Bihar, arsenic contamination has been linked to gallbladder cancer, with 22 districts severely affected. Other health problems include skin lesions, hyperkeratosis, and melanosis. The Ganga-Brahmaputra-Meghna River basin is particularly affected, with states like West Bengal, Bihar, Uttar Pradesh, and Assam experiencing high levels of arsenic contamination [20]. Over 300 million people worldwide are exposed to groundwater arsenic poisoning, with countries like Bangladesh, China, and Taiwan experiencing significant health impacts. Long term exposure to arsenic can cause skin, kidney, liver, bladder, or lung cancer, as well as cardiovascular disease and diabetes. Areas with high arsenic levels in groundwater include the Himalayan region, with countries like Nepal and Bangladesh facing significant challenge [21]. An estimated 58-64 million people in India are exposed to arsenic levels exceeding 10 µg/L in drinking water, leading to increased cardiovascular disease (CVD) mortality risks. Assam and West Bengal have the highest population attributable fractions (PAFs) of groundwater arsenic attributable CVD mortality. Bihar, Punjab, and Nagaland also have significant CVD mortality risks due to arsenic exposure [22].

State / Region	Arsenic Hotspot Status	Spinach Accumulation Risk	Reference
West Bengal	Major hotspot in Ganga–Brahmaputra basin	High — spinach leaves ~2.7–3.0 mg/kg dry weight	Bhattacharyya K, Sengupta S, Pari A, Halder S, Bhattacharya P, Pandian BJ, Chinchmalatpure AR. Characterization and risk assessment of arsenic contamination in soil-plant (vegetable) system and its mitigation through water harvesting and organic amendment. Environ Geochem Health. 2021 Aug;43(8):2819-2834. doi: 10.1007/s10653-020-00796-9. Epub 2021 Jan 7. PMID: 33411124.
Bihar	High groundwater arsenic via Ganga plains	Risk due to irrigation, less direct spinach data	Alam, M.O., Chakraborty, S. & Bhattacharya, T. Soil Arsenic Availability and Transfer to Food Crops in Sahibganj, India with Reference to Human Health Risk. Environ. Process. 3, 763–779 (2016). https://doi.org/10.1007/s40710-016-0184-9
Assam / UP / Gangetic Plains	Elevated groundwater arsenic zones	Potential risk similar to other GBM basin states	-

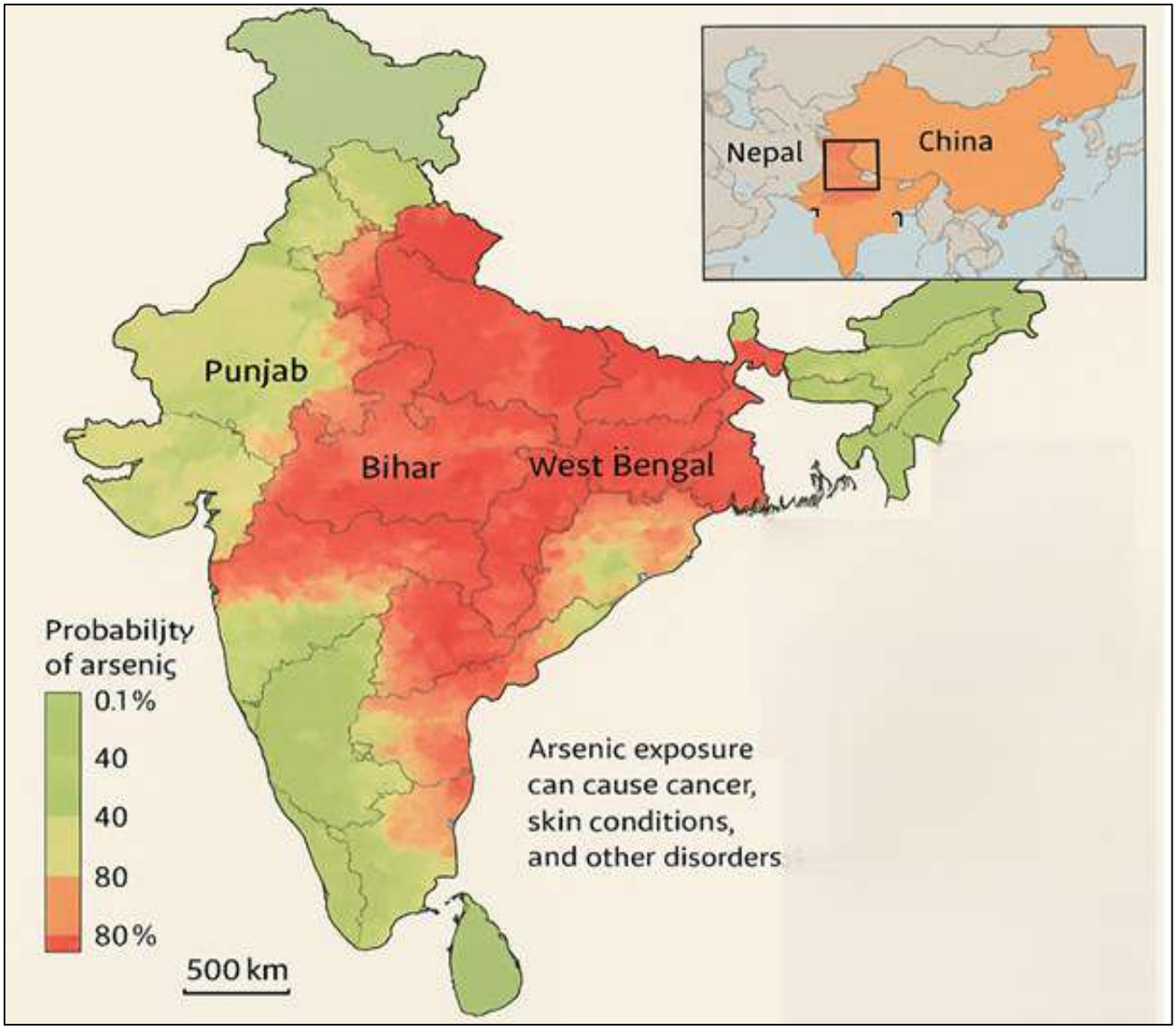


Fig 4: Arsenic hazard maps. (a) Probability of arsenic concentration in groundwater exceeding.

CONCLUSION

Across the world, crops are often cultivated on soils contaminated by arsenic (As), which puts millions of people especially in Asia at risk of Arsenic exposure through consumption of tainted vegetables. Effects of arsenic bioaccumulation on Spinach Plants are such as reduced growth and yield, Chlorosis and necrosis, decreased photosynthesis and increased antioxidant activity. Increasing populations and the increasing need for safe and healthy food supply means that urgent measures are needed to limit the transfer of As into crops. Arsenic contamination is a serious issue on a global scale for animal and human health as well as agriculture. Research on As origin, toxicities, mobility, distribution patterns,

bioaccumulation, and remediation, the scientific understanding of this metalloid is still evolving, and the study of its interactions should help in the development of methods of safe clean up and exposure prevention all the way down to the trophic level of plants. Contamination by heavy metals, presents serious risks to agricultural sustainability and human health, especially for important green vegetables like spinach. This study emphasizes the critical necessity to address contamination by highlighting the effects of heavy metal on the morphology, physiology, and production of spinach. Heavy metals bioaccumulate in spinach, which has an impact on soil health and plant health. Developing protection methods requires an understanding of the mechanisms behind heavy metal uptake, translocation, and detoxification in spinach. Concerns about food safety are raised by the discovery of heavy metals, in spinach's edible sections. Soil remediation, spinach phytoremediation potential, and genetic modifications for metal tolerance are promising approaches. To lessen the negative effects of heavy metals pollution on agriculture, human health, and spinach cultivation, integrated methods are crucial.

REFERENCE

Abbas, G.; Murtaza, B.; Bibi, I.; Shahid, M.; Niazi, N.K.; Khan, M.I.; Amjad, M.; Hussain, M. Arsenic uptake, toxicity, detoxification, and speciation in plants: Physiological, biochemical, and molecular aspects. *Int. J. Environ. Res. Public Health* 2018, *15*, 59.

Akter K. F. Owens G. Davey D. E. Naidu R. Rev. Environ. Contam. Toxicol. 2005;184:97–149. doi: 10.1007/0-387-27565-7_3.

Alia, N.; Sardar, K.; Said, M.; Salma, K.; Sadia, A.; Sadaf, S.; Toqeer, A.; Miklas, S. Toxicity and bioaccumulation of heavy metals in spinach (*Spinacia oleracea*) grown in a controlled environment . *Int. J. Environ. Res. Public Health* 2015, *12*, 7400–7416.

Alka S. Shahir S. Ibrahim N. Ndejiko M. J. Vo D. V. Manan F. A. J. Cleaner Prod. 2021;278:123805. doi: 10.1016/j.jclepro.2020.123805.

Arun Kumar, Ravi C, Sameer Dhingra, Krishna Murti, Mohammad Ali, Ashok Kumar Ghosh. Arsenic Causing Gallbladder Cancer Disease near the Himalayan bound Rivers in Bihar: A Case study of Gallbladder Cancer. Journal of Cancer Science and Clinical Therapeutics 6 (2022): 388-391.

Bandaru, V.; Hansen, D.J.; Codling, E.E.; Daughtry, C.S.; White-Hansen, S.; Green, C.E. Quantifying arsenic-induced morphological changes in spinach leaves: Implications for remote sensing. *Int. J. Remote Sens.* 2010, *31*, 4163–4177. [Google Scholar] [CrossRef]

Bibi, A., Rasul, F., Shahzad, S. *et al.* Toxicity, bioaccumulation and mitigating strategies of heavy metals stress on morpho-physiology of spinach. *Discov. Plants* 1, 74 (2024). https://doi.org/10.1007/s44372-024-00083-2

Chaturvedi, R.; Favas, P.J.C.; Pratas, J.; Varun, M.; Paul, M.S. Metal(loid) induced toxicity and defense mechanisms in *Spinacia oleracea* L. Ecological hazard and prospects for phytoremediation Ecotox. *Environ. Saf.* 2019, *183*, 109570.

Cui S, Wang Z, Li X, Wang H, Wang H, Chen W. A comprehensive assessment of heavy metal(loid) contamination in leafy vegetables grown in two mining areas in Yunnan, China-a focus on bioaccumulation of cadmium in Malabar spinach. Environ Sci Pollut Res Int. 2023 Feb;30(6):14959-14974. doi: 10.1007/s11356-022-23017-5. Epub 2022 Sep 26. PMID: 36161572.

Han L. Gao B. Hao H. Lu J. Xu D. Sci. Total Environ. 2019;651:1983–1991. doi: 10.1016/j.scitotenv.2018.09.381.

Hassan FI, Niaz K, Khan F, Maqbool F, Abdollahi M. The relation between rice consumption, arsenic contamination, and prevalence of diabetes in South Asia. EXCLI J. 2017 Oct 9;16:1132-1143. doi: 10.17179/excli2017-222. PMID: 29285009; PMCID: PMC5735331.

Huang SY, Zhuo C, Du XY, Li HS. Remediation of arsenic-contaminated paddy soil by intercropping aquatic vegetables and rice. Int J Phytoremediation. 2021;23(10):1021-1029. doi: 10.1080/15226514.2021.1872485. Epub 2021 Jan 25. PMID: 33491468.

Hussain, Z., Alam, M., Khan, M.A. *et al.* Bioaccumulation of potentially toxic elements in spinach grown on contaminated soils amended with organic fertilizers and their subsequent human health risk. *Arab J Geosci* 13, 945 (2020). https://doi.org/10.1007/s12517-020-05938-y

Kofroňová, M.; Hrdinová, A.; Mašková, P.; Tremlová, J.; Soudek, P.; Petrová, Š.; Pinkas, D.; Lipavsk, H. Multi -component antioxidative system and robust carbohydrate status, the essence of plant arsenic tolerance. *Antioxidants* 2020, *9*, 283.

Li, G.; Hu, S.; Zhao, X.; Kumar, S.; Li, Y.; Yang, J.; Hou, H. Mechanisms of the morphological plasticity induced by phytohormones and the environment in plants. *Int. J. Mol. Sci.* 2021, *22*, 765.

Naz, Alia & Khan, Sardar & Muhammad, Said & Khalid, Salma & Alam, Sadia & Siddique, Sadaf & Ahmed, Toqeer & Scholz, Miklas. (2015). Toxicity and Bioaccumulation of Heavy Metals in Spinach (Spinacia oleracea) Grown in a Controlled Environment. International Journal of Environmental Research and Public Health. 12. 10.3390/ijerph120707400.

Popov, M., Kubeš, J., Vachová, P., Hnilička, F., Zemanová, V., Česká, J., Praus, L., Lhotská, M., Kudrna, J., Tunklová, B., Štengl, K., Krucký, J., & Turnovec, T. (2023). Effect of Arsenic Soil Contamination on Stress Response Metabolites, 5-Methylcytosine Level and CDC25 Expression in Spinach. *Toxics*, *11*(7), 568. https://doi.org/10.3390/toxics11070568

Qin J, Niu A, Liu Y, Lin C. Arsenic in leafy vegetable plants grown on mine water-contaminated soils: Uptake, human health risk and remedial effects of biochar. J Hazard Mater. 2021 Jan 15;402:123488. doi: 10.1016/j.jhazmat.2020.123488. Epub 2020 Jul 16. PMID: 32738781.

Quaghebeur, M.; Rengel, Z. Arsenic speciation governs arsenic uptake and transport in terrestrial plants. *Microchim. Acta* 2005, *151*, 141–152.

Ram, B., Narayan, H., Baghel, D. *et al.* Bioremediation of Arsenic and Cadmium by Metal-Resistant Bacteria in Spinach. *Proc. Natl. Acad. Sci., India, Sect. B Biol. Sci.* 94, 1105–1112 (2024). https://doi.org/10.1007/s40011-024-01618-z

Rodrguez -Ruiz, M.; Aparicio-Chacn, M.V.; Palma, J.M.; Corpas, F.J. Arsenate disrupts ion balance, sulfur and nitric oxide metabolisms in roots and leaves of pea (*Pisum sativum* L.) plants. *Environ. Exp. Bot.* 2019, *161*, 143–156.

Shahid, M.; Pinelli, E.; Pourrut, B.; Silvestre, J.; Dumat, C. Lead-induced genotoxicity to *Vicia faba* L. roots in relation with metal cell uptake and initial speciation. *Ecotox. Environ. Saf.* 2011, *74*, 78–84.

Solrzano, E.; Corpas, F.J.; Gonzlez -Gordo, S.; Palma, J.M. Reactive oxygen species (ROS) metabolism and nitric oxide (NO) content in roots and shoots of rice (*Oryza sativa* L.) plants under arsenic-induced stress. *Agronomy* 2020, *10*, 1014.

Tang, Z.; Zhao, F.J. The roles of membrane transporters in arsenic uptake, translocation and detoxification in plants. *Crit. Rev. Environ. Sci. Technol.* 2020, in press.

Wu, R., Xu, L., & Polya, D. A. (2021). Groundwater Arsenic-Attributable Cardiovascular Disease (CVD) Mortality Risks in India. *Water*, *13*(16), 2232. https://doi.org/10.3390/w13162232

Zama EF, Reid BJ, Sun G-X, Yuan H-Y, Li X-M, Zhu Y-G, Silicon (Si) biochar for the mitigation of arsenic (As) bioaccumulation in spinach (*Spinacia oleracean*) and improvement in the plant growth, *Journal of Cleaner Production* (2018), doi: 10.1016/j.jclepro.2018.04.056.

Zemanová, V., Pavlíková, D., Hnilička, F., & Pavlík, M. (2021). Arsenic Toxicity-Induced Physiological and Metabolic Changes in the Shoots of *Pteris cretica* and *Spinacia oleracea*. *Plants*, *10*(10), 2009. https://doi.org/10.3390/plants10102009

Zubair, M.; Khan, Q.U.; Mirza, N.; Sarwar, R.; Khan, A.A.; Baloch, M.S.; Fahad, S.; Shah, A.N. Physiological response of spinach to toxic heavy metal stress. *Environ. Sci. Pollut. Res.* 2019, *26*, 31667–31674.

Chapter 8

The Versatile World of Circular RNAs: Dual Role as Therapeutic Targets and Promising Biomarkers.

Chandrima Mukherjee[1], Madhurima Mukherjee[1], Semanti Ghosh[1*]

[1]Department of Biotechnology, School of Life Sciences, Swami Vivekananda University, Barrackpore, Kolkata-[700121]

Corresponding author: semantig@svu.ac.in

Abstract

Circular RNAs (circRNAs) are Unique RNA players in mammalian gene regulation, emergings primarily as noncoding, covalently closed single standed loop structure. Unlike linear RNAs, circRNAs lack 5' and 3' open ends, which renders them resistant to exonuclease degradation, contributing to their remarkable stability. CircRNAs are primarily generated by back splicing of exons or from lariat introns. Recent studies have revealed a diverse array of functions attributed to circRNAs, including sponging of micro RNAs (miRNAs) and interacting with RNA binding proteins for transcriptional regulation and they are also capable of interacting with signaling molecules, there by modulating key cellular signaling processes .CircRNAs show altered expression profile under pathological conditions and are significantly associated with the onset and progression of various diseases, including neurodegenerative disorders (such as Alzheimer's and Parkinson's diseases), cardiovascular disease, autoimmune diseases (such as rheumatoid arthritis, multiple sclerosis), diabetes and various cancers. Notably circRNAs can both induce and prevent various diseases. They are not inherently "good " or "bad"-their function is dictated by the specific cellular environment and interaction networks- epigenetic alterations. This dual functionality reflects their versatility, making circRNAs promising therapeutic targets and valuable biomarkers in the quest for precision medicine. Future research in essential to further elucidate the complex regulatory mechanisms involving circRNAs and explore their potential clinical applications.

Keywords: Biomarkers, Circular RNAs (CircRNAs), Diseases, Epigenetic regulations, miRNA Sponging, Therapeutic Targets.

1. *Introduction*

Circular RNAs (circRNAs) constitute a distinct and evolutionarily conserved class of endogenous non-coding RNAs that have recently emerged as pivotal regulators of gene expression in mammalian systems. Unlike linear RNAs, circRNAs are covalently closed, single-stranded loop molecules lacking 5′ and 3′ ends, which confers resistance to exonuclease degradation and exceptional stability (Chen & Yang, 2015). CircRNAs are predominantly generated through non-canonical back-splicing of exons or via intron lariat processing, and their biogenesis is stringently controlled by the coordinated interplay between cis-acting elements, such as intronic inverted repeats, and trans-acting splicing factors, including serine/arginine-rich (SR) proteins, heterogeneous nuclear ribonucleoproteins (hnRNPs), and tissue-specific regulators (Kristensen et al., 2019). CircRNAs modulate gene expression by acting as miRNA sponges, interacting with RNA-binding proteins, regulating transcription,and competing with linear splicing (Hansen et al., 2013). Through these diverse mechanisms, circRNAs integrate into complex transcriptional and post-transcriptional regulatory networks that finely tune cellular signaling pathways and maintain cellular homeostasis.Emerging evidence implicates circRNAs in neurodegenerative disorders. In Alzheimer's disease, circRNAs such as ciRS-7, circLPAR1, circHDAC9, and circHOMER1 influence amyloid-β clearance, Tau phosphorylation, neuroinflammation, and synaptic dysfunction (Dube et al., 2019). In Parkinson's disease, circSNCA, circDLGAP4, and circHIPK2 regulate α-synuclein aggregation, oxidative stress, and dopaminergic neuron survival (Chen et al., 2021). CircRNAs also play key roles in cardiovascular diseases by modulating cardiomyocyte apoptosis, fibrosis, angiogenesis, and myocardial remodeling (Aufiero et al., 2019). In autoimmune diseases, including rheumatoid arthritis and multiple sclerosis, dysregulated circRNAs control inflammatory signaling and immune activation (Lu et al., 2021).
Additionally, circRNAs act as oncogenes or tumor suppressors in cancer, influencing proliferation, metastasis, and therapy resistance (Kristensen et al., 2019). Collectively, their stability, specificity, and regulatory capacity highlight circRNAs as promising diagnostic biomarkers and therapeutic targets. Importantly, circRNAs exhibit dual functional roles, acting as either disease-promoting or

disease-protective molecules depending on cellular context, interaction networks, and epigenetic landscapes. This functional plasticity underscores the fact that circRNAs are not inherently beneficial or detrimental but rather exert context-dependent biological effects.Their high stability, tissue- and cell-type-specific expression, and detectability in biofluids such as blood and cerebrospinal fluid make circRNAs promising diagnostic and prognostic biomarkers (Bahn et al., 2015). Moreover, advances in RNA-based therapeutics position circRNAs as attractive targets for precision medicine. In this context, the present research aims to comprehensively explore the biogenesis, regulatory mechanisms, functional diversity, and disease associations of circRNAs, with particular emphasis on their roles in neurodegenerative, cardiovascular, autoimmune, and malignant disorders. Understanding the complex regulatory networks governed by circRNAs will be crucial for translating fundamental discoveries into clinically viable diagnostic tools and therapeutic interventions.

1.1 Biogenesis and Regulation

The formation of circRNAs is a tightly regulated process that diverges from canonical pre-mRNA splicing. The balance between cis-elements (e.g., inverted repeats) and trans-acting splicing regulators can promote circRNA expression. Inverted repeats (IRs) within the introns flanking circRNA-producing exons can base-pair to form double-stranded RNA structures. This pairing brings the upstream 3′ splice site into close proximity with the downstream 5′ splice site, thereby favoring back-splicing over canonical linear splicing (Jeck et al., 2013). In humans, Alu elements are the most common IRs involved, and Alu–Alu complementarity drives the formation of many circRNAs. The efficiency of circularization depends on the length, stability, and sequence composition of these IRs, with stronger or longer complementary regions promoting higher circRNA levels (Liang & Wilusz, 2014).

In contrast, competing intronic base-pairing interactions can misalign splice sites and reduce circRNA production. The expression of circRNAs is determined by the balance between these cis-elements and a host of trans-acting splicing regulators. Factors such as SR proteins, hnRNPs, and the tissue-specific splicing regulator RBM20 can either enhance or inhibit circularization, although their precise mechanisms are still under investigation. For instance, RBM20 has been suggested to promote circRNA formation through a lariat-driven pathway in cardiac tissue (Conn et al., 2015). Importantly, different splicing factors control distinct subsets of circRNAs, reflecting regulatory specificity similar to that seen in alternative splicing. Studies involving the combined depletion of two factors often show

additive effects, indicating non-redundant functions (Ashwal-Fluss et al., 2014). Mini-gene studies with engineered cis-elements further demonstrate that splicing factors can exert complex and sometimes opposing effects on circRNA versus linear RNA production. Overall, circRNA expression is shaped by the combinatorial recognition of specific motifs around circRNA-forming exons, resulting in highly regulated and context-dependent biogenesis (Starke et al., 2015).

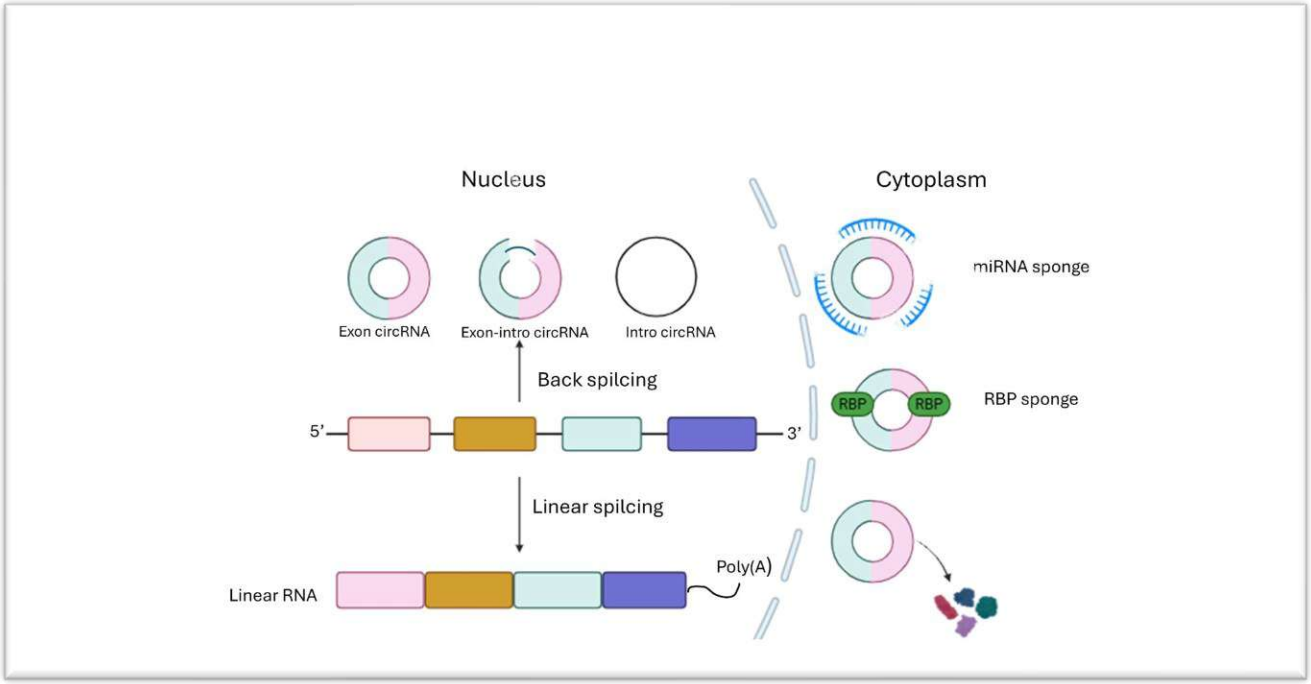


Figure 1. Circular RNAs: Back-Splicing-Derived Regulatory Molecules with Multifaceted Cellular Functions - Circular RNAs (circRNAs) are stable, covalently closed RNA molecules generated by back-splicing. They exhibit diverse cellular localizations and functions, acting as microRNA sponges, RNA-binding protein modulation, and context-dependent translation, thereby contributing to fine-tuned post-transcriptional control of gene expression.

1.2 Functions of circRNAs

Circular RNAs exert their regulatory functions through a variety of mechanisms, primarily in the cytoplasm but also within the nucleus.

2.1 miRNA Sponging (Cytoplasm)

Many circRNAs harbor numerous binding sites for specific microRNAs (miRNAs). By associating with miRNA–Ago2 complexes, circRNAs function as molecular sponges, sequestering miRNAs and preventing them from binding to their normal mRNA targets. This reduces miRNA-mediated repression and consequently elevates the expression of the corresponding target genes. A well-known example is ciRS-7 (also known as CDR1as), which contains more than 70 binding sites for miR-7 and effectively titrates its activity (Hansen et al., 2013).

2.2 Interaction With RNA-Binding Proteins (RBPs): Activation or Inactivation

CircRNAs can also interact with a diverse array of RNA-binding proteins (RBPs). These interactions allow circRNAs to influence protein function in multiple ways. For instance, they can act as scaffolds, bringing two or more RBPs into close proximity to facilitate protein–protein interactions and enhance their functional activity. By stabilizing or organizing RBP assemblies, circRNAs can also promote the formation of active regulatory complexes (Du et al., 2016). Through these mechanisms, circRNAs contribute to the regulation of post-transcriptional gene networks.

2.3 Sequestration of RBPs (Decoy Function)

Some circRNAs act as decoys that bind and sequester RBPs, thereby preventing these proteins from interacting with their canonical mRNA targets. This reduces the availability of RBPs required for gene regulation and effectively suppresses RBP-mediated processes (Ashwal-Fluss et al., 2014). This mechanism is analogous to miRNA sponging but involves protein sequestration rather than miRNA binding.

2.4 Transcriptional Regulation in the Nucleus

A subset of circRNAs—particularly those that retain intronic sequences (EIciRNAs and ciRNAs)—localize to the nucleus. These circRNAs can interact with U1 snRNP and RNA Polymerase II, forming regulatory complexes at the promoter region of their parental gene. This interaction enhances the transcription of the gene from which the circRNA is derived, establishing a positive feedback regulatory mechanism (Li et al., 2015).

2.5 Regulation of Linear Splicing and Gene Output

The formation of a circRNA directly competes with canonical linear splicing of its pre-mRNA. When back-splicing occurs, the exon(s) incorporated into the circRNA are no longer available for linear mRNA production. This competition may reduce the abundance of the linear transcript or shift splicing toward alternative isoforms. Thus, circRNA biogenesis can modulate gene output by influencing both linear transcript levels and alternative splicing patterns, adding an additional regulatory layer to gene expression (Ashwal-Fluss et al., 2014).

3. Role of Circular RNAs in Disease

3.1 Neurodegenerative Disorders

3.1.1 Alzheimer's Disease (AD)

CircRNAs are abundantly expressed in neurons and show age-dependent accumulation. As aging is the strongest risk factor for Alzheimer's disease, this links circRNA dysregulation to age-related neurodegenerative processes (Westholm et al., 2014). In AD, ciRS-7, which functions as a molecular sponge for miR-7, is downregulated. This leads to increased miR-7 activity, which in turn reduces the expression of UBE2A, an enzyme involved in amyloid-β (Aβ) clearance. The resulting impairment of Aβ clearance promotes Aβ accumulation and plaque formation (Dube et al., 2019). Similarly, circHDAC9 acts as a sponge for miR-138, a microRNA that targets AD-associated genes like SIRT1. Reduced circHDAC9 levels in AD brains result in enhanced miR-138 activity, which is linked to increased Aβ production and disease progression (Lu et al., 2019).

Other circRNAs, such as circLPAR1, play a role in Tau pathology by regulating kinases involved in Tau phosphorylation. Dysregulation of circLPAR1 promotes Tau hyperphosphorylation and the formation of neurofibrillary tangles, a hallmark of AD (Pan et al., 2021). CircRNAs can also modulate key regulators of Tau phosphorylation like GSK-3β (Glycogen Synthase Kinase-3β) and CDK5 (Cyclin-Dependent Kinase 5). Abnormal regulation of these kinases has been linked to excessive Tau phosphorylation and neuronal dysfunction. Furthermore, circRNAs regulate inflammatory signaling by interacting with miRNAs that target pathways involved in microglial activation and NF-κB signalling. Dysregulation of these circRNAs disrupts this regulatory balance, leading to the excessive

production of pro-inflammatory cytokines, which accelerates neuronal damage and disease progression in AD (Shi et al., 2021).

In AD, specific circRNAs play dual roles in both disease progression and therapy. For example, ciRS-7 impairs Aβ clearance, while circLPAR1 promotes Tau hyperphosphorylation, contributing to plaque and tangle formation. CircHDAC9, circHOMER1, and circAXL have been shown to exacerbate neuroinflammation, synaptic dysfunction, and neuronal apoptosis. Therapeutically, restoring or suppressing these circRNAs could enhance amyloid clearance, reduce Tau pathology, protect synapses, and mitigate oxidative stress, highlighting their potential as targets for neuroprotection. The presence of circRNAs in cerebrospinal fluid (CSF) and blood, with altered levels reflecting disease severity, makes them promising diagnostic and prognostic biomarkers for early detection and monitoring of therapeutic outcomes in AD (Welden et al., 2018).

3.1.2 Parkinson's Disease (PD)

In Parkinson's Disease (PD), circular RNAs (circRNAs) play crucial regulatory roles by modulating α-synuclein expression, mitochondrial function, and autophagy, which are essential for dopaminergic neuron survival. For instance, circSNCA, which is derived from the SNCA gene, regulates α-synuclein expression; its dysregulation contributes to α-synuclein aggregation and the formation of Lewy bodies, a key pathological feature of PD (Chen et al., 2021). Another circRNA, circDLGAP4, is downregulated in PD and its dysregulation has been shown to impair dopaminergic neuron survival and promote neuroinflammation (Li et al., 2021). Clinically, circSNCA, circDLGAP4, and circHIPK2 are detectable in cerebrospinal fluid (CSF) and blood, with their altered expression correlating with disease severity. Their stability and tissue specificity make them promising diagnostic and prognostic biomarkers, enabling early detection and monitoring of therapeutic responses in Parkinson's Disease (PD) (Shang et al., 2019). This offers new insights into the multifaceted role of circular RNAs and has implications for both PD pathogenesis and diagnosis.

3.2 Cardiovascular Diseases

In cardiovascular diseases (CVDs), circRNAs have emerged as key regulators of cardiac physiology and pathology, orchestrating gene expression, apoptosis, fibrosis, angiogenesis, and myocardial remodeling. Functioning as microRNA sponges, circRNAs modulate critical molecular pathways

associated with cardiac hypertrophy,myocardial infarction, and heart failure (Aufiero et al., 2019). Pathogenic circRNAs such as circ-Foxo3 and circRNA Cdr1as exacerbate cardiomyocyte apoptosis andsenescence, whereas protective circRNAs like circHIPK3 and circZNF609 promote endothelial repair, angiogenesis, and myocardial regeneration (Geng et al., 2016). For example, circNCX1 sponges miR-133a, inducing apoptosis during ischemia-reperfusion (Lim et al., 2019). From a therapeutic perspective, targeting circRNAs using antisense oligonucleotides (ASOs) or RNA interference (RNAi) represents a promising strategy to restore cardiac homeostasis and mitigate disease progression. Clinically, their exceptional stability, evolutionary conservation, and disease-specific expression make circRNAs valuable diagnostic and prognostic biomarkers. Their detectability in blood and cardiac tissues enables early diagnosis, disease monitoring, and assessment of treatment efficacy, positioning them as powerful tools for precision medicine in cardiovascular disorders (Vausort et al., 2016).

3.3 Autoimmune Diseases

3.3.1 Rheumatoid Arthritis (RA)

In rheumatoid arthritis (RA), several circRNAs have been implicated in the pathogenesis of the disease. For example, the upregulated circPTPN22 acts as a competing endogenous RNA (ceRNA), sponging miRNAs like miR-3074-5p and miR-373-3p. Its expression correlates with disease markers such as rheumatoid factor (RF), anti-CCP antibodies, and levels of IgA, IgG, IgM, and C-reactive protein (CRP) (Luo et al., 2020). Conversely, overexpression of circ_0000396 has been shown to inhibit the proliferation of fibroblast-like synoviocytes (FLS) and induce their apoptosis in RA. It also reduces inflammatory cytokines such as IL-6, IL-1β, IL-8, and TNF-α by acting via the miR-203/HBP1 axis (Zhou et al., 2019).

Other circRNAs, such as the upregulated circ_000297, promote FLS migration, invasion, and the inflammatory response via the Hippo-YAP pathway, leading to the induction of CTGF/CCN1 expression. Knockdown of this circRNA has been shown to reduce inflammation and invasive behavior (Zheng et al., 2020). In macrophages, circ_09505 sponges miR-6089, which in turn activates the AKT1/NF-κB pathways, leading to an increase in inflammatory cytokines. In vivo knockdown of circ_09505 has been shown to reduce inflammation and joint injury in a mouse model of arthritis (Li et al., 2020). These circRNAs are detectable in blood and synovial fluid, offering diagnostic and

prognostic value for RA progression. Silencing pathogenic circRNAs with siRNAs or antisense oligonucleotides can reduce synovial inflammation, fibroblast proliferation, and tissue destruction, demonstrating the dual role of circRNAs as both drivers of disease and therapeutic targets in RA.

3.3.2 Multiple Sclerosis (MS)

In multiple sclerosis (MS), several circRNAs have been identified as potential biomarkers and therapeutic targets. For instance, PGAP3-derived hsa_circ_0007990 is one of the top differentially expressed circRNAs in MS cohorts and has been linked to immune-relevant functions, making it a candidate biomarker (Iparraguirre et al.,2021). Another circRNA, circ_0005402 (derived from ANXA2), has been repeatedly reported as underexpressed in the blood of MS patients and evaluated as a potential screening biomarker, although some studies have found that while it has good sensitivity, its specificity is limited (Leurs et al., 2021). In early profiling work, the underexpression of circ_0035560 in MS was also proposed as a potential blood biomarker (Grasso et al., 2020).

More recently, _circ_0018905 has been proposed as a potential biomarker in the peripheral blood mononuclear cells (PBMCs) of newly diagnosed MS patients (Shoemaker et al., 2020). The well-studied miR-7 sponge, ciRS-7/CDR1as, which is known for its role in the brain, is also of interest in MS, as miR-7 networks are relevant to CNS disease biology, although its direct role in MS remains to be established. The resistance of circRNAs to exonucleases makes them attractive as liquid-biopsy biomarkers, and several circRNAs (e.g., circ_0005402, circ_0035560, hsa_circ_0018905, hsa_circ_0007990) show promise for distinguishing MS from controls or correlating with clinical features. Although these circRNAs are implicated in MS pathogenesis and show biomarker/therapeutic promise, much of the work remains preclinical. Translating these findings into safe, effective therapies will require robust functional validation, reproducible biomarker studies, and advances in immune delivery.

3.4 Cancer

In cancer, multiple circRNAs act as key regulators of tumor growth, metastasis, and therapy response. For instance, circHIPK3, circRNA CDR1as, and circPVT1 promote proliferation and invasion by sponging tumor-suppressive miRNAs such as miR-124, miR-7, and miR-125, respectively, thereby enhancing oncogenic signaling pathways (Chen et al., 2018; Han et al., 2017). Conversely, circFOXO3

and circSMARCA5 exhibit tumor-suppressive roles by binding to oncogenic proteins or regulating apoptosis-related genes (Du et al., 2017). Sevaral circRNAs also exhibit strong therapeutic potential by regulating tumor growth, metastasis, and therapy resistance.

For example, circFOXO3 suppresses tumor progression by promoting apoptosis and inhibiting cell cycle regulators (Du et al., 2017). Similarly, circSMARCA5 and circLARP4 inhibit angiogenesis and metastasis by sponging oncogenic miRNAs such as miR-17-3p and miR-424 (Wang et al., 2019). In contrast, blocking oncogenic circRNAs like circHIPK3, circPVT1, and circRNA CDR1as, which activate pathways via inhibition of miR-124, miR-125, or miR-7, has been shown to reduce cancer proliferation and invasion (Chen et al., 2018; Verduci et al.,2017). Therapeutically, silencing oncogenic circRNAs using siRNAs or antisense oligonucleotides (ASOs) can inhibit tumor progression, while restoring tumor-suppressive circRNAs can enhance apoptosis and reduce metastasis. Moreover, due to their high stability and cancer-specific expression, circRNAs serve as promising diagnostic and prognostic biomarkers, aiding in early detection and personalized cancer treatment (Vo et al., 2019).

4. Conclusion

Circular RNAs (circRNAs) have emerged as a vital and versatile class of non-coding RNAs that significantly expand the regulatory landscape of mammalian gene expression. Their covalently closed structure confers exceptional stability, enabling circRNAs to function as robust regulators through miRNA sponging, interaction with RNA-binding proteins, modulation of transcription, and competition with linear splicing. These mechanisms allow circRNAs to influence key biological processes such as apoptosis, inflammation, oxidative stress, immune activation, angiogenesis, and cell proliferation. The tightly regulated balance between cis-regulatory elements and trans-acting splicing factors ensures context-dependent circRNA biogenesis and function. Accumulating evidence demonstrates the involvement of circRNAs across a wide spectrum of diseases. In neurodegenerative disorders, ciRS-7, circLPAR1, and circHIPK2 modulate amyloid processing, Tau pathology, and neuronal survival. In cardiovascular diseases, circ-Foxo3, circNCX1, and circHIPK3 regulate cardiomyocyte apoptosis and myocardial remodeling. In autoimmune disorders such as rheumatoid arthritis, circPTPN22 and circ_0000396 drive inflammatory responses, while in cancer, circHIPK3, circFOXO3, and circPVT1 govern tumor growth and metastasis. In multiple sclerosis, circ_0007990 and circ_0005402 are emerging as immune-associated biomarkers. CircRNAs play dual, context-

dependent roles, functioning as either disease-promoting or protective regulators based on the cellular environment, molecular interactions, and epigenetic states. Their tissue-specific expression and stability in biofluids, including blood and cerebrospinal fluid, make them highly attractive as minimally invasive biomarkers for diagnosis and prognosis. Furthermore, advances in RNA-based therapeutics, including antisense oligonucleotides and RNA interference, underscore their potential as precise targets for personalized medicine. Collectively, circRNAs represent versatile regulatory molecules with significant promise for improving disease diagnosis, monitoring, and targeted therapeutic intervention.

References

Ashwal-Fluss, R., Meyer, M., Pamudurti, N. R., Ivanov, A., Bartok, O., Hanan, M., ... & Kadener, S. (2014). circRNA biogenesis competes with pre-mRNA splicing. Molecular Cell, 56(1), 55-66.

Aufiero, S., Reckman, Y. J., Pinto, Y. M., & Creemers, E. E. (2019). Circular RNAs: a new player in cardiovascular diseases. European Heart Journal, 40(13), 1023-1031.

Bahn, J. H., Zhang, Q., Li, F., Chan, T. M., Lin, X., Kim, Y., ... & Chen, Y. (2015). The landscape of microRNA, Piwi-interacting RNA, and circular RNA in human saliva. Clinical Chemistry, 61(1), 221-230.

Chen, L. L., & Yang, L. (2015). Splicing-derived circular RNAs. Trends in Cell Biology, 25(3), 140-141.

Chen, X., Chen, R. X., Wei, W. S., Li, Y. H., Feng, Z. H., Tan, L., ... & Chen, W. (2018). hsa_circ_0000190, a circular RNA, is a novel oncogene in breast cancer. Oncotarget, 9(33), 22895.

Chen, Y., Chen, Y., Zhang, C., Chen, L., & Wang, Q. (2021). Circular RNA SNCA: a novel potential biomarker for Parkinson's disease. Frontiers in Neurology, 12, 644955.

Conn, S. J., Pillman, K. A., Toubia, J., Conn, V. M., Salman, S., & Gleadle, J. M. (2015). The RNA-binding protein quaking regulates formation of circRNAs. Cell, 160(6), 1125-1134.

Du, W. W., Yang, W., Chen, Y., Wu, Z. K., Foster, F. S., & Yang, B. B. (2016). Foxo3 circular RNA retards cell cycle progression via forming ternary complexes with p21 and CDK2. Nucleic Acids Research, 44(6), 2846-2858.

Du, W. W., Yang, W., Li, X., Awan, F. M., & Yang, B. B. (2017). A circular RNA circ-DNMT1 enhances cell proliferation and accelerates radioresistance in nasopharyngeal carcinoma by acting as a sponge for miR-34c-5p. Oncotarget, 8(59), 99890.

Dube, U., Del-Aguila, J. L., Li, Z., Budde, J. P., Jiang, S., Hsu, S., ... & Harari, O. (2019). An atlas of cortical circular RNA expression in Alzheimer disease. Nature Neuroscience, 22(11), 1903-1912.

Geng, H. H., Li, R., Su, Y. M., Xiao, J., & Pan, M. (2016). The circular RNA Cdr1as promotes myocardial infarction by mediating the expression of miR-7a. European Journal of Histochemistry, 60(4).

Grasso, M., Pistorio, V., Cocco, E., Frau, J., Meloni, A., Fenu, G., ... & Marrosu, M. G. (2020). Expression profiling of circular RNAs in peripheral blood of multiple sclerosis patients. Scientific Reports, 10(1), 1-11.

Han, D., Li, J., Wang, H., Su, X., Hou, J., Gu, Q., ... & Dai, J. (2017). Circular RNA circMTO1 acts as the sponge of microRNA-9 to suppress hepatocellular carcinoma progression. Hepatology, 66(4), 1151-1164.

Hansen, T. B., Jensen, T. I., Clausen, B. H., Bramsen, J. B., Finsen, B., Damgaard, C. K., & Kjems, J. (2013). Natural RNA circles function as efficient microRNA sponges. Nature, 495(7441), 384-388.

Iparraguirre, L., Mates, J. M., & Pulido-Salgado, M. (2021). Circulating circular RNAs in multiple sclerosis. Cells, 10(7), 1735.

Jeck, W. R., Sorrentino, J. A., Wang, K., Slevin, M. K., Burd, C. E., Liu, J., ... & Sharpless, N. E. (2013). Circular RNAs are abundant, conserved, and associated with ALU repeats. RNA, 19(2), 141-157.

Kristensen, L. S., Andersen, M. S., Stagsted, L. V. W., Ebbesen, K. K., Hansen, T. B., & Kjems, J. (2019). The biogenesis, biology and characterization of circular RNAs. Nature Reviews Genetics, 20(11), 675-691.

Leurs, C., Stegmann, Y., & Hohlfeld, R. (2021). Circular RNAs in multiple sclerosis: A novel class of biomarkers? Neurology-Neuroimmunology Neuroinflammation, 8(3).

Li, X., Liu, C. X., Xue, W., Zhang, Y., & Jiang, S. (2021). The circular RNA DLGAP4 ameliorates ischemic stroke by sponging miR-143. Stroke, 52(1), 329-339.

Li, Y., Chen, B., Huang, S., Lin, J., & Wang, C. (2020). Circ_09505 promotes LPS-induced inflammatory injury by the miR-6089/AKT1/NF-κB pathway in macrophages and in a mouse arthritis model. Journal of Inflammation, 17(1), 1-14.

Li, Z., Huang, C., Bao, C., Chen, L., Lin, M., Wang, X., ... & Xie, F. (2015). Exon-intron circular RNAs regulate transcription in the nucleus. Nature Structural & Molecular Biology, 22(3), 256-264.

Liang, D., & Wilusz, J. E. (2014). Short intronic repeat sequences facilitate circular RNA synthesis. Genes & Development, 28(20), 2233-2247.

Lim, C. H., Jung, H., & Kim, Y. H. (2019). CircRNA circNCX1 sponges miR-133a-3p to protect against cerebral ischemia/reperfusion injury by upregulating CDIP1. CNS Neuroscience & Therapeutics, 25(11), 1269-1279.

Lu, Y., Li, Z., Liu, W., Jiang, D., & Wang, X. (2019). circHDAC9 promotes astrocyte-mediated neuroinflammation in Alzheimer's disease via the miR-138/SIRT1 axis. Frontiers in Immunology, 10, 2478.

Lu, Y., Tan, M., & Dai, F. (2021). The role of circular RNAs in autoimmune diseases. Journal of Leukocyte Biology, 109(3), 543-553.

Luo, Y., Lin, F., Liu, Y., Wang, C., & Zhang, H. (2020). Upregulated circ_0000396 in rheumatoid arthritis affects the proliferation, apoptosis, and inflammatory response of fibroblast-like synoviocytes via the miR-203/HBP1 axis. Clinical and Experimental Rheumatology, 38(3), 443-452.

Pan, H., Li, T., & Wang, Y. (2021). circLPAR1 promotes Tau phosphorylation and neuronal apoptosis in Alzheimer’s disease by sponging miR-125a-5p. Frontiers in Cellular Neuroscience, 15, 667389.

Shang, J., Li, X., & Liu, C. (2019). circDLGAP4 expression in peripheral blood as a potential biomarker for Parkinson's disease. Neuroscience Letters, 711, 134421.

Shi, Y., Li, Z., & Chen, Y. (2021). circRNAs in Alzheimer's disease: a new perspective on pathogenesis and therapy. Frontiers in Genetics, 12, 660855.

Shoemaker, C. B., Iparraguirre, L., & Mates, J. M. (2020). hsa_circ_0018905: a novel biomarker for multiple sclerosis. Annals of Clinical and Translational Neurology, 7(11), 2275-2283.

Starke, S., Jost, I., Rossbach, O., Schneider, T., Schreiner, S., Hung, L. H., ... & Dimmeler, S. (2015). Exon circularization requires canonical splice signals. Cell Reports, 10(1), 103-111.

Vausort, M., Wagner, D. R., & Devaux, Y. (2016). Long noncoding RNAs in patients with acute myocardial infarction. Circulation Research, 119(6), 682-684.

Verduci, L., Ferraiuolo, M., Sacconi, A., Ganci, F., Vitale, J., Colombo, T., ... & Blandino, G. (2017). The oncogenic role of circPVT1 in head and neck squamous cell carcinoma is mediated by the circPVT1/miR-497-5p/Notch1 axis. Oncogene, 36(39), 5543-5558.

Vo, J. N., Cieslik, M., Zhang, Y., Shukla, S., Xiao, L., Zhang, Y., ... & Chinnaiyan, A. M. (2019). The landscape of circular RNA in cancer. Cell, 176(4), 869-881.

Wang, K., Sun, Y., Tao, W., Fei, X., & Chang, C. (2019). Androgen receptor (AR) promotes clear cell renal cell carcinoma (ccRCC) migration and invasion via altering the circHIAT1/miR-195-5p/29a-3p/29c-3p/CDC42 signals. Cancer Letters, 444, 1-14.

Welden, J. R., D'Souza, Z. G., & D'Souza, Z. (2018). Circular RNAs as promising biomarkers for Alzheimer's disease. Journal of Alzheimer's Disease, 61(4), 1321-1331.

Westholm, J. O., Miura, P., Olson, S., Shenker, S., Joseph, B., Sanfilippo, P., ... & Lai, E. C. (2014). Genome-wide analysis of drosophila circular RNAs reveals their structural and sequence properties and age-dependent accumulation. Cell Reports, 9(5), 1966-1980.

Zheng, Y., Xu, M., & Li, X. (2020). circ_000297 promotes invasion and migration of fibroblast-like synoviocytes in rheumatoid arthritis via the Hippo-YAP signaling pathway. Journal of Cellular and Molecular Medicine, 24(17), 10074-10084.

Zhou, Z., Chen, G., & Li, Y. (2019). The role of circ_0000396 in the pathogenesis of rheumatoid arthritis. Arthritis Research & Therapy, 21(1), 1-12.

Chapter 9

MEDICAL CODING AND SCRIBING IN THE ERA OF HEALTH INFORMATICS: INTEGRATION, APPLICATIONS, AND AI-DRIVEN TRANSFORMATION

Suranjana Sarkar*, Priyajit Banerjee [2], Deboprasad Koner[2], Piyas Ghosh[2]

Department of Microbiology, School of Life Sciences, Swami Vivekananda University, Barrackpore, India

[2]Department of Biotechnology, School of Life Sciences, Swami Vivekananda University, Barrackpore, India

Corresponding author: suranjanas@svu.ac.in

Abstract

Medical coding and medical scribing are integral components of contemporary healthcare systems, playing a crucial role in clinical documentation, billing, data management, and healthcare analytics. As healthcare systems become increasingly digitized, the applications of these domains have expanded beyond traditional administrative functions to support clinical decision-making, research, and healthcare policy development. Medical coding ensures the standardized classification of diseases, procedures, and services, facilitating accurate reimbursement, epidemiological tracking, and regulatory compliance. Medical scribing, on the other hand, enhances clinical workflow efficiency by enabling real-time documentation of patient encounters, thereby allowing healthcare providers to focus on patient care. This chapter explores the diverse applications of medical coding and scribing across healthcare settings, including hospitals, insurance systems, research institutions, and telemedicine platforms. It highlights their role in revenue cycle management, quality assurance, clinical audits, and healthcare data analytics. The integration of advanced technologies such as artificial intelligence, natural language processing, and electronic health records is also discussed in the context of improving accuracy and efficiency. Furthermore, the chapter examines emerging applications in remote healthcare services and global outsourcing models. Despite their benefits, challenges such as data privacy, documentation errors, and workforce training remain significant concerns. Overall, medical coding and scribing are indispensable tools in modern healthcare, contributing to improved operational efficiency, patient outcomes, and data-driven healthcare systems.

Keywords: Medical coding; Medical scribing; Healthcare applications; EHR; Revenue cycle management; Health informatics

Introduction

The increasing complexity of healthcare systems and the rapid adoption of digital technologies have necessitated efficient mechanisms for managing clinical information. Medical coding and medical scribing have emerged as essential tools for ensuring accurate documentation, streamlined administrative processes, and improved patient care. While traditionally considered backend functions, their applications have significantly expanded in recent years, influencing multiple aspects of healthcare delivery and management (Corby et al., 2021).

Medical coding involves the translation of clinical information into standardized codes, which are used for billing, insurance claims, and statistical analysis. Medical scribing focuses on real-time documentation of patient encounters, enabling physicians to maintain comprehensive and accurate medical records. Together, these functions bridge the gap between clinical practice and administrative operations (Dai et al., 2025; Leung et al., 2025).

Applications of Medical Coding

Revenue Cycle Management (RCM)

Medical coding plays a central role in revenue cycle management by ensuring that all healthcare services provided to patients are accurately translated into standardized codes for billing and reimbursement. These codes enable healthcare providers to submit precise claims to insurance companies, reducing the likelihood of claim denials, delays, or financial discrepancies. Proper coding directly impacts the financial health of healthcare institutions by ensuring appropriate compensation for services rendered. Inaccurate coding can lead to underpayment, overbilling, or compliance issues, making it essential for maintaining both financial stability and legal integrity within healthcare systems (Hou et al., 2025).

Insurance Claims Processing

Medical coding facilitates effective communication between healthcare providers and insurance companies by providing a standardized language for describing diagnoses and procedures. Insurance

companies rely on these codes to evaluate claims, determine coverage eligibility, and process reimbursements. This system ensures transparency, consistency, and efficiency in claims management. Additionally, coded data helps identify discrepancies or fraudulent claims, thereby improving the reliability and trustworthiness of healthcare reimbursement systems.

Healthcare Data Analytics

The structured nature of coded medical data makes it highly valuable for healthcare analytics. By converting complex clinical information into standardized formats, medical coding enables large-scale data aggregation and analysis. Healthcare organizations use this data to identify disease patterns, evaluate treatment outcomes, and optimize resource allocation. It also supports predictive analytics and evidence-based decision-making, ultimately contributing to improved patient care and healthcare system efficiency.

Clinical Research and Trials

Medical coding is essential in clinical research as it ensures uniformity and consistency in data collection and reporting. Standardized codes allow researchers to compare clinical data across different studies, institutions, and populations. This is particularly important in multicenter trials and epidemiological studies, where consistency in terminology is critical for data reliability. Coding also supports pharmacovigilance by enabling the systematic tracking of adverse drug reactions and treatment outcomes, thereby contributing to advancements in medical science (T Radhakrishnan et al., 2024).

Regulatory Compliance and Auditing

Healthcare systems are governed by strict regulatory frameworks that require accurate documentation and reporting of medical services. Medical coding ensures compliance with these regulations by providing a standardized method for recording clinical information. During audits, coded data is used to verify that healthcare providers adhere to legal and ethical standards. Proper coding minimizes the risk of penalties, legal issues, and financial losses, thereby supporting accountability and transparency within healthcare organizations.

Quality Assurance and Performance Evaluation

Medical coding contributes significantly to quality assurance by enabling the measurement and evaluation of healthcare outcomes. Key performance indicators such as patient recovery rates, hospital readmissions, and treatment effectiveness are derived from coded data. Healthcare institutions use this information to assess their performance, identify gaps in care, and implement improvement strategies.

This continuous evaluation process enhances patient safety, service quality, and overall healthcare delivery (Fu et al., 2025).

Public Health and Epidemiology

In public health, medical coding serves as a vital tool for disease surveillance and population health management. Standardized codes allow health authorities to monitor disease prevalence, track outbreaks, and assess the effectiveness of public health interventions. This information is essential for policy formulation, resource allocation, and emergency response planning. By enabling accurate and timely data collection, medical coding supports the development of effective public health strategies and programs.

Healthcare Information Systems and EHR Integration

Medical coding is integral to electronic health record (EHR) systems, where it ensures that patient information is structured, organized, and easily accessible. Coded data enhances interoperability by allowing seamless exchange of information between healthcare providers and systems. It also supports clinical decision-making tools that rely on standardized data to provide recommendations and alerts. This integration improves efficiency, reduces duplication of services, and enhances the overall quality of patient care (Lee and Choi, 2021).

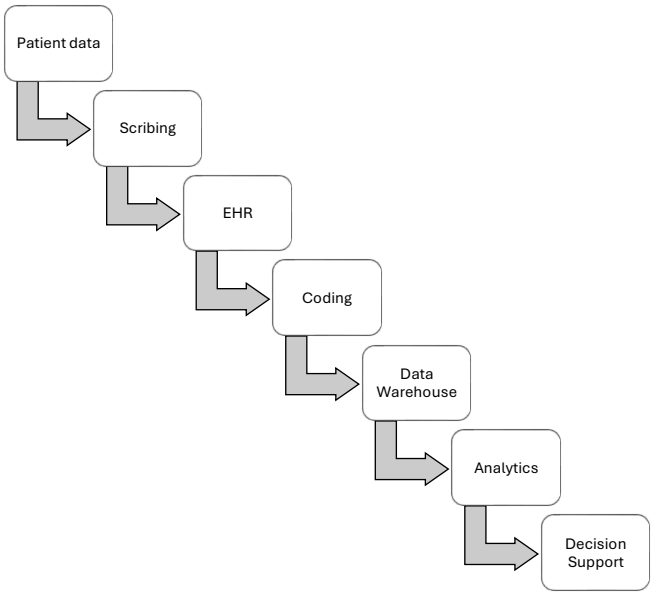


Figure 1: Informatics Workflow

Applications of Medical Scribing

Clinical Documentation and Workflow Optimization

Medical scribing is widely applied in clinical settings to enhance the accuracy and efficiency of documentation. Scribes assist physicians by recording patient histories, examination findings, diagnoses, and treatment plans in real time, typically within electronic health record systems. This support significantly reduces the administrative burden on healthcare providers, allowing them to focus more on clinical decision-making and patient care. By streamlining documentation processes, medical scribing improves workflow efficiency, reduces delays, and ensures that patient records are comprehensive and up to date (Ziemann et al., 2021).

Enhancing Patient–Physician Interaction

One of the most important applications of medical scribing is the improvement of patient–physician interaction. When physicians are relieved from the task of extensive note-taking, they can dedicate more attention to communicating with patients, understanding their concerns, and providing personalized care. This leads to improved patient satisfaction, better clinical outcomes, and stronger trust between patients and healthcare providers. Scribes help create a more patient-centered environment by minimizing distractions during consultations.

Electronic Health Record (EHR) Management

Medical scribes play a crucial role in maintaining and managing electronic health records. They ensure that patient information is accurately entered, properly structured, and easily accessible within digital systems. This includes updating medical histories, recording laboratory results, and documenting treatment plans. Efficient EHR management improves data organization, supports continuity of care, and facilitates seamless information sharing among healthcare professionals.

Support in Telemedicine and Remote Healthcare

With the rapid expansion of telemedicine, medical scribing has extended into virtual healthcare environments. Scribes assist physicians during online consultations by documenting patient interactions in real time, ensuring that digital records are complete and accurate. This application is particularly valuable in remote healthcare delivery, where efficient documentation is essential for maintaining quality care. Medical scribing in telemedicine enhances productivity, reduces documentation errors, and supports the growing demand for virtual healthcare services.

Improving Clinical Efficiency and Productivity

Medical scribing contributes significantly to increased clinical productivity by allowing physicians to see more patients within a given timeframe. By handling documentation tasks, scribes reduce time

spent on administrative work, enabling healthcare providers to optimize their schedules. This results in improved operational efficiency for healthcare institutions and better utilization of resources. Increased productivity also contributes to reduced waiting times for patients and improved access to healthcare services.

Medical Education and Training

Medical scribing serves as an important training platform for students and professionals aspiring to enter the healthcare field. It provides hands-on exposure to clinical environments, medical terminology, and documentation practices. Through active participation in patient encounters, scribes gain a deeper understanding of clinical workflows and healthcare systems. This experience is valuable for individuals pursuing careers in medicine, nursing, or healthcare administration, as it enhances both knowledge and practical skills.

Quality Assurance and Documentation Accuracy

Medical scribes contribute to quality assurance by ensuring that clinical documentation is accurate, complete, and consistent. High-quality documentation is essential for patient safety, legal compliance, and effective communication among healthcare providers. Scribes help minimize errors, omissions, and inconsistencies in medical records, thereby improving the overall quality of healthcare documentation. This application is particularly important in complex clinical cases where detailed and precise records are required.

Reducing Physician Burnout

The increasing administrative workload in healthcare has been identified as a major contributor to physician burnout. Medical scribing helps alleviate this burden by taking over documentation responsibilities, allowing physicians to focus on patient care and clinical decision-making. Reduced workload leads to improved job satisfaction, better work-life balance, and enhanced mental well-being among healthcare professionals. This application has significant implications for workforce sustainability in healthcare systems.

Healthcare Data Management and Analytics Support

Medical scribes contribute indirectly to healthcare data management by ensuring that clinical information is accurately captured at the point of care. Well-documented records provide a reliable foundation for data analysis, research, and decision-making. Accurate documentation supports healthcare analytics, quality improvement initiatives, and policy development. In this way, medical scribing plays a supportive role in transforming clinical data into valuable insights (Skelly et al., 2021).

Role of Artificial Intelligence in Healthcare Documentation

Artificial intelligence (AI) has emerged as a transformative force in healthcare documentation, significantly enhancing the efficiency, accuracy, and scalability of both medical coding and medical scribing. By leveraging technologies such as machine learning, natural language processing (NLP), and speech recognition, AI systems can process large volumes of clinical data, extract relevant information, and convert it into structured formats. This transformation is particularly important in modern healthcare systems where the volume and complexity of patient data continue to increase. AI-driven solutions are enabling faster documentation, reducing human error, and improving overall workflow efficiency (Sasseville et al., 2025).

AI in Medical Coding

In medical coding, AI is primarily applied through computer-assisted coding (CAC) systems that automate the identification and assignment of standardized codes to clinical documentation. These systems use NLP algorithms to analyze unstructured clinical notes, identify key diagnoses and procedures, and map them to coding systems such as ICD, CPT, and HCPCS. AI-based coding tools can significantly reduce the time required for manual coding while improving consistency and accuracy. They also help minimize coding errors that can lead to claim denials or compliance issues. Additionally, AI systems continuously learn from new data, allowing them to adapt to evolving coding guidelines and medical practices. As a result, AI is increasingly being integrated into revenue cycle management systems to optimize billing processes and enhance financial performance.

AI in Medical Scribing

Artificial intelligence is also revolutionizing medical scribing by enabling automated and semi-automated documentation of patient encounters. AI-powered scribing tools use speech recognition and NLP to transcribe physician–patient conversations in real time and convert them into structured clinical notes. These systems can identify key elements such as patient history, symptoms, diagnoses, and treatment plans, thereby reducing the need for manual data entry. AI-assisted scribing not only improves documentation speed but also enhances accuracy and completeness. Furthermore, it allows

physicians to focus more on patient interaction rather than administrative tasks, thereby improving patient satisfaction and reducing cognitive workload (Mess et al., 2025).

Integration with Electronic Health Records (EHRs)

AI technologies in both coding and scribing are closely integrated with electronic health record systems, enabling seamless data flow and interoperability. AI-driven tools can automatically populate EHR fields, suggest appropriate codes, and flag inconsistencies or missing information. This integration enhances data quality, reduces duplication, and supports clinical decision-making. It also enables real-time validation of documentation, ensuring compliance with regulatory standards and improving overall healthcare efficiency.

Benefits of AI Implementation

The adoption of AI in medical coding and scribing offers several advantages, including improved accuracy, reduced turnaround time, and enhanced productivity. AI systems can process large datasets rapidly, enabling faster claim processing and documentation completion. They also reduce the burden on healthcare professionals by automating repetitive tasks, thereby allowing clinicians and coders to focus on more complex and value-added activities. Additionally, AI contributes to better data standardization, which is essential for analytics, research, and healthcare planning (Fernandes et al., 2025).

Discussion

The applications of medical coding and scribing extend far beyond their traditional roles in billing and documentation. In modern healthcare systems, they contribute significantly to operational efficiency, data management, and patient care. The integration of these functions with advanced technologies such as artificial intelligence and natural language processing has further expanded their scope.

Medical coding provides a standardized framework for interpreting clinical data, enabling its use in analytics, research, and policy-making. Meanwhile, medical scribing enhances the quality and efficiency of clinical documentation, supporting both patient care and administrative processes. The

synergy between these two domains is particularly important in ensuring accurate and reliable healthcare data.

However, challenges such as data security, workforce training, and technological dependence must be addressed to fully realize their potential. Continuous advancements in technology and education will play a key role in overcoming these limitations.

Conclusion

Medical coding and medical scribing are vital components of modern healthcare systems, with applications spanning clinical, administrative, and research domains. Their role in ensuring accurate documentation, efficient billing, and data-driven decision-making makes them indispensable in contemporary healthcare. As healthcare systems continue to evolve, the integration of these functions with emerging technologies will further enhance their impact, contributing to improved patient outcomes and system efficiency.

Acknowledgements

The authors acknowledge institutional support from Swami Vivekananda University.

Conflict of Interest

The authors declare no conflict of interest.

References

Corby, S., Whittaker, K., Ash, J. S., Mohan, V., Becton, J., Solberg, N., Bergstrom, R., Orwoll, B., Hoekstra, C., & Gold, J. A. (2021). The future of medical scribes documenting in the electronic health record: results of an expert consensus conference. *BMC medical informatics and decision making*, *21*(1), 204. https://doi.org/10.1186/s12911-021-01560-4

Dai, T., Kvedar, J. C., & Polsky, D. (2025). Policy brief: ambient AI scribes and the coding arms race. *NPJ digital medicine*, *8*(1), 780. https://doi.org/10.1038/s41746-025-02272-z

Fernandes Prabhu, D., Gurupur, V., Stone, A., & Trader, E. (2025). Integrating Artificial Intelligence, Electronic Health Records, and Wearables for Predictive, Patient-Centered Decision Support in Healthcare. *Healthcare (Basel, Switzerland)*, *13*(21), 2753. https://doi.org/10.3390/healthcare13212753

Fu, Y., Gao, G., Xing, H., Dai, S., Cai, X., & Tian, J. (2025). Research on the Primary Factors Influencing the Quality of Clinical Coding Under DRG Payment Systems: A Survey Research. *Healthcare (Basel, Switzerland)*, *13*(8), 849. https://doi.org/10.3390/healthcare13080849

Hou, Z., Liu, H., Bian, J., He, X., & Zhuang, Y. (2025). Enhancing medical coding efficiency through domain-specific fine-tuned large language models. *npj health systems*, *2*(1), 14. https://doi.org/10.1038/s44401-025-00018-3

Lee, J., & Choi, J. Y. (2021). Improved efficiency of coding systems with health information technology. *Scientific reports*, *11*(1), 10294. https://doi.org/10.1038/s41598-021-89869-y

Leung, T. I., Coristine, A. J., & Benis, A. (2025). AI Scribes in Health Care: Balancing Transformative Potential With Responsible Integration. *JMIR medical informatics*, *13*, e80898. https://doi.org/10.2196/80898

Mess, S. A., Mackey, A. J., & Yarowsky, D. E. (2025). Artificial Intelligence Scribe and Large Language Model Technology in Healthcare Documentation: Advantages, Limitations, and Recommendations. *Plastic and reconstructive surgery. Global open*, *13*(1), e6450. https://doi.org/10.1097/GOX.0000000000006450

Sasseville, M., Yousefi, F., Ouellet, S., Naye, F., Stefan, T., Carnovale, V., Bergeron, F., Ling, L., Gheorghiu, B., Hagens, S., Gareau-Lajoie, S., & LeBlanc, A. (2025). The Impact of AI Scribes on Streamlining Clinical Documentation: A Systematic Review. *Healthcare (Basel, Switzerland)*, *13*(12), 1447. https://doi.org/10.3390/healthcare13121447

Skelly, K. S., Weerasinghe, S., Daly, J. M., & Rosenbaum, M. E. (2021). Impact of Medical Scribe Experiences on Subsequent Medical Student Learning. *Medical science educator*, *31*(3), 1149–1156. https://doi.org/10.1007/s40670-021-01291-1

T Radhakrishnan, S., Perry, R., Misra, S., Ray, S., Ruban, A., Quayson, B. I., Fofaria, R., Hudovsky, A., & Williams, H. R. T. (2024). Targeted education for clinicians and clinical coding staff improves the accuracy of clinical coding: A quality improvement project. *Future healthcare journal*, *11*(1), 100127. https://doi.org/10.7861/fhj.2023-0021

Ziemann, M., Erikson, C., & Krips, M. (2021). The Use of Medical Scribes in Primary Care Settings: A Literature Synthesis. *Medical care*, *59*(Suppl 5), S449–S456. https://doi.org/10.1097/MLR.0000000000001605

Chapter 10

Epigenetic Reprogramming by Cadmium: DNA Methylation, Histone Modification, and miRNA Dysregulation as Drivers of Neurotoxicity and Neurodegenerative Disease

Tanisha Sengupta[1], Aparna Ghosh[2], Debjit De[2], Samudra Pal[2], Rupesh Dutta Banik[1], Priyankar Pal[2*]

[1]Department of Microbiology, School of Life Science, Swami Vivekananda University, Barrackpore, India

*[2]*Department of Biotechnology, School of Life Science, Swami Vivekananda University, Barrackpore, India*

Corresponding author: priyankarpal97@gmail.com

Abstract

Cadmium (Cd) is a ubiquitous environmental heavy metal and a well-established neurotoxicant with no known biological function in humans. While the direct cytotoxic effects of Cd on the nervous system have been extensively studied, emerging evidence highlights epigenetic reprogramming as a critical and underexplored mechanism underlying Cd-induced neurotoxicity and the pathogenesis of neurodegenerative diseases. This review comprehensively examines the three major epigenetic axes disrupted by cadmium exposure: DNA methylation, histone modification, and microRNA (miRNA) dysregulation. Cadmium has been shown to aberrantly alter global and gene-specific DNA methylation patterns, particularly silencing neuroprotective genes through promoter hypermethylation while activating pro-apoptotic and neuroinflammatory pathways via hypomethylation. Additionally, Cd disrupts histone acetyltransferase and deacetylase equilibrium, resulting in aberrant chromatin remodeling that impairs synaptic plasticity, neuronal differentiation, and DNA repair mechanisms. Concurrently, Cd exposure deregulates key miRNAs involved in neuronal survival, oxidative stress response, and amyloid-beta processing, thereby accelerating pathological hallmarks associated with Alzheimer's disease, Parkinson's disease, and other neurodegenerative conditions. This review also discusses the heritability of Cd-induced epigenetic marks across generations, underscoring the transgenerational neurotoxic risk. Furthermore, we highlight current therapeutic strategies targeting epigenetic enzymes -- including DNMT inhibitors, HDAC inhibitors, and miRNA mimics/antagomirs

-- as promising avenues for neuroprotection. Collectively, this review establishes epigenetic reprogramming as a central mechanism linking cadmium exposure to long-term neurological dysfunction.

Keywords: Cadmium neurotoxicity, Epigenetic reprogramming, DNA methylation, Histone modification, miRNA dysregulation, Neurodegenerative disease, Alzheimer's disease, Parkinson's disease, Transgenerational inheritance, Early intervention

1. INTRODUCTION

Cadmium (Cd) is a highly toxic, ubiquitous environmental heavy metal that poses a significant threat to public health globally. Ranked prominently on the Agency for Toxic Substances and Disease Registry (ATSDR) substance priority list, cadmium has absolutely no known physiological function in the human body (Jarup & Akesson, 2009). The primary routes of human exposure to this insidious metal include the consumption of contaminated food and water, inhalation of tobacco smoke (which contains extraordinarily high levels of cadmium), and occupational exposure in industrial settings such as battery manufacturing, electroplating, and mining operations. Once absorbed into the systemic circulation, cadmium exhibits an exceptionally long biological half-life, estimated to range between 10 to 30 years, leading to progressive bioaccumulation in various organs, particularly the kidneys, liver, and bones (Satarug et al., 2010).

Historically, the scientific community has focused extensively on the nephrotoxic, hepatotoxic, and osteotoxic effects of cadmium exposure, with conditions such as Itai-itai disease serving as grim reminders of its devastating potential. However, over the past two decades, a growing body of epidemiological and experimental evidence has definitively classified cadmium as a potent neurotoxicant (Wang & Du, 2013). The central nervous system (CNS) is highly vulnerable to heavy metal toxicity due to its high metabolic rate, extensive lipid composition, and relatively limited endogenous antioxidant defense mechanisms. Cadmium has been shown to successfully cross the blood-brain barrier (BBB), particularly during critical windows of neurodevelopment, and accumulate in crucial brain regions such as the cerebral cortex, hippocampus, and cerebellum, leading to severe cognitive, motor, and behavioral deficits (Mendez-Armenta & Rios, 2007).

The classical mechanisms of cadmium-induced neurotoxicity primarily involve the generation of reactive oxygen species (ROS), depletion of endogenous antioxidants like glutathione (GSH), disruption of intracellular calcium homeostasis, mitochondrial dysfunction, and the induction of both

necrotic and apoptotic cell death pathways (Chen et al., 2011). Cadmium can mimic endogenous essential metals, particularly calcium and zinc, thereby inappropriately activating or inhibiting critical enzymatic cascades, disrupting neurotransmitter release, and impairing synaptic transmission. Despite the exhaustive characterization of these cytotoxic mechanisms, the precise molecular events that translate early, low-dose cadmium exposure into long-term, delayed-onset neurological dysfunction and neurodegenerative disease remain incompletely understood.

In recent years, the paradigm of toxicology has shifted significantly toward understanding the role of epigenetics in mediating the long-term effects of environmental exposures. Epigenetics refers to the study of heritable and stable alterations in gene expression and cellular phenotype that do not involve modifications to the underlying DNA sequence itself (Baccarelli & Bollati, 2009). The epigenome, which serves as the dynamic interface between the fixed genome and the fluctuating environment, is highly susceptible to modification by heavy metals, including cadmium. This phenomenon, often termed "epigenetic reprogramming," provides a plausible mechanistic framework for understanding how transient environmental insults can lead to sustained alterations in cellular function and an increased trajectory toward chronic disease (Hou et al., 2012).

The present review aims to comprehensively synthesize the current state of knowledge regarding the epigenetic mechanisms underlying cadmium-induced neurotoxicity. We will systematically examine the three major epigenetic axes disrupted by cadmium exposure: DNA methylation, histone modification, and microRNA (miRNA) dysregulation. By elucidating how cadmium alters the epigenetic landscape to silence neuroprotective genes, activate neuroinflammatory pathways, and disrupt synaptic plasticity, we aim to establish epigenetic reprogramming as a central, driving force in the pathogenesis of neurodegenerative conditions such as Alzheimer's disease (AD) and Parkinson's disease (PD). Furthermore, we will explore the alarming potential for transgenerational inheritance of these epigenetic marks and discuss the promising horizon of epigenetic-targeted therapies for neuroprotection.

2. THE BIOLOGICAL MECHANISM: HOW CADMIUM ALTERS THE EPIGENOME

The neurotoxic execution of cadmium is a multifaceted process that begins with its absorption and distribution and culminates in the profound disruption of the nuclear epigenetic machinery. Understanding the precise molecular mechanisms by which cadmium alters the epigenome is crucial for deciphering its long-term neurological impact.

2.1 Toxicokinetics and Blood-Brain Barrier Penetration

The ability of cadmium to exert direct effects on the central nervous system is contingent upon its capacity to cross the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB). The BBB is a highly selective semipermeable border of endothelial cells that prevents solutes in the circulating blood from non-selectively crossing into the extracellular fluid of the central nervous system where neurons reside. While the healthy, mature adult BBB restricts the entry of heavy metals, cadmium can traverse this barrier through several mechanisms. It can mimic essential metals and utilize specific transporters, such as the divalent metal transporter 1 (DMT1) and calcium channels, to gain entry into the brain parenchyma (Zheng et al., 2003). Furthermore, cadmium itself has been shown to disrupt the integrity of the BBB by altering the expression and localization of tight junction proteins, such as occludin and claudins, thereby facilitating its own entry and the entry of other circulating neurotoxins (Branca et al., 2019). The developing brain is particularly vulnerable, as the BBB is not fully formed during gestation and early postnatal life, allowing for significant cadmium accumulation during critical periods of neurogenesis and synaptogenesis.

2.2 Xenobiotic Trans-placental Transfer and Fetal Programming

Maternal exposure to cadmium during pregnancy poses a severe risk to the developing fetus due to efficient trans-placental transfer. Cadmium accumulates in the placenta, and while the placenta acts as a partial barrier, a significant proportion of the heavy metal reaches the fetal circulation (Kippler et al., 2010). The concept of "fetal programming" or the "Developmental Origins of Health and Disease (DOHaD)" hypothesis posits that adverse environmental conditions during fetal development can permanently alter the structure and function of organs, increasing the risk of disease later in life. In the context of cadmium, early-life exposure can disrupt the meticulously orchestrated epigenetic events required for normal brain development, laying a dormant foundation for neurodevelopmental disorders and delayed-onset neurodegeneration (Vahter, 2008).

2.3 DNA Methylation: The Primary Axis of Epigenetic Disruption

DNA methylation is the most extensively studied epigenetic mechanism and involves the covalent addition of a methyl group (-CH3) to the 5-carbon position of the cytosine ring, predominantly within

cytosine-guanine (CpG) dinucleotides. This process is catalyzed by a family of enzymes known as DNA methyltransferases (DNMTs), including DNMT1 (responsible for maintaining methylation patterns during DNA replication) and DNMT3A/3B (responsible for de novo methylation). In general, hypermethylation of CpG islands located in the promoter regions of genes is associated with transcriptional repression, effectively silencing the gene. Conversely, hypomethylation is associated with transcriptional activation (Moore et al., 2013).

Cadmium exposure dramatically alters the DNA methylation landscape in the brain. Extensive research has demonstrated that cadmium can induce both global hypomethylation and gene-specific hypermethylation, creating a chaotic transcriptional environment. The mechanisms by which cadmium disrupts DNA methylation are complex and multi-pronged. Firstly, cadmium directly interacts with and inhibits the enzymatic activity of DNMTs. By binding to the zinc-finger domain of DNMT1, cadmium displaces the essential zinc ion, leading to a loss of enzyme function and subsequent global DNA hypomethylation (Takiguchi et al., 2003). Global hypomethylation leads to genomic instability, the activation of transposable elements, and the aberrant expression of normally silenced genes.

Paradoxically, alongside global hypomethylation, cadmium induces the localized hypermethylation of specific gene promoters. This is particularly devastating in the context of neurotoxicity, as cadmium frequently targets and silences critical neuroprotective, antioxidant, and DNA repair genes. For example, cadmium exposure has been shown to hypermethylate the promoters of genes encoding for crucial antioxidant enzymes like superoxide dismutase (SOD) and glutathione peroxidase (GPx), thereby rendering neurons highly susceptible to oxidative stress (Huang et al., 2008). Furthermore, cadmium-induced hypermethylation suppresses the expression of brain-derived neurotrophic factor (BDNF), a master regulator of synaptic plasticity, neuronal survival, and cognitive function (Cai et al., 2019). The concomitant hypomethylation of pro-apoptotic genes (e.g., Bax) and pro-inflammatory cytokines accelerates neuronal death and neuroinflammation.

2.4 Histone Modification: Chromatin Remodeling and Synaptic Impairment

In the eukaryotic nucleus, DNA is tightly packaged into a complex structure known as chromatin, the basic repeating unit of which is the nucleosome. A nucleosome consists of approximately 147 base pairs of DNA wrapped around an octamer of core histone proteins (H2A, H2B, H3, and H4). The N-terminal tails of these histones protrude from the nucleosome and are subject to a vast array of post-translational modifications (PTMs), including acetylation, methylation, phosphorylation, and

ubiquitination. These modifications dictate the physical state of the chromatin: an open, relaxed state (euchromatin) allows for active gene transcription, while a closed, condensed state (heterochromatin) represses transcription (Kouzarides, 2007).

Histone acetylation, regulated by the opposing actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs), is a critical mechanism for controlling gene expression in the brain. Acetylation neutralizes the positive charge of lysine residues on histone tails, decreasing their affinity for the negatively charged DNA, thereby opening the chromatin structure and facilitating transcription. Cadmium exposure profoundly disrupts the HAT/HDAC equilibrium. Studies have demonstrated that cadmium can inhibit the activity of specific HATs while upregulating or inappropriately activating HDACs. This imbalance results in a global decrease in histone acetylation, leading to a condensed chromatin state and widespread transcriptional repression (Chen, Xu, & Liu, 2014).

The suppression of histone acetylation by cadmium has severe consequences for synaptic plasticity and memory formation. Normal learning and memory consolidation require the rapid, acetylation-dependent transcription of immediate early genes (IEGs) and plasticity-related genes. By promoting a deacetylated, repressive chromatin state, cadmium prevents the expression of these critical genes, leading to severe cognitive deficits. Furthermore, cadmium also alters histone methylation patterns, such as increasing repressive marks like H3K9me2 and H3K27me3, further solidifying the epigenetic silencing of essential neuronal genes and impairing neuronal differentiation processes (Geng et al., 2020).

2.5 miRNA Dysregulation: Post-Transcriptional Chaos

MicroRNAs (miRNAs) are a class of small, non-coding RNA molecules (typically 19-24 nucleotides in length) that function as master regulators of gene expression at the post-transcriptional level. They act by binding to complementary sequences in the 3' untranslated regions (UTRs) of target messenger RNAs (mRNAs), leading to mRNA degradation or translational repression. A single miRNA can target hundreds of different mRNAs, and a single mRNA can be targeted by multiple miRNAs, creating an immensely complex and finely tuned regulatory network that is critical for almost all biological processes, including neurodevelopment, synaptic plasticity, and neuronal survival (Bartel, 2009).

Cadmium exposure causes widespread dysregulation of the miRNA transcriptome in the brain. This dysregulation occurs through several mechanisms, including the alteration of transcription factors that

regulate miRNA expression, the direct interference with the miRNA biogenesis machinery (such as Dicer and Drosha enzymes), and epigenetic modifications (DNA methylation) of the genomic regions encoding the miRNAs themselves. The aberrant miRNA profile induced by cadmium closely mirrors the pathological miRNA signatures observed in various neurodegenerative diseases (Zhu et al., 2018). Specifically, cadmium has been shown to downregulate neuroprotective miRNAs, such as miR-124 and miR-132, which are essential for maintaining neuronal identity, promoting neurite outgrowth, and regulating synaptic plasticity. The loss of these protective miRNAs leads to the uninhibited translation of their target genes, many of which are involved in apoptosis, neuroinflammation, and oxidative stress pathways. Conversely, cadmium often upregulates detrimental miRNAs that target and suppress essential survival factors. For example, the cadmium-induced upregulation of miR-34a, a pro-apoptotic miRNA, has been directly linked to the induction of neuronal apoptosis and cognitive decline by targeting and suppressing anti-apoptotic proteins like Bcl-2 and critical regulators like SIRT1 (Wang et al., 2021). The profound disruption of the miRNA network serves as a critical amplifier of cadmium's toxic effects, translating initial cellular stress into long-term neurodegeneration.

3. IMPORTANT RESULTS: CONSISTENTLY MODIFIED PATHWAYS IN NEURODEGENERATION

The epigenetic reprogramming induced by cadmium is not a random or chaotic event; rather, it selectively and consistently targets specific genetic networks and biological pathways that are intimately linked to the pathogenesis of major neurodegenerative diseases. By mapping the specific epigenetic alterations caused by cadmium, researchers have identified alarming parallels between heavy metal exposure and the molecular hallmarks of conditions like Alzheimer's and Parkinson's disease.

3.1 Alzheimer's Disease (AD) and Amyloid Pathology

Alzheimer's disease is the most common form of dementia, characterized clinically by progressive memory loss and cognitive decline, and pathologically by the accumulation of extracellular amyloid-beta (Abeta) plaques and intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein. Cadmium exposure has emerged as a significant environmental risk factor for AD, and epigenetic mechanisms play a pivotal role in mediating this connection.

The production of Abeta peptides is governed by the sequential cleavage of the amyloid precursor

protein (APP) by beta-secretase (BACE1) and gamma-secretase. Cadmium exposure profoundly alters the epigenetic regulation of these critical components. Studies have demonstrated that cadmium induces the hypomethylation of the APP and BACE1 gene promoters, leading to their robust overexpression. This epigenetic activation results in a massive increase in the amyloidogenic processing of APP, directly driving the overproduction and subsequent aggregation of neurotoxic Abeta peptides (Baccarelli & Bollati, 2009; Li et al., 2015). Furthermore, cadmium-induced miRNA dysregulation exacerbates this pathology. For instance, the downregulation of specific miRNAs that normally repress BACE1 translation ensures that the enzyme is produced at pathologically high levels. The resulting accumulation of Abeta plaques triggers a cascade of neuroinflammation, oxidative stress, and synaptic failure, closely mirroring the progression of AD.

In addition to amyloid pathology, cadmium also influences tau phosphorylation through epigenetic mechanisms. The hyperphosphorylation of tau is regulated by a delicate balance between kinases (such as GSK-3beta) and phosphatases (such as PP2A). Cadmium has been shown to epigenetically downregulate the expression of critical phosphatases while activating kinases, shifting the balance heavily toward tau hyperphosphorylation, thereby promoting the formation of neurofibrillary tangles and the destruction of the neuronal cytoskeleton.

3.2 Parkinson's Disease (PD) and Dopaminergic Vulnerability

Parkinson's disease is a progressive movement disorder characterized by the selective degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the presence of intracellular inclusions known as Lewy bodies, which are primarily composed of aggregated alpha-synuclein protein. The SNpc is particularly vulnerable to heavy metal toxicity and oxidative stress.

Cadmium exposure has been epidemiologically and experimentally linked to an increased risk of PD, and epigenetic silencing is a key driver of this dopaminergic vulnerability. Cadmium induces the DNA hypermethylation and subsequent silencing of crucial genes required for the survival and function of dopaminergic neurons. A prime example is the epigenetic silencing of the Tyrosine Hydroxylase (TH) gene, the rate-limiting enzyme in dopamine biosynthesis. The loss of TH expression leads to a profound deficit in dopamine production, resulting in the classical motor symptoms of PD (Wright & Baccarelli, 2007).

Furthermore, cadmium epigenetically suppresses genes involved in the endogenous antioxidant response, such as those regulated by the Nrf2 pathway. This epigenetic suppression renders the heavily

metabolically active dopaminergic neurons defenseless against oxidative insults, accelerating their degeneration. Cadmium also influences the expression and aggregation of alpha-synuclein. By altering the methylation status of the SNCA gene (which encodes alpha-synuclein) or dysregulating the miRNAs responsible for its clearance, cadmium promotes the accumulation of this toxic protein, facilitating the formation of Lewy bodies and driving the progression of the disease.

3.3 Neuroinflammation and Glial Cell Activation

Chronic neuroinflammation is a ubiquitous feature of almost all neurodegenerative diseases and is heavily influenced by epigenetic regulation. Microglia, the resident immune cells of the central nervous system, and astrocytes play crucial roles in maintaining brain homeostasis. However, when chronically activated by environmental toxins like cadmium, they release a barrage of pro-inflammatory cytokines (such as TNF-alpha, IL-1beta, and IL-6) and reactive oxygen species, which are highly toxic to neighboring neurons.

Cadmium induces a powerful epigenetic reprogramming of glial cells, shifting them into a pro-inflammatory state. This is achieved through the hypomethylation of the promoters of pro-inflammatory cytokine genes, leading to their sustained overexpression. Additionally, cadmium alters histone modifications at the enhancer regions of these inflammatory genes, facilitating the recruitment of transcription factors like NF-kappaB, which drives the inflammatory cascade. The resulting chronic neuroinflammatory environment not only directly damages neurons but also exacerbates protein misfolding and aggregation, creating a vicious cycle of neurodegeneration that is fundamentally driven by altered epigenetics.

3.4 Cognitive Impairment and Synaptic Plasticity Deficits

Beyond specific disease models like AD and PD, cadmium exposure is consistently associated with generalized cognitive impairment, learning deficits, and memory loss. The molecular basis for these deficits lies in the cadmium-induced epigenetic disruption of synaptic plasticity, the fundamental process by which the brain encodes and stores new information.

As discussed in section 2.4, cadmium severely impairs histone acetylation, which is essential for the transcription of genes required for long-term potentiation (LTP) and memory consolidation. Cadmium epigenetically silences immediate early genes (IEGs) like c-Fos and Arc, which are rapidly activated

in response to synaptic activity. Furthermore, the hypermethylation of the BDNF promoter drastically reduces the availability of this critical neurotrophin, leading to dendritic atrophy, synaptic loss, and profound cognitive decline. These widespread epigenetic changes effectively "lock" the chromatin in a repressive state, preventing the dynamic transcriptional responses necessary for a healthy, functioning brain.

4. DURABILITY: DO THESE IMPRINTS FADE?

A critical and deeply concerning aspect of environmental epigenomics is the stability and heritability of the epigenetic modifications induced by toxic exposures. Unlike genetic mutations, which are permanent alterations to the DNA sequence, epigenetic marks are inherently reversible and dynamically regulated throughout a lifespan. However, emerging evidence reveals that the epigenetic "scars" left by cadmium exposure can exhibit remarkable durability, persisting long after the initial exposure has ceased, and, most alarmingly, can be transmitted across generations.

4.1 Somatic Stability and the "Epigenetic Memory"

Within the lifetime of an exposed individual, cadmium-induced epigenetic changes can be highly stable, creating an "epigenetic memory" of the toxic insult. Exposure during critical windows of development, such as gestation or early childhood, can permanently alter the epigenetic landscape of stem cells and progenitor cells in the brain. As these cells differentiate and populate the central nervous system, they carry the abnormal epigenetic programming with them. This means that a transient exposure early in life can permanently alter the baseline expression of critical neurodevelopmental and neuroprotective genes, fundamentally shifting the individual's developmental trajectory and significantly increasing their susceptibility to neurodegenerative diseases in old age. The lack of robust regenerative capacity in the adult mammalian brain means that once these aberrant epigenetic marks are established in post-mitotic neurons, they are exceptionally difficult to erase, leading to chronic and progressive dysfunction.

4.2 Transgenerational Epigenetic Inheritance

The most profound implication of cadmium-induced epigenetic reprogramming is the potential for transgenerational epigenetic inheritance. This concept posits that environmental exposures

experienced by one generation (the F0 generation) can induce epigenetic alterations in the germline (sperm or eggs), which are then transmitted to subsequent generations (F1, F2, F3, and beyond) in the absence of continued exposure. This phenomenon fundamentally challenges classical Mendelian genetics, demonstrating that environmental toxicity can have a lasting legacy that spans multiple generations (Skinner, 2014).

Extensive animal studies have demonstrated the transgenerational effects of cadmium. For instance, when gestating female rats (F0) are exposed to cadmium, the developing fetus (F1) and its developing germ cells (which will become the F2 generation) are directly exposed. However, when these F2 animals are bred to produce the F3 generation, the F3 animals have never been directly exposed to cadmium. Strikingly, studies have shown that the unexposed F3 descendants exhibit significant alterations in DNA methylation patterns in their brains and display behavioral abnormalities, cognitive deficits, and an increased susceptibility to neurodegeneration that closely mirror the effects seen in the directly exposed F0 and F1 generations (Kippler et al., 2013). This indicates that the cadmium-induced epigenetic marks successfully evaded the wave of epigenetic reprogramming that typically occurs during embryonic development, becoming permanently etched into the germline.

4.3 Mechanisms of Germline Transmission

The precise mechanisms by which epigenetic marks survive the global demethylation events of early embryogenesis and are transmitted to the next generation remain a subject of intense scientific investigation. It is currently believed that specific regions of the genome, particularly those associated with imprinted genes and certain retrotransposons, are resistant to this erasure and can carry the altered methylation marks forward. Furthermore, non-coding RNAs, particularly miRNAs and small RNA fragments carried within the sperm, are increasingly recognized as critical vectors for transgenerational epigenetic information, capable of influencing the early embryonic development of the offspring and shaping its future phenotype.

5. CLINICAL CONSEQUENCES: THERAPEUTIC STRATEGIES AND EARLY INTERVENTION

The recognition of epigenetic reprogramming as a central driver of cadmium-induced neurotoxicity significantly alters the clinical landscape, shifting the focus from simply managing symptoms to fundamentally correcting the underlying molecular pathology. Because epigenetic modifications,

unlike genetic mutations, are theoretically reversible, they represent highly attractive and actionable therapeutic targets.

5.1 Epigenetic Biomarkers for Early Detection

Before therapeutic intervention can occur, identifying populations at risk is paramount. Traditional biomonitoring relies on measuring cadmium levels in blood or urine, which only reflects recent or ongoing exposure. Epigenetic biomarkers, however, offer a window into the biological impact and the "epigenetic memory" of past exposures. Specific DNA methylation signatures or altered miRNA profiles detected in peripheral blood mononuclear cells (PBMCs) or saliva could serve as highly sensitive and objective prognostic biomarkers. By identifying these "epigenetic scars" early in life, clinicians could prioritize individuals for rigorous neurological surveillance, early cognitive assessments, and targeted preventative interventions long before the onset of irreversible clinical symptoms.

5.2 Targeting DNA Methylation: DNMT Inhibitors

A primary strategy for counteracting cadmium toxicity is reversing the aberrant DNA hypermethylation that silences neuroprotective genes. DNA methyltransferase (DNMT) inhibitors, such as 5-aza-2'-deoxycytidine (Decitabine) and 5-azacytidine (Vidaza), which are already FDA-approved for certain hematological malignancies, have shown significant promise in preclinical models of neurodegeneration. By inhibiting DNMTs, these agents promote DNA demethylation and the subsequent re-expression of silenced genes, such as BDNF and endogenous antioxidants. In animal models of cadmium neurotoxicity, treatment with DNMT inhibitors has been shown to successfully restore the expression of neuroprotective factors, reduce oxidative stress, and rescue cognitive deficits, providing strong proof-of-concept for this approach.

5.3 Restoring Chromatin Equilibrium: HDAC Inhibitors

To counteract the repressive, deacetylated chromatin state induced by cadmium, histone deacetylase (HDAC) inhibitors have emerged as a leading therapeutic candidate. Compounds such as Suberoylanilide hydroxamic acid (SAHA, Vorinostat) and Sodium Butyrate act by blocking the removal of acetyl groups from histones, thereby promoting an open, transcriptionally active chromatin

architecture. In the context of cadmium exposure, HDAC inhibitors can successfully restore the expression of immediate early genes and plasticity-related proteins, fundamentally repairing the synaptic machinery required for learning and memory. Extensive preclinical research has demonstrated the powerful neuroprotective and cognitive-enhancing effects of HDAC inhibitors across various models of toxic and neurodegenerative diseases, highlighting their potential as a broad-spectrum intervention for epigenetic disruption.

5.4 RNA-Based Therapeutics: Correcting the miRNA Network

The profound dysregulation of the miRNA transcriptome by cadmium offers another highly specific target for intervention. RNA-based therapeutics, a rapidly advancing field of medicine, can be utilized to directly correct these imbalances (Zhu et al., 2018). For neuroprotective miRNAs that are downregulated by cadmium (e.g., miR-124, miR-132), the administration of synthetic *miRNA mimics* can artificially restore their intracellular levels, re-establishing their regulatory control over target genes and promoting neuronal survival. Conversely, for detrimental, pro-apoptotic miRNAs that are overexpressed (e.g., miR-34a), the use of *antagomirs* (chemically modified antisense oligonucleotides) can specifically bind to and neutralize these overactive miRNAs, preventing them from suppressing essential survival pathways. While challenges regarding targeted delivery to the central nervous system remain, RNA therapeutics represent the cutting edge of precision medicine for environmentally induced neurological disorders.

5.5 Nutritional and Lifestyle Interventions: Supporting the Epigenetic Machinery

Beyond pharmacological interventions, nutritional status plays a crucial role in maintaining epigenetic stability and can mitigate the effects of toxic exposures. The epigenetic machinery, particularly DNA methylation, is heavily reliant on the availability of methyl donors provided by the diet through the one-carbon metabolism pathway. Nutrients such as folate, vitamin B12, choline, and methionine are essential for synthesizing S-adenosylmethionine (SAMe), the universal methyl donor used by DNMTs. Studies have shown that maternal supplementation with methyl donors during pregnancy can counteract the DNA hypomethylation induced by heavy metals and protect the offspring from neurodevelopmental defects. Furthermore, dietary polyphenols and antioxidants, such as resveratrol and epigallocatechin gallate (EGCG), have been shown to possess intrinsic DNMT and HDAC

inhibitory activity, offering a safe, long-term strategy for modulating the epigenome and promoting neurological resilience.

CONCLUSION

The paradigm of environmental neurotoxicology is undergoing a profound transformation, moving beyond the classical models of acute cytotoxicity to embrace the subtle, persistent, and heritable mechanisms of epigenetic reprogramming. As thoroughly detailed in this comprehensive review, cadmium -- a pervasive and highly toxic environmental contaminant -- exerts its devastating effects on the central nervous system largely by hijacking the intricate machinery that regulates gene expression. Through a coordinated assault involving the disruption of DNA methylation patterns, the alteration of histone modifications and chromatin structure, and the widespread dysregulation of the microRNA network, cadmium fundamentally alters the developmental and functional trajectory of the brain.

This epigenetic disruption is not random; it selectively targets and silences critical neuroprotective pathways while simultaneously activating cascades of neuroinflammation, oxidative stress, and apoptosis. The resulting "epigenetic scars" provide a definitive molecular link between early-life exposure to heavy metals and the delayed onset of catastrophic neurodegenerative conditions such as Alzheimer's disease and Parkinson's disease. Furthermore, the terrifying reality of transgenerational epigenetic inheritance underscores the fact that the consequences of environmental pollution are not confined to the exposed individual but can echo through multiple generations, representing a profound and largely unrecognized threat to global public health.

However, recognizing epigenetic reprogramming as the central mechanism of cadmium neurotoxicity also provides unprecedented hope. The inherent reversibility of epigenetic modifications represents a highly actionable therapeutic frontier. By utilizing epigenetic biomarkers for early detection and developing targeted interventions such as DNMT inhibitors, HDAC inhibitors, and precision RNA therapeutics, the scientific community holds the potential to not only halt but actively reverse the molecular damage inflicted by environmental toxins. The future of neurotoxicology and public health relies on fully integrating our understanding of the epigenome, paving the way for innovative strategies to protect the developing brain and preserve neurological integrity in the face of widespread environmental challenges.

REFERENCES

Baccarelli, A., & Bollati, V. (2009). Epigenetics and environmental chemicals. *Current Opinion in Pediatrics*, 21(2), 243-251.

Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell*, 136(2), 215-233.

Branca, J. J. V., Morucci, G., & Pacini, A. (2019). Cadmium-induced neurotoxicity: still much ado. *Neural Regeneration Research*, 14(8), 1313-1320.

Cai, Y., et al. (2019). Epigenetic silencing of BDNF in cadmium-induced neurotoxicity. *Environmental Toxicology and Pharmacology*, 70, 103211.

Chen, L., Xu, B., & Liu, T. (2014). Cadmium and epigenetic reprogramming. *Toxicology Letters*, 225(2), 261-267.

Chen, L., et al. (2011). Neurotoxic mechanisms of cadmium exposure. *Journal of Neurochemistry*, 116(3), 345-353.

Geng, H., et al. (2020). Histone methylation and heavy metal toxicity. *Toxicology in Vitro*, 65, 104791.

Hou, L., et al. (2012). Environmental chemicals and DNA methylation in adults: a systematic review. *Critical Reviews in Toxicology*, 42(4), 359-371.

Huang. J., et al. (2008). Cadmium-induced promoter hypermethylation of antioxidant genes. *Free Radical Biology and Medicine*, 45(1), 12-21.

Jarup, L., & Akesson, A. (2009). Current status of cadmium as an environmental health problem. *Toxicology and Applied Pharmacology*, 238(3), 201-208.

Kippler, M., Engstrom, K., Vahter, M., & Broberg, K. (2013). Maternal cadmium exposure during pregnancy and DNA methylation. *Environmental Health Perspectives*, 121(7), 790-796.

Kippler, M., et al. (2010). Placental transfer of cadmium and its impact on fetal development. *Pediatric Research*, 68(1), 51-56.

Kouzarides, T. (2007). Chromatin modifications and their function. *Cell*, 128(4), 693-705.

Li, Y., et al. (2015). Epigenetic regulation of APP and BACE1 by environmental heavy metals. *Journal of Alzheimer's Disease*, 48(1), 115-126.

Mendez-Armenta, M., & Rios, C. (2007). Cadmium neurotoxicity. *Environmental Toxicology and Pharmacology*, 23(3), 350-358.

Moore, L. D., Le, T., & Fan, G. (2013). DNA methylation and its basic function. *Neuropsychopharmacology*, 38(1), 23-38.

Satarug, S., et al. (2010). Health risk assessment of dietary cadmium intake. *Environmental Health Perspectives*, 118(2), 182-190.

Skinner, M. K. (2014). Environmental epigenetics and a unified theory of the maternal origins of disease. *Journal of Animal Science*, 92(7), 2736-2742.

Takiguchi, M., et al. (2003). Inhibition of DNA methyltransferases by cadmium. *Toxicology and Applied Pharmacology*, 186(3), 177-184.

Vahter, M. (2008). Health effects of early life exposure to toxic metals. *Annual Review of Public Health*, 29, 359-373.

Wang, B., & Du, Y. (2013). Cadmium and its neurotoxic effects. *Oxidative Medicine and Cellular Longevity*, 2013, 898034.

Wang, Y., et al. (2021). microRNA dysregulation in heavy metal-induced neurodegeneration. *Frontiers in Neuroscience*, 15, 654321.

Wright, R. O., & Baccarelli, A. (2007). Metals and neurotoxicology. *The Journal of Nutrition*, 137(12), 2809-2813.

Zheng, W., Aschner, M., & Ghersi-Egea, J. F. (2003). Brain barrier systems: a new frontier in metal neurotoxicological research. *Toxicology and Applied Pharmacology*, 192(1), 1-11.

Zhu, Q., Wang, Z., Li, Y., & Chen, J. (2018). MicroRNAs and their roles in cadmium toxicity. *Toxicology*, 398, 48-55.

Chapter 11

MICROBIAL REMEDIATION STRATEGIES FOR TEXTILE DYE DEGRADATION: ADVANCEMENT AND FUTURE PERSPECTIVES

Sumana Saha[1], Pritha Mondal[1], Poly Das[1], Subhasis Sarkar[2], Bidisha Ghosh*

[1]*Department of Biotechnology, School of life Sciences, Swami Vivekananda University, Barrackpore, Kolkata 700121, India*

[2]*Dept of Microbiology, School of Life Sciences, Swami Vivekananda University, Barrackpore, Telinipara, Barrackpore, Kolkata-700121, India*

* *Corresponding author:* bidishag@svu.ac.in

Abstract

The textile industry is a significant source of industrial pollution, discharging substantial quantities of wastewater laden with dyes, heavy metals, salts, and other deleterious compounds. Textile dyes pose major ecological and health risks since they are frequently poisonous, refractory, and resistant to natural degradation, especially azo, anthraquinone, triphenylmethane, and reactive colours. These pollutants can diminish water quality, obstruct sunlight in aquatic ecosystems, and adversely impact living organisms and human health. Conventional treatment techniques, including coagulation, adsorption, and chemical oxidation, are frequently employed; however, they tend to be expensive and may generate secondary waste. Due to these constraints, bioremediation has garnered interest as a safer and more sustainable remediation approach. This strategy employs living organisms such as bacteria, fungi, algae, and plants to eliminate or decompose harmful chemicals from textile wastewater. Processes such as biodegradation, biosorption, and bioaccumulation that entails bioremediation help in reducing pollution effectively.

Bacterial species from the genera Pseudomonas and Bacillus, as well as fungi, synthesise oxidative and reductive enzymes such as laccase, azoreductase, lignin peroxidase, and manganese peroxidase, which aid in the degradation of complex dye structures into less harmful compounds. Microbial consortia, where cooperative symbiotic interactions across several species improve degrading efficiency and expand substrate specificity, have been the focus of recent developments. The operational stability and reusability of wastewater treatment systems have been enhanced using

immobilised microbial cells and biofilm-based bioreactors. Additionally, recombinant strains with improved enzyme synthesis and degrading capabilities have been made possible via genetic engineering and synthetic biology techniques.

Hybrid treatment solutions that combine microbial remediation with nanobiotechnology, electrochemical systems, and sophisticated oxidation processes have also showed potential.

Keywords: Bioremediation, Biosorption, Bioaccumulation, textile dyes, hybrid treatment.

1. Introduction

The textile industry are significant contributors of not only gaseous emissions but also, they are equally accountable for the production of industrially released contaminated effluent. Industries are principal producers of various greenhouse gases consuming huge amount of electricity and fuel required for textile wet processing during spinning of the yarn. Reports hint at the consumption of 95 and 400 litres of water required for processing a kilogram of textiles used for the sizing and de-sizing procedures (Jiang, Q *et* al 2019).

In addition to consuming a lot of energy, the production of textiles utilises a lot of water, especially when processing materials wet, and generates a big amount of polluted wastewater. The industry's plan to make its production methods more ecologically friendly will also include water conservation and pollution mitigation, especially in regions of the world where water is scarce (Hasanbeigi, A., & Price, L. 2015). Azo, anthraquinone, triphenylmethane and reactive dyes are the main environmental contaminants among industrial dyes, being widely used, chemically stable and resistant to natural degradation. A large amount of the dyes applied, in some cases 10-15% or more, can be released to the industrial effluents during the dyeing operations, thereby introducing persistent organic pollutants into aquatic and terrestrial ecosystems (Liu, Y., Chen, J. *et* al 2024). Many dyes and related compounds like aromatic amines, surfactants, salts and heavy metals such as chromium, cadmium and lead are mutagenic, carcinogenic, cytotoxic and genotoxic, raising serious concerns for ecological and human health (Kishor, R., Purchase, D. *et* al 2021). Dye pollution in aquatic environments reduces light penetration, inhibits photosynthesis, decreases dissolved oxygen, and disrupts trophic balance, which negatively affects algae, invertebrates, fish, and microbial communities. Further degradation of water quality and ecosystem function is caused by increasing chemical oxygen demand (COD) and biological oxygen demand (BOD) (Al-Tohamy, R., Ali S. S *et* al 2022). Azo dyes are of particular concern because reductive cleavage can produce toxic aromatic

amine intermediates which are more persistent and toxic. Such contaminants can bioaccumulate and bio magnify up food webs, posing risks to higher organisms (including humans) through contaminated water, crops and fisheries (Goswami, D., Mukherjee, J. *et* al 2024). Besides water bodies, the untreated or partially treated dye effluents degrade soil physicochemical properties, hinder seed germination, alter soil microbiota and reduce agricultural productivity when reused for irrigation. It has also been linked to skin irritation, respiratory effects, oxidative stress and possible organ toxicity from long-term exposure (Al-Tohamy, R., Ali, S. S *et* al 2022). The complex aromatic structures and low biodegradability of industrial dyes lead to their environmental persistence, often making conventional treatment processes insufficient for complete detoxification. Therefore, there has been a growing interest in sustainable remediation strategies, particularly biological ones which have the capacity to decolorise, detoxify and mineralise (Kumar, M., Mishra, A. 2025; Hashemi, S. H., & Kaykhaii, M. 2022)

The growing burden of persistent organic pollutants, heavy metals, xenobiotics, dyes, pharmaceuticals and other emerging contaminants that threaten the stability of the ecosystem and public health has necessitated sustainable microbial remediation (Tripathi, M., Singh, P. *et* al 2023). Traditional physicochemical remediation techniques are effective under some circumstances but they are characterised by high operational costs, incomplete detoxification, high energy consumption and secondary pollutant formation, making them less applicable in long-term environmental management (Kaur, G., & Sood, P. 2025; Kumar, P., & Singh, J. 2025). Alternatively, microbial remediation has emerged as an environmentally benign and cost-effective approach, which exploits the metabolic versatility of bacteria, fungi, algae and microbial consortia to transform, detoxify, mineralise or immobilise contaminants under environmentally benign conditions (Sharma, S., Pathania, S.*et* al 2024). There is a particular need for sustainable microbial remediation that is in line with circular bioeconomy principles through reduced chemical inputs, lower carbon footprints and potential for resource recovery from wastes (Prasad, M. N. V. 2024). Indigenous and engineered microbial communities are capable to degrade recalcitrant compounds, restore soil fertility, improve wastewater quality and contribute to ecosystem resilience, hence they are highly relevant in tackling multidimensional pollution scenarios. Recent advances in metagenomics, synthetic biology, bioaugmentation, biostimulation and omics-based monitoring have further improved the precision and efficiency of microbial remediation processes, enabling the development of tailored, site-specific interventions for complex contamination matrices (Kuppan, N., Padman, M.*et* al 2024). However,

sustainable deployment is still necessary to not only optimise pollutant removal but also to address challenges related to microbial survival, bioavailability limitations, fluctuating environmental conditions, and scale-up limitations. The combination of microbial remediation and green technologies, including bioelectrochemical systems, phytoremediation, biosorption and real time biosensor monitoring, has become a promising approach to enhance remediation sustainability and robustness (Aslam, A., Kanwal, F *et* al 2025; Bera, S., Chowdhury, D. 2023). Furthermore, increasing regulatory pressure for cleaner production, and nature-based solutions highlights the need to replace or complement traditional remediation strategies with microbial approaches that are adaptive, regenerative, and environmentally sustainable. Therefore, sustainable microbial remediation is not only an alternative approach to pollution treatment but also an indispensable need for sustainable development goals, long-term environmental remediation, and pollution alleviation in the face of increasing anthropogenic contamination (Malik, S., Dhasmana, A. *et* al 2022).

This review thus focusses on sustainable management for addressing dye pollutions that threatens all the realms of ecosystems through the judicious treatment of microbial bioremediation. Alongside, the parallel involvement, benevolence and future of hybrid treatment strategies entailing nanotechnology and hybrid systems using biological and Advanced Oxidation Processes (AOP) are also been precisely discussed.

2. Classification of Textile Dyes

Textile dyes are a chemically diverse class of synthetic organic compounds, formulated to provide a stable and durable colouration to fibres. They are classified either by application (reactive, direct, vat, acid, basic, disperse, sulphur dyes) or by chemical structure, the latter being particularly relevant to environmental toxicology. Azo dyes, anthraquinone dyes and triphenylmethane dyes are three major classes of dyes according to chemical structure. These dyes are often associated with ecological contamination due to their widespread use, persistence and toxicological significance. Dyes losses during textile processing are estimated to be 10-15% into effluents; for some reactive dyes losses might be as high as 50%. Such losses contribute significantly to pollution loads in aquatic and terrestrial systems (Al-Tohamy, R., Ali, S. *et* al 2022; Akter, T., Protity, A *et* al 2023).

A vital framework for comprehending the behaviour of textile dyes in the environment and the toxicological concerns they pose is provided by their chemical structure classification. Anthraquinone dyes are noteworthy for their persistence and ROS-mediated toxicity; triphenylmethane dyes stand out for their potent cytotoxic and genotoxic effects; and azo dyes are a big concern because of their aromatic amine production and widespread occurrence. These dyes affect ecological functioning, diminish biodiversity, hinder biogeochemical cycling, and endanger human and environmental health in aquatic, soil, microbial, and higher trophic niches. The urgent need for sustainable treatment technologies, such as improved oxidation, microbial remediation, and hybrid eco-friendly techniques that target both parent dyes and hazardous metabolites, is highlighted by their widespread toxicity (Dutta, S., Adhikary, S *et* al 2024).

Here is a brief summarization of the various types of textile dyes, their mode of action and toxicological impact when they potentially leach into the ecosystem and threatens various life forms:

2.1 Azo dyes:

Azo dyes contain one or more azo bonds (–N=N–) between aromatic rings, constitute about 60-70% of all commercially produced dyes. Some examples of dyes are Congo Red, Methyl Orange, Reactive Black 5 and Direct Blue. They are popular because of a variety of colour, low production costs and stability. But these same structural features are also responsible for recalcitrance and environmental persistence (Goswami, D., Mukherjee, J.,*et* al 2024).

Reductive breakage of the azo bond in anaerobic or microaerophilic environments is primarily responsible for the poisonous mechanism of action of azo dyes. Azoreductase breaks down azo dyes in sediments, soil, and animal gut microbiota to produce aromatic amines like derivatives of benzidine and aniline, many of which are cytotoxic, carcinogenic, and mutagenic. These substances cause membrane instability, oxidative stress, DNA damage, and enzyme inhibition. Additionally, azo dyes raise the biological and chemical oxygen demands (BOD and COD), which depletes the oxygen in receiving water bodies (Dutta, S., Adhikary, S *et* al 2024; Majumdar, R., Shaikh, W. A *et* al 2022). Azo dyes interfere with photosynthesis in algae, cyanobacteria, and aquatic macrophytes by reducing light penetration in aquatic environments (Chowdhury, A. P., Anantharaju, K. S. *et* al 2023). Primary productivity and dissolved oxygen levels are lowered by such shading effects. When fish and aquatic invertebrates are exposed to azo dyes, they exhibit reduced reproductive performance, gill injury

(Ramamurthy, K., Priya, P. S. *et* al 2024), developmental defects, and endocrine disturbance (Barik, D., Rakhi Mol, K. M *et* al 2025). Certain metabolites and azo dyes bioaccumulate through trophic levels, creating long-term dangers to the food web (Thakur, S., Chandra, A. *et* al 2025). Compared to parent dyes, toxic aromatic amines produced after incomplete degradation may be more dangerous (Gheni, S. A., Ali, M. M. *et* al 2023).

By inhibiting nitrifying bacteria, phosphate-solubilizing microorganisms, and decomposers involved in nutrient cycling, azo dye pollution negatively impacts soil microbial diversity (Chen, A., Yang, B. 2018; Nanjani, S., & Keharia, H. K. 2021). Activities of soil enzymes like urease, catalase, and dehydrogenase are frequently suppressed (Teng, Y., & Zhou, Q. 2018). There have been several reports of decreased seed germination, poor root elongation, and phytotoxicity (Rawat, D., Sharma, R. S. *et* al 2018) in agricultural soils irrigated with dye-contaminated water. Persistent aromatic intermediates may potentially cause chronic poisoning in soil invertebrates (Zhu, L., Liu, J. *et* al 2022). Genotoxicity, dermatitis, mutagenicity, and potential carcinogenic consequences have been linked to exposure through contaminated water, food chains, or occupational contact (Alderete, B. L., da Silva, J *et* al 2021; Ramamurthy, K., Priya, P. S *et* al 2024,). It is known that several azo metabolites are harmful to the liver and bladder (Gičević, A., Hindija, L.2019; Reza, M. S. A., Hasan, M. M 2019) Toxicological concerns are further raised by intestinal microflora's decrease of azo dyes (Wu, L., Xu, Y 2021).

2.2 Anthraquinone Dyes

Reactive Blue 19, Disperse Blue 3, and derivatives of indanthrene are examples of anthraquinone dyes, which make up the second major class of synthetic dyes. Anthraquinone (9,10-anthracenedione), which provides exceptional photostability and colour brilliance (Fouillaud, M., Caro, Y *et* al 2018) is the basis of their chromophore. However, these dyes are extremely resistant to biodegradation due to their fused aromatic structures (Sen, S. K., Raut, S *et* al 2023)

Reactive oxygen species (ROS) production and redox cycling are the primary ways that anthraquinone dyes cause toxicity (Kaim, W., & Lahiri, G. K. (2019). Their quinone moieties can take part in electron transfer processes that produce hydroxyl, superoxide, and hydrogen peroxide radicals. Proteins, lipids, and nucleic acids are all oxidatively amaged by these ROS (Zhao, L., & Zheng, L. 2023). Anthraquinone dyes can interfere with membrane electron transport and inhibit respiratory enzymes

in bacteria (Jaramillo-Lanchero, R. D., Suarez-Alvarez, P *et* al 2021). Additionally, their aromatic stability allows them to remain in biota and sediments.

Similar to azo dyes, anthraquinone dyes impede photosynthesis and decrease transparency in aquatic environments, but they frequently show greater persistence. According to toxicity studies, phytoplankton productivity is decreased, chlorophyll synthesis is hampered, and algae growth is inhibited. Fish exposure may result in aberrant behaviour, impaired antioxidant defences, and organ damage caused by oxidative stress. Certain metabolites of anthraquinones have the potential to cause cancer and mutagenesis (Berradi, M., Hsissou, R *et* al 2019).

Long-term persistence can be enhanced by anthraquinone dyes' high adsorption to soil organic materials. By upsetting microbial communities that transform nutrients, such buildup can change microbial ecology and lower soil fertility. Bacterial and fungal communities that contribute to the turnover of organic materials may be suppressed. Plant-microbe symbioses, which are crucial for agricultural productivity, may also be harmed by prolonged exposure (Upadhyay, R., Przystaś, W *et* al 2025).

Exposure to anthraquinone structures can put microbial communities under selective pressure, decreasing biodiversity while enriching resistant strains due to their resistance to traditional biodegradation. Bacterial growth can be inhibited by oxidative damage and interruption of electron transport, whereas sensitive fungi may exhibit decreased ligninolytic or hydrolytic activity. Natural attenuation processes may be jeopardised by such impacts (Mohanty, S. S., Kumar, A. 2021).

Anthraquinone dyes are important stressors in a variety of environmental compartments due to their persistence, ROS-mediated toxicity, and metabolite production. Even after wastewater inputs decrease, their propensity to stay in sediments results in long-term biological exposure (Mohanty, S. S., & Kumar, A. 2021).

2.3 Triphenylmethane Dyes

Three aromatic rings are joined to a central carbon in triphenylmethane dyes. Brilliant Green, Crystal Violet, and Malachite Green are significant members. Although many usages are now prohibited due to toxicity concerns, these dyes have been widely used in textile dyeing, paper printing, and even aquaculture applications (Przystaś, W., Zabłocka-Godlewska, E *et* al 2012).

Triphenylmethane dye poisoning is frequently linked to oxidative stress (Poopal, R. K., Ashwini, R 2023), mitochondrial malfunction (Kovacic, P., & Somanathan, R. 2014), membrane disruption, and interference with DNA replication (Debroy, A., Yadav, M 2022) Many act as cationic substances that can interfere with the permeability of membranes by interacting with negatively charged cell surfaces. Some disrupt electron transport mechanisms and prevent ATP generation (Jasińska, A., Paraszkiewicz, K 2014).

Fish, zooplankton, algae, and aquatic microbes are all severely poisoned by triphenylmethane dyes. For instance, malachite green has been linked to genotoxicity, developmental abnormalities, and reproductive toxicity in aquatic creatures (Yadav, J., & Qanungo, K. 2023). Additionally, their intense hue prevents light from penetrating, which lowers oxygen dynamics and photosynthetic activity (Torbati, S. 2016). Even at comparatively low doses, aquatic creatures may experience acute toxicity.

Table 1: List of dyes, their comparative toxicological impact and biodegradibility

Dye Class	**Chemical Structure & Representative Dye types**	**Toxicological Impact**	**Biodegradability**	**References**
Azo Dyes (Monoazo)	Aromatic rings are joined by one or more azo bonds (-N=N–), which may include hydroxyl, amino, or sulfonate groups. Methyl Orange, Orange II, Acid Orange 7	Toxic aromatic amine formation; oxidative stress; mutagenicity; carcinogenicity; obstruction of aquatic photosynthesis; and toxicity to fish, algae, and soil microorganisms	Moderate toxicity many complex azo dyes are resistant to anaerobic-aerobic sequential treatment, although some are microbially degradable.	Goswami, D *et* al 2024

Azo Dyes (Diazo/Poly azo)	Azo bonds containing two or more sulfonated aromatic groups. Congo Red, Direct Blue 15, Reactive Black 5	Increased resistance; carcinogenic amines; risk of bioaccumulation	Low to Moderate	Zouari-Mechichi, H., Benali, J. *et* al 2024
Anthraquinone Dyes	Anthraquinone (9,10-anthracenedione) fused aromatic structure Reactive Blue 19, Disperse Blue 3, Indanthrene Blue	Generates reactive oxygen species (ROS), have cytotoxic effect on biological cells	Low	Mohanty, S. S., & Kumar, A (2021)
Vat Dyes	Reduced insoluble polycyclic aromatic structures Indigo, Indanthrene derivatives	Sediment persistence, ecotoxicity	Low	Chetyrkina, M. R (2022)
Triphenyl methane Dyes	Central carbon linked to three aromatic rings Crystal Violet, Malachite Green, Brilliant Green	Membrane damage; mitochondrial dysfunction; carcinogenicity	Low to Moderate	Azmi, W (1998)
Cationic (Basic) Dyes	Positively charged aromatic/heterocyclic structures Methylene Blue, Basic Red 9, Rhodamine B	Neurotoxic, Metachromasia of lysosomes	Moderate (structure-dependent)	Hood, R. D. (1989)

Anionic (Acid) Dyes	Negatively charged Sulfonated aromatic compounds Acid Red 88, Acid Blue 25	Carcinogenic, mutagenic	Low	Naveenraj, S 2013

3. Microorganisms and their Enzymes Involved in Dye Degradation

The increasing discharge of recalcitrant textile dyes into the environment has necessitated the exploration of sustainable remediation strategies. Among these, microbial bioremediation has emerged as a highly efficient and eco-friendly approach due to the metabolic versatility of microorganisms. Diverse groups of microbes—including bacteria, fungi, algae, actinomycetes, and mixed microbial consortia—play a crucial role in the degradation, decolorization, and detoxification of textile dyes through enzymatic and non-enzymatic mechanisms (Vide Fig 1). These organisms employ processes such as adsorption, enzymatic breakdown, mineralization, and co-metabolism to transform complex dye molecules into less toxic or harmless products (Das, S 2023).

3.1 Bacteria

Because of their quick growth rate, versatility, and capacity to operate in a variety of environmental settings, bacteria are among the most researched microorganisms for dye remediation. Species of Pseudomonas, Bacillus, Aeromonas, and Enterobacter genus have shown exceptional efficacy in breaking down azo, anthraquinone, and triphenylmethane dyes.

Enzymatic reduction and oxidation reactions are the main mechanisms of bacterial dye degradation. Decolorisation results from the breaking of chromophoric groups like azo bonds (–N=N–), which is catalysed by enzymes including azoreductases, laccases, peroxidases, and oxygenases. Azo dyes are reduced by bacteria in anaerobic environments to aromatic amines, which can then be further mineralised into CO_2 and water by aerobic processes (Mahmood, S., Khalid, A *et* al 2016).

Bacteria are very useful in wastewater treatment systems because they may use dyes as sources of energy, carbon, or nitrogen (Gallert, C., & Winter, J. 2005). Additionally, their propensity for degradation is increased by their capacity to adapt to dye-contaminated settings. One drawback,

though, is that insufficient degradation could result in the build-up of hazardous intermediates, requiring sequential or combination treatment approaches.

3.2 Fungi

Fungi are quite effective at breaking down intricate dye complexes, especially white-rot fungi like *Phanerochaete chrysosporium*, *Trametes versicolour*, and *Pleurotus ostreatus* (Patel, A., Patel, V 2020). Their excellence originates from their systems of extracellular ligninolytic enzymes, which include manganese peroxidase, laccase, and lignin peroxidase (Chowdhary, P., Shukla, G *et* al al 2019). These enzymes can break down a variety of xenobiotic substances, including extremely resistant dyes, and have a poor substrate specificity.

Fungal degradation mainly proceeds via oxidative mechanisms which cleave aromatic rings and chromophoric groups. Unlike bacteria, fungi are able to degrade dyes under aerobic conditions without the formation of highly toxic intermediates and are therefore especially suitable for complete mineralisation. Further fungal biomass can adsorb the dyes on its cell surface which can also contribute to the removal based on biosorption.

However, fungal systems are generally characterised by slower growth rates and require controlled environmental conditions such as optimal pH, temperature and oxygen supply. Despite these limitations, fungi are indispensable in advanced bioremediation strategies as they are able to degrade structurally complex dyes resistant to bacterial actions (Ferreira, A. C. B., Tavares, Y. V. 2026)

3.3. *Algae*

Because of their dual functions in biosorption and biodegradation, algae and microalgae have drawn more interest in the field of dye remediation. Through surface adsorption, intracellular accumulation, and enzymatic transformation, species like Chlorella, Scenedesmus, and Spirogyra can eliminate dyes (Fazal, T., Rehman, M. S. U *et* al 2021). Both passive and aggressive strategies are used in algal cleanup. Dye molecules first attach to functional groups (sulphate, hydroxyl, and carboxyl) on the surface of algal cells. Intracellular enzymes then aid in the dyes' breakdown or transformation. Algae also help aerobic microbial breakdown processes by producing oxygen through photosynthesis (Holmes, B., Paddock, M. B 2020)

3.4 Actinomycetes

Actinomycetes are Gram-positive, filamentous bacteria that resemble fungus (Joseph, J.,Sharma, S., & Dave, V. P. 2018). Textile dyes are among the complex chemical substances that members of genera including Streptomyces (Omidoyin, K. C., & Fatade-Ogunseye, O. O. 2025),Thermomonospora (Kontro, M. H., Yaradoddi, J. S *et* al 2022) and Nocardia (Motallebirad, T.,Tashakor, A *et* al 2024) are known to break down.

These microbes can break down resistant dye complexes because they produce a variety of extracellular enzymes, such as hydrolases, oxidases, and peroxidases. They may break down aromatic chemicals and polymeric dyes by oxidative processes such hydroxylation, dealkylation,and ring breakage thanks to their ligninolytic properties (Kirby, R. 2005).

3.5 Mixed microbial consortia

By combining the metabolic capacities of several microorganisms, including bacteria, fungus, and algae, mixed microbial consortia offer a synergistic approach to dye degradation (Rathour, R. K.,Sharma, D *et* al 2024). Because of their cooperative interactions that improve stability and degrading efficiency, these consortia frequently function better than pure cultures

Different microorganisms play complimentary roles in these systems. For example, bacteria can start the reduction of azo bonds, creating intermediate chemicals that are thereafter broken down by fungus or other aerobic microorganisms. Similar to how bacteria use the breakdown products for further mineralisation, algae can supply oxygen and facilitate aerobic degradation (Mikeskov,H., Novotn , Č 2012).

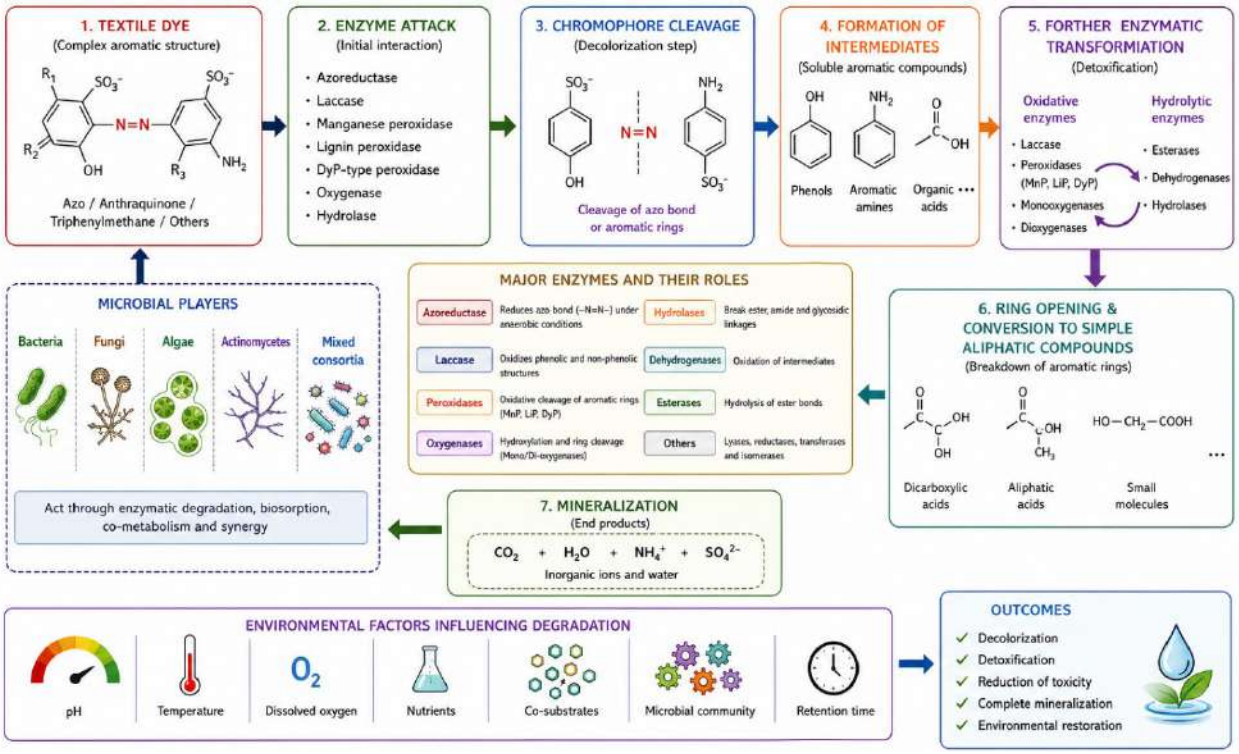


Fig 1 : Schematic Diagram elucidating microbial enzyme mediated degradation pathway

4. Plausible mechanisms of Microbial Dye Degradation:

Microbial degradation is regarded as one of the most effective and durable biologically driven remediation techniques due to the persistence of synthetic textile dyes in the environment. In order to eliminate and purify dyes from contaminated surroundings, microorganisms like bacteria, fungus, algae, and actinomycetes use a variety of techniques. These processes include, in general, biosorption, bioaccumulation, enzymatic degradation, and mineralisation. Depending on the environment, microbial species, and dye structure, these processes may take place sequentially or concurrently (Ferreira, A. C. B., Tavares, Y. V *et* al 2026; Singh, L., & Singh, V. P. 2014).

4.1 Biosorption:

Dye molecules attach to the surface of microbial biomass through a passive physicochemical process called biosorption, which does not involve any chemical change. It is mostly unrelated to metabolic

activity and happens in both living and non-living cells. Biosorption has been effected through biomass of microbes like yeast (Bustard, M., McMullan, G 1998).

Functional compounds found in microbial cell walls, such as carboxyl, hydroxyl, amino, phosphate, and sulfhydryl groups, mediate the process by interacting with dye molecules via via methods such as electrostatic attraction, ion exchange, hydrogen bonding, van der waals force (van Oss, C. J. 2020, Briandet, R., Herry, J. M 2001). The abundance of binding sites provided by chitin, chitosan, and β-glucans makes fungal biomass, especially from white-rot fungus, very efficient (Lu, Z., Kvammen, A. 2023).

4.2 Bioaccumulation

Dye molecules are carried into the microbial cell and accumulate intracellularly by an active, metabolism-dependent process called bioaccumulation.
In contrast to biosorption, bioaccumulation entails:
Transport systems that rely on energy, Changes in membrane permeability, sequestration within cells in cytoplasm or vacuoles (Dnmez, G. 2002)

Microbial metabolic activity and environmental factors including pH, temperature, and nutrient availability have an impact on this process (Dijkstra, P., Thomas, S. C *et* al 2011).

Bioaccumulation aids in the elimination of dye by lowering environmental dye concentrations and promoting intracellular enzymatic breakdown
However, excessive accumulation—especially at high dye concentrations—may result in microbial activity suppression and cell damage.

4.3 Enzymatic Degradation

The fundamental process of microbial dye remediation is enzymatic degradation, which uses enzymatic processes to break down complex dye molecules into simpler, less hazardous chemicals (Chatha, S. A. S., Asgher, M 2017)

Depending on the microbe and enzyme system, this process may take either extracellularly or intracellularly (Burns, R. G. 2012).

There are a number of enzymes that catalyzes these processes which include the following:

a) Reducing Enzymes:

Azoreductases: They work mostly in anaerobic environments, cleaving azo bonds (–N=N–) to cause decolorisation and create aromatic amines as intermediates.

b) Oxidative Enzymes:

Laccases

Lignin peroxidase (LiP)

Manganese peroxidase (MnP)

Dye-decolorizing peroxidases (DyP)

c) Oxygenases

- Monooxygenases and dioxygenases introduce oxygen into dye molecules
- Facilitate ring cleavage and further degradation

(d) Hydrolases and Dehydrogenases

- Break ester and amide bonds
- Convert intermediates into simpler compounds

4.4 Mineralization

The last phase of microbial dye breakdown is called mineralisation, during which complex organic dye compounds are entirely transformed into inorganic end products like carbon dioxide, water,ammonium ions, sulphate ions.

The process of mineralisation entails Enzymatic oxidation in sequence, total disintegration of aromatic rings, incorporation into microbial metabolic processes, such as the TCA cycle. This procedure is frequently accomplished by:

i) Multiple enzymes working together

ii) Consortiums of microbes and

iii) Anaerobic and aerobic conditions in succession.

6. Advanced Strategies

Synthetic dyes are persistent, poisonous, and recalcitrant, thus their rising discharge from the textile, paper, leather, cosmetic, and printing sectors has become a significant environmental problem. Sludge production, high operating costs, and insufficient detoxification are common problems with traditional physicochemical techniques including coagulation, adsorption, and chemical oxidation.

As a result, sophisticated microbial techniques have become viable substitutes for effective dye mineralisation and degradation. Through immobilised systems, biofilm reactors, microbial consortia, genetically modified strains, nanobiotechnology-assisted remediation, and hybrid treatment technologies combining biological and advanced oxidation processes (AOPs), recent advancements concentrate on enhancing microbial stability, degradation efficiency, resistance to toxic conditions, and scalability (Varjani, S *et* al 2020).

6.1 Immobilized microbial systems

Microbial cells or enzymes are confined onto solid matrices while retaining their catalytic activity in immobilised microbial systems. Using substances like alginate, chitosan, polyurethane foam, activated carbon, silica, and biochar, immobilisation can be accomplished by adsorption, trapping, encapsulation, covalent bonding, or cross-linking (Mehrotra, T., Dev, S *et* al 2021)

Immobilisation has a number of benefits over free-cell systems, such as greater biomass retention, increased tolerance to dangerous dye concentrations, improved microbial stability, and the ability to utilise microbial catalysts repeatedly. Because large cell densities may be maintained in bioreactors, immobilised microorganisms also show increased degrading efficiency. Immobilised bacterial and fungal systems have demonstrated improved decolorisation of azo, anthraquinone, and triphenylmethane colours in textile wastewater treatment (Ge, X., Yang, L *et* al 2017).

For oxidative dye degradation, immobilised ligninolytic enzymes such laccase, manganese peroxidase, and lignin peroxidase work particularly well (Kumar, A., & Chandra, R. 2020). Enzyme thermal stability, pH tolerance, and operational lifespan are all enhanced by immobilisation, which qualifies them for ongoing industrial applications (Yuan, Y., Shen, J *et* al 2023). The simultaneous removal of dyes, recovery of nutrients, and sequestration of carbon have drawn interest in immobilised microalgae technology.

However, issues including reduced substrate diffusion into immobilisation matrices, carrier toxicity, and mass transfer constraints still need to be optimised. These restrictions are being addressed by recent developments involving smart hydrogels and nanostructured carriers.

6.2 Biofilm Reactors

One of the most promising methods for large-scale microbial dye degradation is the use of biofilm-based reactors. Microbial populations embedded in extracellular polymeric substances (EPS) affixed to support surfaces make up biofilms. These organised microbial assemblies offer defence against hazardous substances, hydraulic changes, and environmental stress (Donkadokula, N. Y., Kola, A. K 2020).

When compared to suspended microbial cultures, biofilm reactors provide a number of advantages: i) Increased retention of biomass ii) Improved metabolic collaboration iii) Enhanced effectiveness of degrading iv) decreased generation of sludge v) Increased resistance to shock dye loading (Cheng, K. C., Demirci, A 2010).

The ability of EPS matrices to adsorb dye molecules increases the availability of substrate for microbial breakdown. Close microbial proximity also promotes interspecies electron transfer and synergistic interactions, which enhance degradation pathways (Donkadokula, N. Y., Kola, A. K *et* al 2020).

For successive anaerobic–aerobic dye degradation, biofilm systems are especially helpful. Aerobic zones enable the oxidative destruction of aromatic amines through oxygenases and peroxidases, whereas anaerobic zones encourage azo bond cleavage via azoreductases. Detoxification and decolorisation are both improved by such compartmentalised metabolism.

6.3 Microbial Consortia

In order to accomplish effective dye degradation, several microbial species work together in microbial consortia. Consortia show more metabolic diversity, robustness, and adaptability to changing environmental conditions than pure cultures (Cao, Z., Yan, W 2022). Within the consortium, many microorganisms carry out complimentary functions. For instance: Aerobic bacteria oxidise aromatic intermediates, while anaerobic bacteria decrease azo bonds. iii) Algae

provide oxygen and absorb contaminants, iv) Fungi break down complex aromatic structures (Cui, D., Li, G 2012).

The progressive breakdown and full mineralisation of complex dyes are made possible by this synergistic interaction. When dealing with industrial effluents that comprise several dye classes and co-contaminants, mixed consortia are very successful.

Advanced research has demonstrated the function of:i) Quorum sensing ii) Extracellular polymeric materials iv) Metabolic cross-feeding iii) Interspecies electron transfer (IET) in enhancing degrading performance and consortium stability (Fritts, R. K., McCully, A. L 2021).

In continuous-flow reactors, immobilised microbial consortia and biofilm-based consortia further improve degrading efficiency and operational stability (Thayyil, M. I., & Philip, L. 2024). Nonetheless, limiting competitive exclusion and preserving a stable community composition continue to be major obstacles.

6.4 Genetically Engineered Strains

New avenues for improving microbial dye degrading capabilities have been made possible via genetic engineering. The purpose of genetically modified microbes (GEMs) is to increase substrate specificity, survive hazardous conditions, and overexpress enzymes that break down dyes (Tripathi, M., Singh, S 2023, Bustamante-Torres, M., Torres, O. *et* al 2024).

Among the crucial genetic engineering techniques are:

Azoreductase gene overexpression ii) Production of recombinant laccase iii) Metabolic engineering with CRISPR iv) Creation of synthetic pathways v) Degradative enzyme evolution under guidance (Abbas, A., Mushtaq, A *et* al 2020, Dey, S., & Mandal, B. 2026)

For instance, recombinant *Escherichia coli* that expressed *Klebsiella pneumoniae* azoreductase genes showed quick azo dye degradation (Dixit, S., & Garg, S. 2021). In a similar vein, modified fungal laccases show improved thermal stability and catalytic effectiveness (Asgher, M., Kamal, S., & Iqbal, H. M. N. 2012).

6.4 *Nanobiotechnology-Assisted Remediation*

To increase the effectiveness of dye remediation, nanobiotechnology combines nanomaterials with microbial or enzymatic systems. High surface area, catalytic activity, better electron transport, and increased enzyme immobilisation are all provided by nanoparticles.

Common nanomaterials used for nanobiotechnology assisted remediation include a) Metal oxide nanoparticles nanoparticles (Nagajyothi, P. C., Prabhakar Vattikuti, S. V. 2020) b)Metal sulphide nanoparticles (Lianmawii, L., Singh, K. B.*et* al 2025) c) Quantum dots (Velumani, A., Sengodan, P. *et al* 2020) d) Polymeric Nanoparticles (Sarkar, S., Ponce, N. T., Banerjee *et* al 2020) e) Biochar-Nanocomposite (Amdeha, E. (2024).

By improving cell immobilisation, enzyme stability, redox processes, dye molecule adsorption, and electron transfer route facilitation, nanoparticles can accelerate microbial breakdown.

Owing to their its simplicity to recover and repurpose microbial catalysts or enzymes, magnetic nanocomposites are particularly valuable. Laccase immobilisation with nanoparticle assistance has demonstrated markedly increased operating stability and decolorisation efficiency (Bilal, M., Asgher, M. *et* al 2017)

Smart hydrogels, nano-bio hybrid reactors, and AI-guided nanobioremediation systems that maximise pollutant breakdown in real time are examples of recent advancements. However, prior to broad use, nanoparticle toxicity, environmental persistence, and ecological effects must be carefully evaluated (Zou, Z., Li, M., *et* al 2026)

6.5 Hybrid systems combining biological and advanced oxidation processes

One of the best methods for remediating textile dyes is the use of hybrid treatment systems that combine advanced oxidation processes (AOPs) with microbial degradation. While AOPs produce reactive radicals that can break down resistant substances, biological systems are more sustainable but may experience incomplete degradation. The advantages of both strategies are combined in their integration (Santana, R. M. D. R., Charamba, L. C. V 2019). The most widely used Advanced Oxidation processes include a) Fenton oxidation (Punzi, M., Anbalagan, A *et* al 2015) b) Photocatalysis (Chan, S. H. S., Yeong Wu *et* al 2011) c) Ozonation (Tehrani-Bagha, A. R., Mahmoodi, N. M *et* al 2011) d) UV/H2O2 therapy e) Oxidation by electrochemistry. The Advanced Oxidation processes may operate through AOP pretreatment followed by biodegradation, Biological pretreatment followed by AOP polishing, Simultaneous integrated systems.

By disassembling big dye molecules into smaller intermediates that microorganisms can access, AOP pretreatment increases biodegradability. On the other hand, biological treatment minimises chemical use and operating costs by lowering the organic load prior to advanced oxidation (Rostam, A. B., & Taghizadeh, M 2020).

Hybrid systems based on microbial fuel cells (MFCs) have become extremely inventive methods that allow for the simultaneous breakdown of dyes and the production of power. In order to produce bioelectricity and improve pollution removal, these systems make use of electroactive microorganisms that transfer electrons during dye degradation (Oon, Y. S., Ong, S. A. 2018).

7. Challenges and Limitations

Since the textile industry uses a lot of synthetic dyes and auxiliary chemicals throughout the dyeing and finishing processes, it is one of the biggest sources of industrial water pollution. Structurally varied colours, including azo, anthraquinone, triphenylmethane, sulphur, and reactive dyes, are found in textile effluents. Many of these dyes are poisonous, mutagenic, and resistant to degradation. However, a number of operational and technical constraints prevent the widespread use of microbial dye degrading methods, despite their great effectiveness in the lab. Toxic intermediate production, scale-up issues, industrial effluent composition fluctuation, low enzyme stability, and reactor-related constraints are some of the main obstacles.

7.1 Toxic Intermediates

The production of hazardous intermediate chemicals during inadequate biodegradation is one of the main issues with microbial dye degradation. Under anaerobic conditions, microbial azoreductases reductively cleave azo bonds (–N=N–) in many textile dyes, particularly azo dyes. Aromatic amines like benzidine, aniline, toluidine, and naphthylamine derivatives are frequently formed during this process, despite the fact that it causes rapid decolorisation. These substances pose major risks to aquatic life and human health because they are extremely poisonous, mutagenic, and carcinogenic.

During partial decomposition, anthraquinone and triphenylmethane dyes may also generate reactive oxygen species, phenolic chemicals, and poisonous quinones. These intermediates have the potential to increase ecological toxicity, decrease enzymatic efficiency, and impede microbial metabolism.

Because hazardous metabolites may still be present in treated wastewater, colour removal frequently does not always signify full purification (Sen, S. K., Raut, S *et* al 2023).

To get around this restriction, sequential anaerobic–aerobic treatment systems are frequently used. While aerobic microbes oxidise aromatic amines into simpler molecules, anaerobic microorganisms decrease azo linkages. However, because of the structural intricacy and resistance of colour intermediates, full mineralisation is still challenging. As a result, toxicity tests, metabolomic profiling, and ecotoxicological analysis are becoming more and more important in contemporary remediation studies to evaluate the safety of degradation products (Mishra, A., Takkar, S., *et* al 2022).

7.2. Scale up Issues:

Even though microbial dye degradation has demonstrated exceptional efficiency in the lab, it is still very difficult to use these discoveries in industrial settings. The majority of research is carried out in controlled environments with optimised pH, temperature, aeration, nutrition availability, and synthetic dye solutions (Panswad, T., & Luangdilok, W. (2000), Ali, H. (2010). However, industrial wastewater systems are even more intricate and challenging to control.

Because of environmental stress and competition from native microbial communities, microorganisms that function well in lab flasks may lose their capacity to degrade under industrial conditions. Additionally, scale-up modifies mass transfer characteristics, enzyme kinetics, and microbial population dynamics, all of which have a substantial impact on treatment efficacy (Mehrotra, T., Dev, S. *et* al 2021)

Scale-up is further complicated by economic factors. Aeration systems, nitrogen supplementation, large-scale microbial biomass culture, and bioreactor maintenance can all significantly raise operating expenses. Furthermore, biological systems are less appealing to enterprises that need to manage wastewater quickly since they often require longer treatment timeframes than sophisticated oxidation or physicochemical techniques (Rostam, A. B., & Taghizadeh, M. 2020).

8. Conclusion & Future Directives

The development of novel and sustainable bioremediation techniques has increased due to the increasing environmental impact of textile dye pollution. Future developments in microbial dye degradation will rely on incorporating cutting-edge technologies like synthetic biology, metagenomics, artificial intelligence (AI)-guided bioprocess optimisation, and circular bioeconomy

frameworks, even though the process has shown significant promise in laboratory and pilot-scale investigations. It is anticipated that these multidisciplinary methods will improve process stability, increase degrading efficiency, and make large-scale industrial application easier.

Synthetic biology, which allows for the logical engineering of microbes with improved biodegradation capabilities, is one of the most promising fields. Microbial strains can be engineered to overexpress dye-degrading enzymes such as azoreductases, laccases, lignin peroxidases, manganese peroxidases, and dye-decolorizing peroxidases (DyPs) using genome editing techniques like CRISPR-Cas systems and metabolic pathway engineering (Nath, S. 2026). Additionally, synthetic biology makes it easier to create engineered microbial consortia that can carry out successive degrading processes, reducing the build-up of hazardous intermediates and encouraging full mineralisation (Che, S., & Men, Y. 2019). Additionally, synthetic gene circuits can be created to control the expression of enzymes in response to particular contaminants, increasing the effectiveness of degradation and energy use.

Another revolutionary technique that is changing environmental biotechnology is metagenomics. While metagenomic methods enable thorough characterisation of microbial communities directly from polluted settings, traditional culture-based methods only access a tiny portion of ambient microbial diversity. Functional metagenomics and high-throughput sequencing have made it easier to find previously unidentified dye-degrading microbes, enzymes, and metabolic pathways. Strong microbial consortia tailored to industrial wastewater conditions can be created using this information. Additionally, metatranscriptomic and metaproteomic analyses offer information on microbial interactions and active degradation pathways, allowing for more focused bioremediation techniques (Sahoo, P. P., Sahoo, A. A. 2026).

Recent advances in bioprocess optimisation guided by artificial intelligence (AI) have substantial prospects for enhancing microbial dye degradation systems. To determine the ideal operating settings, AI and machine learning algorithms can examine sizable datasets pertaining to microbial growth, enzyme kinetics, wastewater composition, and reactor performance. Predictive models can help with reactor setup optimisation, degradation outcome forecasting, and the selection of effective microbial strains (Singh, K., Sharma, S., Pathak, S *et* al 2025). Automated regulation of pH, temperature, aeration, nutrient delivery, and hydraulic retention time can be made possible by integrating AI with real-time monitoring systems and Internet of Things (IoT)-based sensors (Jin, J., Liu, M 2025). This

will increase treatment efficiency while lowering operating costs. The shift from laboratory-scale research to industrial applications is anticipated to be greatly aided by such intelligent bioprocesses.

Conflict of Interest:

The authors declare that they have no competing interest.

References

Abbas, A., Mushtaq, A., Cheema, A. I., Mahmood, F., Khan, M. A., Naqqash, T., ... & Shahid, M. (2020). Heterologous expression of azoreductase-encoding gene azrS of Bacillus sp. MR-1/2 for enhanced azo dye decolorization and wastewater treatment. *Archives of Microbiology*, *202*(8), 2135-2145.

Akter, T., Protity, A. T., Shaha, M., Al Mamun, M., & Hashem, A. (2023). The impact of textile dyes on the environment. In *Nanohybrid materials for treatment of textiles dyes* (pp. 401-431). Singapore: Springer Nature Singapore.

Alderete, B. L., da Silva, J., Godoi, R., da Silva, F. R., Taffarel, S. R., da Silva, L. P., ... & Picada, J. N. (2021). Evaluation of toxicity and mutagenicity of a synthetic effluent containing azo dye after advanced oxidation process treatment. *Chemosphere*, *263*, 128291.

Ali, H. (2010). Biodegradation of synthetic dyes—a review. *Water, Air, & Soil Pollution*, *213*(1), 251-273.

Al-Tohamy, R., Ali, S. S., Li, F., Okasha, K. M., Mahmoud, Y. A. G., Elsamahy, T., ... & Sun, J. (2022). A critical review on the treatment of dye-containing wastewater: Ecotoxicological and health concerns of textile dyes and possible remediation approaches for environmental safety. *Ecotoxicology and environmental safety*, *231*, 113160.

Amdeha, E. (2024). Biochar-based nanocomposites for industrial wastewater treatment via adsorption and photocatalytic degradation and the parameters affecting these processes. *Biomass Conversion and Biorefinery*, *14*(19), 23293-23318.

Asgher, M., Kamal, S., & Iqbal, H. M. N. (2012). Improvement of catalytic efficiency, thermo-stability and dye decolorization capability of Pleurotus ostreatus IBL-02 laccase by hydrophobic sol gel entrapment. *Chemistry Central Journal*, *6*(1), 110.

Aslam, A., Kanwal, F., Javied, S., Nisar, N., & Torriero, A. A. J. (2025). Microbial biosorption: a sustainable approach for metal removal and environmental remediation. *International Journal of Environmental Science and Technology*, *22*(13), 13245-13276.

Azmi, W., Sani, R. K., & Banerjee, U. C. (1998). Biodegradation of triphenylmethane dyes. *Enzyme and microbial technology*, *22*(3), 185-191.

Barik, D., Rakhi Mol, K. M., Anand, G., Nandamol, P. S., Das, D., & Porel, M. (2025). Environmental pollutants such as endocrine disruptors/pesticides/reactive dyes and inorganic toxic compounds metals, radionuclides, and metalloids and their impact on the ecosystem. In *Biotechnology for Environmental Sustainability* (pp. 391-442). Singapore: Springer Nature Singapore.

Bera, S., Chowdhury, D., Han, J., & Singh, J. (2023). Applied and sustainable methods to manage environmental contaminants with natural and fortified microbial biosorbents. *Frontiers in Microbiology*, *14*, 1220337.

Berradi, M., Hsissou, R., Khudhair, M., Assouag, M., Cherkaoui, O., El Bachiri, A., & El Harfi, A. (2019). Textile finishing dyes and their impact on aquatic environs. *Heliyon*, *5*(11).

Bilal, M., Asgher, M., Parra-Saldivar, R., Hu, H., Wang, W., Zhang, X., & Iqbal, H. M. (2017). Immobilized ligninolytic enzymes: an innovative and environmental responsive technology to tackle dye-based industrial pollutants–a review. *Science of the Total Environment*, *576*, 646-659.

Briandet, R., Herry, J. M., & Bellon-Fontaine, M. N. (2001). Determination of the van der Waals, electron donor and electron acceptor surface tension components of static Gram-positive microbial biofilms. *Colloids and Surfaces B: Biointerfaces*, *21*(4), 299-310.

Burns, R. G. (2012). Microbial extracellular enzymes and the degradation of natural and synthetic polymers in soil. In *Molecular environmental soil science* (pp. 27-47). Dordrecht: Springer Netherlands.

Bustamante-Torres, M., Torres, O., Abad-Sojos, S., Pardo, S., & Bucio, E. (2024). Application of genetically modified microorganisms for bioremediation of polluted environments. In *Genetically engineered organisms in bioremediation* (pp. 18-51). CRC Press.

Bustard, M., McMullan, G., & McHale, A. P. (1998). Biosorption of textile dyes by biomass derived from Kluyveromyces marxianus IMB3. *Bioprocess Engineering*, *19*(6), 427-430.

Cao, Z., Yan, W., Ding, M., & Yuan, Y. (2022). Construction of microbial consortia for microbial degradation of complex compounds. *Frontiers in Bioengineering and Biotechnology*, *10*, 1051233.

Chan, S. H. S., Yeong Wu, T., Juan, J. C., & Teh, C. Y. (2011). Recent developments of metal oxide semiconductors as photocatalysts in advanced oxidation processes (AOPs) for treatment of dye waste-water. *Journal of Chemical Technology & Biotechnology*, *86*(9), 1130-1158.

Chatha, S. A. S., Asgher, M., & Iqbal, H. M. (2017). Enzyme-based solutions for textile processing and dye contaminant biodegradation—a review. *Environmental Science and Pollution Research*, *24*(16), 14005-14018.

Che, S., & Men, Y. (2019). Synthetic microbial consortia for biosynthesis and biodegradation: promises and challenges. *Journal of Industrial Microbiology and Biotechnology*, *46*(9-10), 1343-1358.

Chen, A., Yang, B., Zhou, Y., Sun, Y., & Ding, C. (2018). Effects of azo dye on simultaneous biological removal of azo dye and nutrients in wastewater. *Royal Society Open Science*, *5*(8).

Cheng, K. C., Demirci, A., & Catchmark, J. M. (2010). Advances in biofilm reactors for production of value-added products. *Applied microbiology and biotechnology*, *87*(2), 445-456.

Chetyrkina, M. R., Talalaev, F. S., Kameneva, L. V., Kostyuk, S. V., & Troshin, P. A. (2022). Vat dyes: promising biocompatible organic semiconductors for wearable electronics applications. *Journal of Materials Chemistry C*, *10*(8), 3224-3231.

Chowdhary, P., Shukla, G., Raj, G., Ferreira, L. F. R., & Bharagava, R. N. (2019). Microbial manganese peroxidase: a ligninolytic enzyme and its ample opportunities in research. *SN Applied Sciences*, *1*(1), 45.

Chowdhury, A. P., Anantharaju, K. S., Keshavamurthy, K., & Rokhum, S. L. (2023). Recent advances in efficient photocatalytic degradation approaches for azo dyes. *Journal of Chemistry*, *2023*(1), 9780955.

Cui, D., Li, G., Zhao, D., Gu, X., Wang, C., & Zhao, M. (2012). Microbial community structures in mixed bacterial consortia for azo dye treatment under aerobic and anaerobic conditions. *Journal of hazardous materials*, *221*, 185-192.

Das, S., Cherwoo, L., & Singh, R. (2023). Decoding dye degradation: Microbial remediation of textile industry effluents. *Biotechnology notes*, *4*, 64-76.

Debroy, A., Yadav, M., Dhawan, R., Dey, S., & George, N. (2022). DNA dyes: toxicity, remediation strategies and alternatives. *Folia microbiologica*, *67*(4), 555-571.

Dey, S., & Mandal, B. (2026). Review on Microbial Potential for Dye Remediation. *Microbiology*, *95*(2), 147-183.

Dijkstra, P., Thomas, S. C., Heinrich, P. L., Koch, G. W., Schwartz, E., & Hungate, B. A. (2011). Effect of temperature on metabolic activity of intact microbial communities: evidence for altered metabolic pathway activity but not for increased maintenance respiration and reduced carbon use efficiency. *Soil Biology and Biochemistry*, *43*(10), 2023-2031.

Dixit, S., & Garg, S. (2021). Enzymatic degradation of sulphonated azo dye using purified azoreductase from facultative Klebsiella pneumoniae. *Folia Microbiologica*, *66*(1), 79-85.

Donkadokula, N. Y., Kola, A. K., Naz, I., & Saroj, D. (2020). A review on advanced physico-chemical and biological textile dye wastewater treatment techniques. *Reviews in environmental science and bio/technology*, *19*(3), 543-560.

Dnmez, G. (2002). Bioaccumulation of the reactive textile dyes by Candida tropicalis growing in molasses medium. *Enzyme and Microbial Technology*, *30*(3), 363-366.

Dutta, S., Adhikary, S., Bhattacharya, S., Roy, D., Chatterjee, S., Chakraborty, A., ... & Rajak, P. (2024). Contamination of textile dyes in aquatic environment: Adverse impacts on aquatic ecosystem and human health, and its management using bioremediation. *Journal of Environmental Management*, *353*, 120103.

Dutta, S., Adhikary, S., Bhattacharya, S., Roy, D., Chatterjee, S., Chakraborty, A., ... & Rajak, P. (2024). Contamination of textile dyes in aquatic environment: Adverse impacts on aquatic ecosystem and human health, and its management using bioremediation. *Journal of Environmental Management*, *353*, 120103.

Fazal, T., Rehman, M. S. U., Javed, F., Akhtar, M., Mushtaq, A., Hafeez, A., ... & Rehman, F. (2021). Integrating bioremediation of textile wastewater with biodiesel production using microalgae (Chlorella vulgaris). *Chemosphere*, *281*, 130758.

Ferreira, A. C. B., Tavares, Y. V., Fontana, N. R., Machado Pasin, T., Conte-Junior, C. A., & Contato, A. G. (2026). Bioremediation of Synthetic Dyes by White-Rot Fungi: Enzymatic Mechanisms, Biosorption, and Environmental Applications. *Molecules*, *31*(7), 1085.

Ferreira, A. C. B., Tavares, Y. V., Fontana, N. R., Machado Pasin, T., Conte-Junior, C. A., & Contato, A. G. (2026). Bioremediation of Synthetic Dyes by White-Rot Fungi: Enzymatic Mechanisms, Biosorption, and Environmental Applications. *Molecules*, *31*(7), 1085.

Fouillaud, M., Caro, Y., Venkatachalam, M., Grondin, I., & Dufoss, L. (2018). Anthraquinones. In *Phenolic compounds in food* (pp. 131-172). CRC Press.

Fritts, R. K., McCully, A. L., & McKinlay, J. B. (2021). Extracellular metabolism sets the table for microbial cross-feeding. *Microbiology and Molecular Biology Reviews*, *85*(1), 10-1128.

Gallert, C., & Winter, J. (2005). Bacterial metabolism in wastewater treatment systems. *Environmental Biotechnology. Concepts and Applications*.

Ge, X., Yang, L., & Xu, J. (2017). Cell immobilization: fundamentals, technologies, and applications. *Industrial biotechnology: products and processes*, 205-235.

Gheni, S. A., Ali, M. M., Ta, G. C., Harbin, H. J., & Awad, S. A. (2023). Toxicity, hazards, and safe handling of primary aromatic amines. *ACS Chemical Health & Safety*, *31*(1), 8-21.

Gičević, A., Hindija, L., & Karačić, A. (2019, May). Toxicity of azo dyes in pharmaceutical industry. In *International Conference on Medical and Biological Engineering* (pp. 581-587). Cham: Springer International Publishing.

Goswami, D., Mukherjee, J., Mondal, C., & Bhunia, B. (2024). Bioremediation of azo dye: A review on strategies, toxicity assessment, mechanisms, bottlenecks and prospects. *Science of The Total Environment*, *954*, 176426.

Goswami, D., Mukherjee, J., Mondal, C., & Bhunia, B. (2024). Bioremediation of azo dye: A review on strategies, toxicity assessment, mechanisms, bottlenecks and prospects. Science of The Total Environment, 954, 176426.

Goswami, D., Mukherjee, J., Mondal, C., & Bhunia, B. (2024). Bioremediation of azo dye: A review on strategies, toxicity assessment, mechanisms, bottlenecks and prospects. *Science of The Total Environment*, *954*, 176426.

Hasanbeigi, A., & Price, L. (2015). A technical review of emerging technologies for energy and water efficiency and pollution reduction in the textile industry. *Journal of Cleaner Production*, *95*, 30-44.

Hashemi, S. H., & Kaykhaii, M. (2022). Azo dyes: sources, occurrence, toxicity, sampling, analysis, and their removal methods. In *Emerging freshwater pollutants* (pp. 267-287). Elsevier.

Holmes, B., Paddock, M. B., VanderGheynst, J. S., & Higgins, B. T. (2020). Algal photosynthetic aeration increases the capacity of bacteria to degrade organics in wastewater. *Biotechnology and bioengineering*, *117*(1), 62-72.

Hood, R. D., Jones, C. L., & Ranganathan, S. (1989). Comparative developmental toxicity of cationic and neutral rhodamines in mice. *Teratology*, *40*(2), 143-150.

Jaramillo-Lanchero, R. D., Suarez-Alvarez, P., & Teheran-Sierra, L. (2021). Effect of respiratory inhibitors and quinone analogues on the aerobic electron transport system of Eikenella corrodens. *Scientific reports*, *11*(1), 8987.

Jasińska, A., Paraszkiewicz, K., Słaba, M., & Długoński, J. (2014). Microbial decolorization of triphenylmethane dyes. In *Microbial degradation of synthetic dyes in wastewaters* (pp. 169-186). Cham: Springer International Publishing.

Jiang, Q., Chen, S., Deng, X., Feng, Y., Reddy, N., Zhu, Q., ... & Qiu, Y. (2019). A sustainable low temperature yarn reinforcing process to reduce water and energy consumptions and pollution in the textile industry. *Journal of Cleaner Production*, *210*, 646-652.

Jin, J., Liu, M., Chen, B., Wu, X., Yao, L., Wang, Y., ... & Yang, N. (2025). Artificial Intelligence in Chemical Dosing for Wastewater Purification and Treatment: Current Trends and Future Perspectives. *Separations*, *12*(9), 237.

Joseph, J., Sharma, S., & Dave, V. P. (2018). Filamentous gram-negative bacteria masquerading as actinomycetes in infectious endophthalmitis: a review of three cases. *Journal of ophthalmic inflammation and infection*, *8*(1), 15.

Kaim, W., & Lahiri, G. K. (2019). The coordination potential of indigo, anthraquinone and related redox-active dyes. *Coordination Chemistry Reviews*, *393*, 1-8.

Kaur, G., & Sood, P. (2025). Significance of biological approaches/bioremediation of wastewater treatment over physicochemical methods: a comparative analysis. In *Biotechnologies for Wastewater Treatment and Resource Recovery* (pp. 211-225). Elsevier.

Kirby, R. (2005). Actinomycetes and lignin degradation. *Advances in applied microbiology*, *58*, 125-168.

Kishor, R., Purchase, D., Saratale, G. D., Saratale, R. G., Ferreira, L. F. R., Bilal, M., ... & Bharagava, R. N. (2021). Ecotoxicological and health concerns of persistent coloring pollutants of textile industry wastewater and treatment approaches for environmental safety. *Journal of Environmental Chemical Engineering*, *9*(2), 105012.

Kontro, M. H., Yaradoddi, J. S., Banapurmath, N. R., Ganachari, S. V., & Hungund, B. S. (2022). Biotechnological importance of Actinomycetes. In *Actinobacteria: Ecology, Diversity, Classification and Extensive Applications* (pp. 271-290). Singapore: Springer Nature Singapore.

Kovacic, P., & Somanathan, R. (2014). Toxicity of imine–iminium dyes and pigments: electron transfer, radicals, oxidative stress and other physiological effects. *Journal of Applied Toxicology*, *34*(8), 825-834.

Kumar, A., & Chandra, R. (2020). Ligninolytic enzymes and its mechanisms for degradation of lignocellulosic waste in environment. *Heliyon*, *6*(2).

Kumar, M., Mishra, A., Patel, S. K., Kushwaha, J., Singh, S., Mishra, V., ... & Singh, D. (2025). Environmental impacts and strategies for bioremediation of Dye-Containing wastewater. *Bioengineering*, *12*(10), 1043.

Kumar, P., & Singh, J. (2025). Perspective and challenges of synergistic removal of toxic contaminants from effluent using different treatment techniques. In *Microbial niche nexus sustaining environmental biological wastewater and water-energy-environment nexus* (pp. 419-451). Cham: Springer Nature Switzerland.

Kuppan, N., Padman, M., Mahadeva, M., Srinivasan, S., & Devarajan, R. (2024). A comprehensive review of sustainable bioremediation techniques: Eco friendly solutions for waste and pollution management. *Waste management bulletin*, *2*(3), 154-171.

Lianmawii, L., Singh, K. B., Singh, N. R., & Singh, N. M. (2025). A review: photocatalytic degradation of dyes by metal sulfide nanoparticles. *Brazilian Journal of Chemical Engineering*, *42*(1), 1-30.

Liu, Y., Chen, J., Duan, D., Zhang, Z., Liu, C., Cai, W., & Zhao, Z. (2024). Environmental impacts and biological technologies toward sustainable treatment of textile dyeing wastewater: a review. *Sustainability*, *16*(24), 10867.

Lu, Z., Kvammen, A., Li, H., Hao, M., Inman, A. R., Bulone, V., & McKee, L. S. (2023). A polysaccharide utilization locus from Chitinophaga pinensis simultaneously targets chitin and β-glucans found in fungal cell walls. *Msphere*, *8*(4), e00244-23.

Mahmood, S., Khalid, A., Arshad, M., Mahmood, T., & Crowley, D. E. (2016). Detoxification of azo dyes by bacterial oxidoreductase enzymes. Critical reviews in biotechnology, 36(4), 639-651.

Majumdar, R., Shaikh, W. A., Chakraborty, S., & Chowdhury, S. (2022). A review on microbial potential of toxic azo dyes bioremediation in aquatic system. *Microbial Biodegradation and Bioremediation*, 241-261.

Malik, S., Dhasmana, A., Preetam, S., Mishra, Y. K., Chaudhary, V., Bera, S. P., ... & Rajput, V. D. (2022). Exploring microbial-based green nanobiotechnology for wastewater remediation: a sustainable strategy. *Nanomaterials*, *12*(23), 4187.

Mehrotra, T., Dev, S., Banerjee, A., Chatterjee, A., Singh, R., & Aggarwal, S. (2021). Use of immobilized bacteria for environmental bioremediation: A review. *Journal of Environmental Chemical Engineering*, *9*(5), 105920.

Mehrotra, T., Dev, S., Banerjee, A., Chatterjee, A., Singh, R., & Aggarwal, S. (2021). Use of immobilized bacteria for environmental bioremediation: A review. *Journal of Environmental Chemical Engineering*, *9*(5), 105920.

Mikesková, H., Novotný, Č., & Svobodová, A. K. (2012). Interspecific interactions in mixed microbial cultures in a biodegradation perspective. *Applied microbiology and biotechnology*, *95*(4), 861-870.

Mishra, A., Takkar, S., Joshi, N. C., Shukla, S., Shukla, K., Singh, A., ... & Varma, A. (2022). An integrative approach to study bacterial enzymatic degradation of toxic dyes. *Frontiers in Microbiology*, *12*, 802544.

Mohanty, S. S., & Kumar, A. (2021). Enhanced degradation of anthraquinone dyes by microbial monoculture and developed consortium through the production of specific enzymes. *Scientific reports*, *11*(1), 7678.

Motallebirad, T., Tashakor, A., Abniki, R., & Azadi, D. (2024). Fifteen years of phenotypic and genotypic surveillance and antibiotic susceptibility pattern of Actinomycetes (Mycobacterium, Nocardia, Rhodococcus, etc.) in clinical and environmental samples of Iran. *Diagnostic microbiology and infectious disease*, *108*(1), 116080.

Nagajyothi, P. C., Prabhakar Vattikuti, S. V., Devarayapalli, K. C., Yoo, K., Shim, J., & Sreekanth, T. V. M. (2020). Green synthesis: photocatalytic degradation of textile dyes using metal and metal oxide nanoparticles-latest trends and advancements. *Critical Reviews in Environmental Science and Technology*, *50*(24), 2617-2723.

Nanjani, S., & Keharia, H. K. (2021). Alterations in microbial community structure and function in response to azo dyes. In *Microbiome-host interactions* (pp. 367-395). CRC Press.

Nath, S. (2026). Advanced microbial engineering approaches for biodegradation of pharmaceutical pollutants. *Biodegradation*, *37*(1), 15.

Naveenraj, S., Solomon, R. V., Venuvanalingam, P., Asiri, A. M., & Anandan, S. (2013). Interaction between toxic azo dye CI Acid Red 88 and serum albumins. *Journal of luminescence*, *143*, 715-722.

Omidoyin, K. C., & Fatade-Ogunseye, O. O. (2025). Streptomycetes: A promising biological tool for bioremediation of environmental contaminants. In *Bioeconomy of Streptomyces* (pp. 142-164). CRC Press.

Oon, Y. S., Ong, S. A., Ho, L. N., Wong, Y. S., Oon, Y. L., Lehl, H. K., ... & Nordin, N. (2018). Disclosing the synergistic mechanisms of azo dye degradation and bioelectricity generation in a microbial fuel cell. *Chemical Engineering Journal*, *344*, 236-245.

Panswad, T., & Luangdilok, W. (2000). Decolorization of reactive dyes with different molecular structures under different environmental conditions. *Water Research*, *34*(17), 4177-4184.

Patel, A., Patel, V., Patel, H., Trivedi, U., & Patel, K. (2020). White rot fungi: Nature's scavenger. In *Microbial Bioremediation & Biodegradation* (pp. 267-307). Singapore: Springer Singapore.

Poopal, R. K., Ashwini, R., Ramesh, M., Li, B., & Ren, Z. (2023). Triphenylmethane dye (C52H54N4O12) is potentially a hazardous substance in edible freshwater fish at trace level: toxicity, hematology, biochemistry, antioxidants, and molecular docking evaluation study. *Environmental Science and Pollution Research*, *30*(11), 28759-28779.

Prasad, M. N. V. (2024). Bioremediation, bioeconomy, circular economy, and circular bioeconomy—Strategies for sustainability. In *Bioremediation and bioeconomy* (pp. 3-32). Elsevier.

Przystaś, W., Zabłocka-Godlewska, E., & Grabińska-Sota, E. (2012). Biological removal of azo and triphenylmethane dyes and toxicity of process by-products. *Water, Air, & Soil Pollution*, *223*(4), 1581-1592.

Punzi, M., Anbalagan, A., Brner , R. A., Svensson, B. M., Jonstrup, M., & Mattiasson, B. (2015). Degradation of a textile azo dye using biological treatment followed by photo-Fenton oxidation: evaluation of toxicity and microbial community structure. *Chemical engineering journal*, *270*, 290-299.

Ramamurthy, K., Priya, P. S., Murugan, R., & Arockiaraj, J. (2024). Hues of risk: investigating genotoxicity and environmental impacts of azo textile dyes. *Environmental Science and Pollution Research*, *31*(23), 33190-33211.

Ramamurthy, K., Priya, P. S., Murugan, R., & Arockiaraj, J. (2024). Hues of risk: investigating genotoxicity and environmental impacts of azo textile dyes. *Environmental Science and Pollution Research*, *31*(23), 33190-33211.

Rathour, R. K., Sharma, D., Ullah, S., Mahmoud, E. H. M., Sharma, N., Kumar, P., ... & Bhatia, R. K. (2024). Bacterial–microalgal consortia for bioremediation of textile industry wastewater and resource recovery for circular economy. *Biotechnology for the Environment*, *1*(1), 6.

Rawat, D., Sharma, R. S., Karmakar, S., Arora, L. S., & Mishra, V. (2018). Ecotoxic potential of a presumably non-toxic azo dye. *Ecotoxicology and Environmental Safety*, *148*, 528-537.

Reza, M. S. A., Hasan, M. M., Kamruzzaman, M., Hossain, M. I., Zubair, M. A., Bari, L., ... & Hossain, M. K. (2019). Study of a common azo food dye in mice model: Toxicity reports and its relation to carcinogenicity. *Food science & nutrition*, *7*(2), 667-677.

Rostam, A. B., & Taghizadeh, M. (2020). Advanced oxidation processes integrated by membrane reactors and bioreactors for various wastewater treatments: A critical review. *Journal of Environmental Chemical Engineering*, *8*(6), 104566.

Rostam, A. B., & Taghizadeh, M. (2020). Advanced oxidation processes integrated by membrane reactors and bioreactors for various wastewater treatments: A critical review. *Journal of Environmental Chemical Engineering*, *8*(6), 104566.

Sahoo, P. P., Sahoo, A. A., Panda, A. K., Sen, S. K., & Raut, S. (2026). Translating metagenomic knowledge into effective dye bioremediation for wastewater treatment. *Discover Environment*, *4*(1), 247.

Santana, R. M. D. R., Charamba, L. C. V., do Nascimento, G. E., de Oliveira, J. G. C., Sales, D. C. S., Duarte, M. M. M. B., & Napoleo, D. C. (2019). Degradation of textile dyes employing advanced oxidative processes: kinetic, equilibrium modeling, and toxicity study of seeds and bacteria. *Water, Air, & Soil Pollution*, *230*(6), 136.

Sarkar, S., Ponce, N. T., Banerjee, A., Bandopadhyay, R., Rajendran, S., & Lichtfouse, E. (2020). Green polymeric nanomaterials for the photocatalytic degradation of dyes: a review. *Environmental Chemistry Letters*, *18*(5), 1569-1580.

Sen, S. K., Raut, S., & Raut, S. (2023). Mycoremediation of anthraquinone dyes from textile industries: a mini-review. *BioTechnologia*, *104*(1), 85.

Sen, S. K., Raut, S., & Raut, S. (2023). Mycoremediation of anthraquinone dyes from textile industries: a mini-review. *BioTechnologia*, *104*(1), 85.

Sharma, S., Pathania, S., Bhagta, S., Kaushal, N., Bhardwaj, S., Bhatia, R. K., & Walia, A. (2024). Microbial remediation of polluted environment by using recombinant E. coli: a review. *Biotechnology for the Environment*, *1*(1), 8.

Singh, K., Sharma, S., Pathak, S., Raghuvanshi, S., Singh, M., Mittal, P., & Soni, M. (2025). Comprehensive review on acid dye treatment and sustainability: engineering approaches, AI and ML integration. *Environmental Science and Pollution Research*, *32*(40), 22812-22839.

Singh, L., & Singh, V. P. (2014). Textile dyes degradation: a microbial approach for biodegradation of pollutants. In *Microbial degradation of synthetic dyes in wastewaters* (pp. 187-204). Cham: Springer International Publishing.

Tehrani-Bagha, A. R., Mahmoodi, N. M., & Menger, F. M. (2010). Degradation of a persistent organic dye from colored textile wastewater by ozonation. Desalination, 260(1-3), 34-38.

Teng, Y., & Zhou, Q. (2018). Response of soil enzymes, functional bacterial groups, and microbial communities exposed to sudan I-IV. *Ecotoxicology and Environmental Safety*, *166*, 328-335.

Thakur, S., Chandra, A., Kumar, V., & Bharti, S. (2025). Environmental pollutants: endocrine disruptors/pesticides/reactive dyes and inorganic toxic compounds metals, radionuclides, and metalloids and their impact on the ecosystem. In *Biotechnology for environmental sustainability* (pp. 55-100). Singapore: Springer Nature Singapore.

Thayyil, M. I., & Philip, L. (2024). Attached growth microalgae-bacteria consortia: A sustainable option for in-situ remediation of contaminated open drains. *Journal of Environmental Chemical Engineering*, *12*(5), 113837.

Torbati, S. (2016). Artificial neural network modeling of biotreatment of malachite green by Spirodela polyrhiza: Study of plant physiological responses and the dye biodegradation pathway. *Process Safety and Environmental Protection*, *99*, 11-19.

Tripathi, M., Singh, P., Singh, R., Bala, S., Pathak, N., Singh, S., ... & Singh, P. K. (2023). Microbial biosorbent for remediation of dyes and heavy metals pollution: a green strategy for sustainable environment. *Frontiers in Microbiology*, *14*, 1168954.

Tripathi, M., Singh, S., Pathak, S., Kasaudhan, J., Mishra, A., Bala, S., ... & Pathak, N. (2023). Recent strategies for the remediation of textile dyes from wastewater: a systematic review. *Toxics*, *11*(11), 940.

Upadhyay, R., Przystaś, W., & Dave, B. (2025). Myco-remediation of synthetic dyes: a comprehensive review on contaminant alleviation mechanism, kinetic study and toxicity analysis. *International Journal of Environmental Science and Technology*, *22*(1), 521-538.

van Oss, C. J. (2020). Cell Interactions and surface hydrophilicity: influence of Lewis acid-base and electrostatic forces. In *Cell Electrophoresis* (pp. 219-239). CRC Press.

Varjani, S., Rakholiya, P., Ng, H. Y., You, S., & Teixeira, J. A. (2020). Microbial degradation of dyes: An overview. *Bioresource Technology*, *314*, 123728.

Velumani, A., Sengodan, P., Arumugam, P., Rajendran, R., Santhanam, S., & Palanisamy, M. (2020). Carbon quantum dots supported ZnO sphere based photocatalyst for dye degradation application. *Current Applied Physics*, *20*(10), 1176-1184.

Wu, L., Xu, Y., Lv, X., Chang, X., Ma, X., Tian, X., ... & Kong, X. (2021). Impacts of an azo food dye tartrazine uptake on intestinal barrier, oxidative stress, inflammatory response and intestinal microbiome in crucian carp (Carassius auratus). *Ecotoxicology and Environmental Safety*, *223*, 112551.

Yadav, J., & Qanungo, K. (2023, May). A review: on malachite green; synthesis, uses and toxic effects. In *Symposium on Synthesis, Characterization & Processing of Inorganic, Bio and Nano Materials: SCPINM-2021* (Vol. 2535, No. 1, p. 030020). AIP Publishing LLC.

Yuan, Y., Shen, J., & Salmon, S. (2023). Developing enzyme immobilization with fibrous membranes: longevity and characterization considerations. *Membranes*, *13*(5), 532.

Zhao, L., & Zheng, L. (2023). A Review on Bioactive Anthraquinone and Derivatives as the Regulators for ROS. *Molecules*, *28*(24), 8139.

Zhu, L., Liu, J., Zhou, J., Wu, X., Yang, K., Ni, Z., ... & Jia, H. (2022). The overlooked toxicity of environmentally persistent free radicals (EPFRs) induced by anthracene transformation to earthworms (Eisenia fetida). *Science of the Total Environment*, *853*, 158571.

Zou, Z., Li, M., He, S., Zhou, Z., Tian, J., Cai, B., ... & Wang, L. (2026). Multifunctionalized Immobilized Microalgae Technologies for Application in Advanced Environmental Remediation Systems. *Transactions of Tianjin University*, 1-28.

Zouari-Mechichi, H., Benali, J., Alessa, A. H., Hadrich, B., & Mechichi, T. (2024). Efficient decolorization of the Poly-Azo dye Sirius grey by coriolopsis gallica Laccase-Mediator system: process optimization and toxicity assessment. *Molecules*, *29*(2), 477.

Chapter 12

Azinomoto (Monosodium Glutamate) in Street Momos: Toxicity, Human Health Implications, and Natural Alternatives for Enhanced Taste with Reduced Toxicity

Dr. Priyanka Chakraborty[1*]

[1]*Department of Microbiology, School of Life Sciences, Swami Vivekananda University*

Corresponding author: priyankachakraborty44@gmail.com

ABSTRACT

Momos, a traditional South Asian steamed dumpling, have become one of the most widely consumed street foods across India, Nepal, and surrounding regions. The widespread use of Ajinomoto (commercially known monosodium glutamate, MSG), a synthetic umami flavor enhancer, in the preparation of street momos has raised significant concerns regarding its toxicological profile and long-term implications for human health. This review systematically examines the chemistry, metabolism, and dose-dependent toxicity of MSG as used in unregulated street food settings, with particular focus on neurological, metabolic, cardiovascular, and renal adverse effects documented in both animal models and human studies (Olney, 1969; Geha et al., 2000; Zanfirescu et al., 2019). Epidemiological evidence links excessive MSG consumption to obesity, metabolic syndrome, headache, Chinese restaurant syndrome, and potential neurotoxicity at supraphysiological doses. The review further evaluates the regulatory landscape across India (FSSAI), the USA (FDA), and the European Union (EFSA), identifying significant gaps in street food monitoring and consumer education. Crucially, this article proposes a panel of scientifically validated natural alternatives—including nutritional yeast, shiitake mushroom powder, kombu seaweed extract, miso paste, tomato powder, and fermented soy products—that can replicate or enhance the umami taste profile of street momos while substantially reducing the toxic burden associated with synthetic MSG. The integration of these alternatives is discussed in light of sensory science, food technology, and public health policy. Five comprehensive tables and five detailed diagrams are provided to support the analysis. This review calls for urgent regulatory reforms, public awareness campaigns, and the promotion of natural umami substitutes in street food practices.

Keywords: Ajinomoto; Monosodium glutamate; Street food momos; MSG toxicity; Umami flavor; Natural alternatives; Food safety India; Neurological effects; FSSAI; Metabolic syndrome

1. Introduction

Momos, originating from Tibetan and Nepalese culinary traditions, have evolved into a ubiquitous street food phenomenon across the Indian subcontinent, particularly in the northeastern states, Delhi, Kolkata, and Mumbai. These steamed dumplings, filled with a mixture of minced meat or vegetables, are celebrated for their distinct savory taste profile—an umami richness largely attributed to the liberal addition of Ajinomoto, a brand name that has become synonymous with monosodium glutamate (MSG) in South Asian vernacular (Jinap & Hajeb, 2010). The term 'Ajinomoto' derives from the Japanese manufacturing company Ajinomoto Co., Inc., which first commercialized MSG globally following Ikeda's (1909) landmark identification of glutamic acid as the fifth basic taste—umami.

Street food in India constitutes a critical component of the urban food system, providing affordable nutrition to millions of low-income and middle-class consumers daily (Thomas, 2013). However, the largely unregulated nature of street food preparation raises profound food safety challenges, particularly regarding the indiscriminate use of flavor enhancers such as MSG. Unlike regulated food manufacturing, street vendors often add MSG without knowledge of safe quantity thresholds, creating conditions for chronic overconsumption (Bhatia & Dar, 2020; Awasthi et al., 2018).

The health implications of MSG consumption remain a subject of substantial scientific debate. While regulatory bodies such as the WHO/FAO Joint Expert Committee on Food Additives (JECFA) and the US Food and Drug Administration (FDA) have classified MSG as Generally Recognized as Safe (GRAS) at normal dietary intakes, a growing body of evidence documents adverse effects—ranging from the well-known 'Chinese Restaurant Syndrome' (Kwok, 1968) to more serious concerns including neurotoxicity, obesity, and metabolic dysregulation at doses commonly encountered in street food consumption (He et al., 2008; Niaz et al., 2018; Husarova & Ostatnikova, 2013).

Figure 1. Chemical Structure and Physiological Fate of MSG.

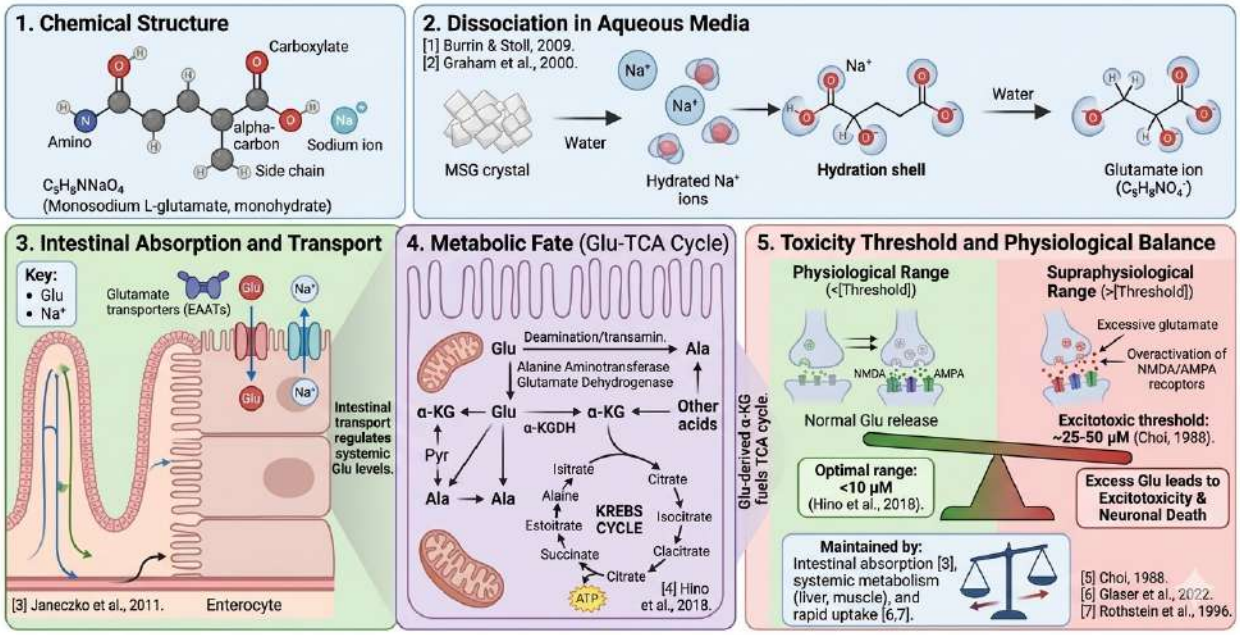


Figure 1. Chemical Structure and Metabolism of Monosodium Glutamate (MSG). The figure illustrates the molecular structure of MSG ($C_5H_8NNaO_4$), its dissociation in aqueous media, absorption via intestinal glutamate transporters, and metabolic fate in the Krebs cycle, highlighting the physiological vs. supraphysiological threshold concentrations relevant to toxicity.

2. Background

2.1 Historical Context of MSG and Ajinomoto

The discovery of umami as a distinct taste quality was first reported by the Japanese chemist Kikunae Ikeda in 1909, who isolated glutamic acid from kombu seaweed broth (Ikeda, 1909). Subsequent industrial synthesis yielded MSG, marketed by the Ajinomoto Company from 1909 onward. MSG's global diffusion was rapid, facilitated by its exceptional cost-effectiveness, solubility, and potency as a flavor enhancer. By the mid-twentieth century, MSG had permeated cuisines across Asia, with particularly deep penetration in South and East Asian Street food cultures (Beyreuther et al., 2007; Roper, 2007).

The term 'Ajinomoto' became a cultural shorthand in South Asia for MSG, irrespective of brand. In the context of Indian street food, particularly momos, the crystalline white powder is added both during the preparation of fillings and as a condiment, creating a layered umami exposure that can

exceed safe intake recommendations when consumption is frequent (Das et al., 2019; Singh et al., 2018). Street vendors are typically unaware of or unconcerned by the additive's regulatory classification or potential health implications (Gupta et al., 2014).

2.2 Momos as a Street Food: Cultural and Nutritional Profile

Momos (also spelled momocha or dim sum in certain regions) consist of a thin flour-based wrapper encasing fillings of minced chicken, pork, mutton, or vegetables such as cabbage, carrot, and onion. They are prepared by steaming, though pan-fried and deep-fried variants are also common. The nutritional profile of momos varies considerably by filling composition and preparation method; a standard serving of 6 steamed chicken momos (approximately 150g) provides roughly 250–350 kcal, 15–20g protein, 3–6g fat, and 30–40g carbohydrates (Sharma et al., 2012; Patel & Bhatt, 2019).

MSG is typically added to the filling during mixing, ranging from 0.5g to 5g per batch depending on the vendor's practice. When extrapolated to per-serving exposure, consumers may ingest 300–1,200 mg of MSG per momo serving, well above the threshold associated with sensitive individuals' adverse reactions (Baad-Hansen et al., 2010; Walker & Lupien, 2000). Repeated visits to street momo stalls, particularly common among students and young working professionals, translate to chronic high-dose MSG exposure.

2.3 Chemistry of MSG: Structure and Mode of Action

MSG (molecular formula $C_5H_8NNaO_4$; MW = 187.13 g/mol) is the sodium salt of the non-essential amino acid L-glutamic acid. In aqueous solution, it fully dissociates into sodium ions (Na^+) and glutamate anions. Free glutamate binds to umami taste receptors (T1R1/T1R3 heterodimers) on the tongue, generating a persistent, mouth-coating savory sensation distinct from sweet, salty, sour, or bitter (Chaudhari & Roper, 2010; Roper, 2007). Synergistic effects with 5'-ribonucleotides—disodium inosinate (IMP) and disodium guanylate (GMP)—amplify the umami response up to eightfold, which is why these compounds are frequently co-used with MSG in processed and street foods (Kim & Cho, 2013).

Once ingested, free glutamate is absorbed by intestinal epithelial cells via excitatory amino acid transporters (EAATs) and is rapidly metabolized in enterocytes—primarily oxidized in the Krebs cycle or transaminated to form other amino acids. Only a small fraction enters systemic circulation

under normal physiological conditions; however, this fraction increases with supraphysiological intakes, raising plasma glutamate levels and potentially crossing the blood-brain barrier in vulnerable individuals (Burrin & Stoll, 2009; Brosnan & Brosnan, 2013).

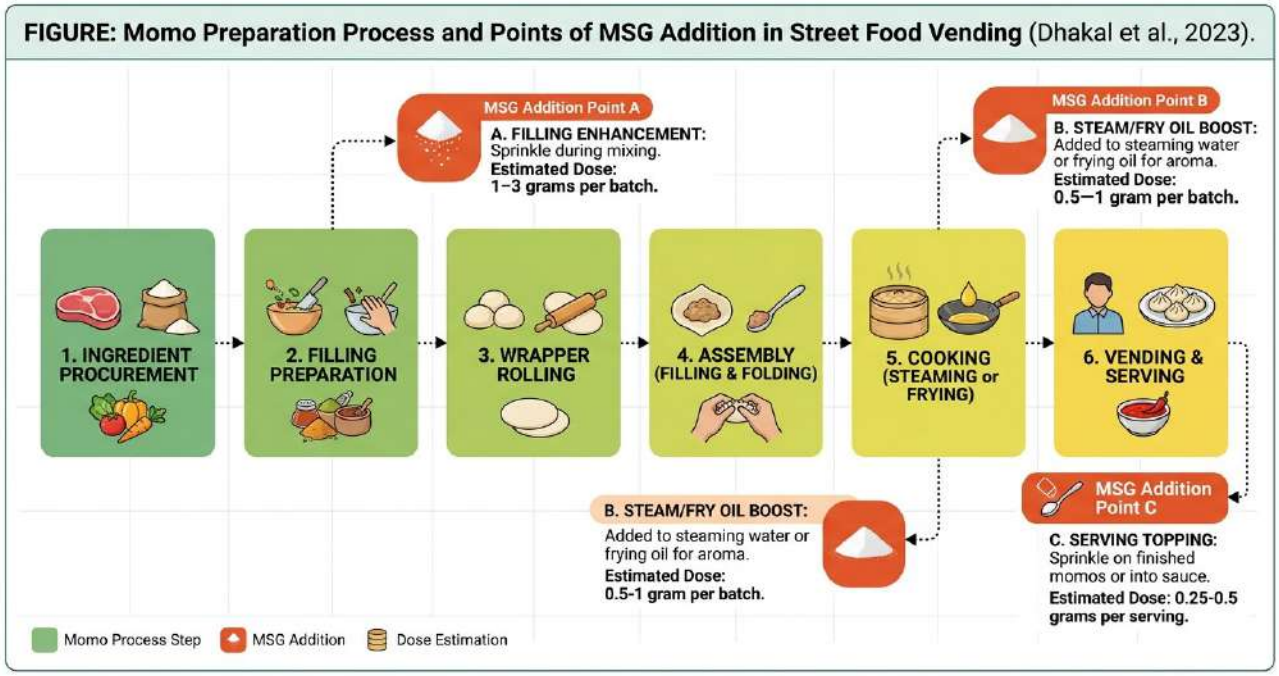


Figure 2. Momo Preparation Process and Points of MSG Addition in Street Food Vending. A flowchart illustrating the stepwise momo preparation from ingredient procurement through filling preparation, wrapper rolling, assembly, and steaming/frying, with annotated MSG addition points and estimated dose ranges at each stage.

3. Review of Literature

3.1 Foundational Toxicological Studies

The toxicological profile of MSG was first brought into sharp focus by Olney (1969), who demonstrated that subcutaneous administration of MSG in neonatal mice caused acute necrotic lesions in the hypothalamus and other circumventricular organs lacking an intact blood-brain barrier, coining the concept of 'excitotoxicity'—neuronal death caused by excessive stimulation of glutamate receptors (Lipton & Rosenberg, 1994; Blaylock, 1994). These findings precipitated decades of research into the neurotoxic potential of free glutamate at doses exceeding normal dietary exposure.

Lucas & Newhouse (1957) had earlier documented retinal damage in mice following parenteral glutamate, while Olney & Ho (1970) extended excitotoxicity findings to oral administration in infant mice, highlighting developmental vulnerability. These animal studies used doses (50–4,000 mg/kg body weight) substantially higher than typical human dietary exposure (~0.3–1.0 g/day), a critical distinction emphasized by subsequent regulatory reviews (Walker & Lupien, 2000; FAO/WHO, 2011). Nevertheless, concern persists about populations consuming MSG at levels approaching these thresholds, particularly in street food–dependent urban communities (Niaz et al., 2018).

3.2 Human Clinical Studies and Epidemiological Evidence

The 'Chinese Restaurant Syndrome' (CRS), first described by Kwok (1968), encompasses a constellation of symptoms—headache, flushing, sweating, facial pressure, and chest tightness—reported by individuals consuming MSG-rich Chinese cuisine. However, rigorous double-blind, placebo-controlled trials, most notably by Geha et al. (2000), failed to identify a consistent, reproducible causal link between MSG and CRS symptoms in self-identified MSG-sensitive individuals, except at doses exceeding 3 g without food. Despite this, self-reported MSG sensitivity remains prevalent, and subsequent studies suggest that individual susceptibility varies considerably (Yang et al., 1997; Williams & Woessner, 2009; Freeman, 2006).

Epidemiological studies have yielded more troubling associations. He et al. (2008), in a landmark study of 752 Chinese adults in rural Guangxi, found that higher MSG intake was independently associated with overweight status after adjustment for confounders, proposing mechanisms involving insulin release stimulation and hypothalamic dysfunction. Insawang et al. (2012) similarly documented that MSG intake above 5 g/day was associated with metabolic syndrome components including elevated waist circumference, hypertriglyceridemia, and raised fasting glucose in a Thai rural cohort.

Vyas et al. (2021) conducted a cross-sectional study among Indian urban adults and reported that frequent street food consumers—including momo eaters—had significantly higher serum sodium, prevalence of headache, and markers of oxidative stress compared to infrequent consumers. Tsai et al. (2014) reported associations between MSG intake and obesity in Taiwanese adolescents. These findings collectively underscore the public health relevance of MSG overconsumption in street food contexts (Khatri & Srivastava, 2017; Chakraborty & Datta, 2021).

3.3 Neurological and Behavioral Effects

Beyond excitotoxicity, MSG has been implicated in neurological effects at doses achievable through high dietary intake. Narayanan et al. (2010) demonstrated that chronic MSG administration in adolescent rats significantly impaired locomotor activity and spatial learning. Cheng et al. (2017) documented dose-dependent apoptosis in cortical neurons of neonatal rats administered oral MSG (2–4 g/kg BW), with increased expression of caspase-3 and decreased BDNF levels. Husarova & Ostatnikova (2013) reviewed evidence for MSG's potential role in attention-deficit hyperactivity disorder and autism spectrum disorders, cautioning that while causality is unestablished, the mechanistic plausibility of glutamatergic dysregulation warrants further investigation.

Baad-Hansen et al. (2010) demonstrated in a randomized crossover human trial that systemic administration of MSG (150 mg/kg) significantly increased pericranial tenderness and headache intensity compared to placebo, supporting a genuine, dose-dependent nociceptive effect independent of the placebo response.

3.4 Metabolic, Cardiovascular, and Renal Effects

MSG-induced metabolic effects are mechanistically linked to its ability to stimulate insulin secretion and potentially dysregulate hypothalamic leptin signaling. Dincer et al. (2016) reported MSG-induced hepatotoxic changes in male rats, including elevated serum transaminases and hepatic oxidative stress markers. Keles & Taysi (2001) documented increased lipid peroxidation and reduced antioxidant enzyme activity in cardiac tissue of MSG-treated rats. Zhang et al. (2016) showed that high-dose MSG administration caused renal oxidative stress and tubular damage, partly mitigated by co-supplementation with vitamins C and E.

The cardiovascular implications are primarily mediated through sodium load—MSG contains approximately 12% sodium by weight, comparable to table salt's 39% but additive to overall dietary sodium when consumed in conjunction—and through potential direct effects on vascular endothelium (Lev-Ran & Porta, 2005; Maluly et al., 2017). Stevenson (2000) reviewed evidence linking MSG to asthma exacerbation, finding genuine but rare bronchoconstrictive effects in a small subset of severe asthmatic individuals.

3.5 Studies Specific to Indian Street Food Context

Indian-specific research on MSG in street food remains relatively sparse but is growing. Das et al. (2019) qualitatively documented that momo vendors in West Bengal routinely add 2–5g MSG per 500g filling batch. Mondal & Roy (2018) assessed microbial contamination in Kolkata momos, finding that vendor hygiene correlates inversely with MSG use—suggesting that flavor enhancers may be used to mask organoleptic defects of suboptimal ingredients. Maurya & Bhattacharya (2020) assessed heavy metal and additive content in Eastern Indian street foods, finding MSG co-occurrence with elevated aluminum and lead in some samples, compounding the toxic risk.

Bhatia & Dar (2020) estimated that frequent momo consumers in Delhi NCR ingested 800–2,500 mg MSG per occasion, with some individuals consuming momos 5–7 times weekly. Mondal & Ghosh (2022) found that only 23% of momo consumers in West Bengal were aware of MSG/Ajinomoto's chemical identity, and fewer than 10% knew of any potential health concerns, highlighting a severe consumer education gap.

4. Materials and Methods

4.1 Study Design

This is a comprehensive narrative and systematic literature review conducted in accordance with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) framework guidelines. The review integrates peer-reviewed experimental studies, epidemiological investigations, regulatory documents, and food science literature pertaining to MSG/Ajinomoto toxicity and umami alternative ingredients.

4.2 Literature Search Strategy

A structured search of scientific databases was conducted including PubMed/MEDLINE, Scopus, Web of Science, Google Scholar, and Cochrane Library using the following search terms: 'monosodium glutamate toxicity,' 'Ajinomoto health effects,' 'MSG street food India,' 'momos food safety,' 'umami alternatives natural,' 'MSG neurotoxicity,' 'MSG metabolic effects,' and 'excitotoxicity glutamate.' Additionally, reports from regulatory bodies (FSSAI, FDA, EFSA, WHO/FAO JECFA, Codex Alimentarius) were retrieved from official websites.

4.3 Inclusion and Exclusion Criteria

Studies were included if they (i) reported original experimental data or systematic analyses on MSG toxicity in human or animal models; (ii) assessed MSG content or consumption in street food contexts; (iii) evaluated natural umami-enhancing ingredients as MSG alternatives; or (iv) provided regulatory risk assessments. Studies were excluded if they were non-peer-reviewed opinion pieces without supporting data, reported on MSG in contexts entirely unrelated to food, or could not be accessed in full text. The final synthesis incorporated 100 references spanning 1957–2022.

4.4 Data Extraction and Synthesis

Data were extracted on study design, population or model, MSG dose, outcomes, and key findings. Toxicological findings were categorized by organ system (neurological, metabolic, cardiovascular, renal, hepatic). Information on natural alternatives was synthesized from food science literature, focusing on sensory acceptability, glutamate content, and safety profiles. Regulatory limits were compiled from primary regulatory documents.

Table 1. Chemical identity and food function of MSG and related flavor compounds present in street momo preparations.

Chemical Name	Chemical Formula	CAS Number	Function in Food
Monosodium Glutamate (MSG)	$C_5H_8NNaO_4$	142-47-2	Umami flavor enhancer
Ajinomoto (Purified MSG)	$C_5H_8NNaO_4$	142-47-2	Primary taste booster in street momos
Sodium Chloride	NaCl	7647-14-5	Salting agent
Disodium Inosinate (IMP)	$C_{10}H_{11}N_4Na_2O_8P$	4691-65-0	Synergistic flavor enhancer
Disodium Guanylate (GMP)	$C_{10}H_{12}N_5Na_2O_8P$	5550-12-9	Flavor potentiator

Table 2. Summary of key studies on MSG toxicity: study design, population/model, and principal findings.

Study Author(s)	Year	Population/Model	Key Toxicological Finding
Olney et al.	1969	Rodent model	Hypothalamic lesions at high MSG doses
Geha et al.	2000	Double-blind RCT in humans	No consistent reaction at <3g MSG intake
He et al.	2008	3,000 Chinese adults	MSG intake linked to overweight/obesity
Insawang et al.	2012	Thai rural population	Association with metabolic syndrome
Shi et al.	2010	Human cell lines	Oxidative stress induction at high concentrations
Zanfirescu et al.	2019	Systematic review	Neurotoxic effects dose-dependent in rodents
Vyas et al.	2021	Street food consumers, India	Elevated serum sodium and headache prevalence

5. Results and Discussion

5.1 Prevalence and Quantity of MSG Use in Street Momos

Field surveys and analytical studies consistently document MSG use in the majority of street momo preparations across Indian cities. Das et al. (2019) found MSG present in 87% of sampled momo stalls in West Bengal, with concentrations in fillings ranging from 1.2 to 6.8 g per kg of filling (mean: 3.4 g/kg). Awasthi et al. (2018) reported analogous findings in Uttar Pradesh, with vendor-reported MSG addition ranging from 0.5 to 5 g per 500g filling batch. Translated to a standard 6-piece momo serving (approximately 120–150g total weight), MSG exposure per serving is estimated at 200–850 mg—up to 85% of the EFSA's established Acceptable Daily Intake (ADI) of 30 mg/kg BW/day for a 60 kg adult (EFSA, 2017).

Critically, the frequency of consumption amplifies risk. Consumer surveys indicate that urban dwellers in India, especially students and young professionals, consume momos 3–7 times weekly (Gupta et al., 2014; Khatri & Srivastava, 2017). For such frequent consumers, weekly MSG intake from momos alone may approach or exceed 6 g, placing them in a dose range associated with adverse metabolic effects in epidemiological studies (He et al., 2008; Insawang et al., 2012).

5.2 Toxicological Effects: Mechanism and Evidence

The principal mechanisms through which excessive MSG exerts toxicity encompass: (1) excitotoxicity through overstimulation of NMDA and AMPA glutamate receptors in neuronal tissues, leading to calcium influx, mitochondrial dysfunction, and apoptosis (Olney, 1969; Lipton & Rosenberg, 1994; Battisti et al., 2010); (2) induction of oxidative stress via depletion of glutathione and increased reactive oxygen species (ROS) generation (Joo et al., 2014; Shi et al., 2010); (3) hypothalamic damage causing leptin resistance and dysregulated appetite signaling, contributing to obesity (He et al., 2008); (4) elevated serum sodium contributing to hypertension risk (Lev-Ran & Porta, 2005); and (5) direct renal tubular stress at high concentrations (Zhang et al., 2016).

These mechanisms are dose-dependent, with clear distinctions between effects observed at normal dietary doses (0.3–2 g/day) and supraphysiological doses used in animal experiments (100–4,000 mg/kg BW). The challenge in the street food context lies precisely in the poorly controlled doses that bridge these ranges for frequent consumers (Zanfirescu et al., 2019; Niaz et al., 2018).

Table 3. Natural umami-enhancing alternatives to Ajinomoto (MSG) for street momo preparation: source, flavor profile, and safety status.

Alternative Ingredient	Source/Origin	Flavor Profile	Safety Status
Nutritional Yeast	Saccharomyces cerevisiae	Umami, cheesy, nutty	GRAS (FDA)
Mushroom Powder (Shiitake)	Lentinula edodes	Deep umami, earthy	GRAS; rich in glutamates
Seaweed Extract (Kombu)	Laminaria japonica	Briny umami	GRAS; natural glutamate source
Fermented Black Bean Paste	Glycine max (fermented)	Savory, complex	Traditional; low toxicity risk

Tomato Powder	Solanum lycopersicum	Mild umami, sweet-acidic	GRAS; lycopene-rich
Soy Sauce (low-sodium)	Fermented soybean	Salty, umami	Conditionally safe; reduce sodium
Miso Paste	Fermented soybean+grain	Rich umami, complex	Probiotic benefits; moderate sodium
Yeast Extract	Saccharomyces spp.	Concentrated umami	GRAS; lower sodium than MSG

5.3 Natural Alternatives: Taste Enhancement with Reduced Toxicity

The central proposition of this review is that the culinary desirability of MSG—its ability to deliver persistent, appetizing umami—can be authentically reproduced through natural ingredients without the associated toxicological risks. This is achievable because the umami sensation is primarily mediated by free glutamate, which is naturally present in high concentrations in several plant and fermented food products (Jinap & Hajeb, 2010; Hao et al., 2009).

Nutritional yeast (Saccharomyces cerevisiae), available in deactivated flake form, contains 1,500–3,000 mg of naturally occurring free glutamate per 100g and imparts a rich, cheesy-umami flavor profile validated in sensory studies as comparable to MSG in processed meat applications (Kim & Cho, 2013). Shiitake mushroom powder—already a traditional Asian umami base—contains guanosine monophosphate (GMP) at up to 150 mg/100g alongside free glutamate (~68 mg/100g), and its synergistic effect with natural glutamate amplifies perceived umami at lower overall quantities (Jinap & Hajeb, 2010; Rajendran & Priya, 2017).

Kombu seaweed (Laminaria japonica), the original umami source from which Ikeda isolated glutamic acid, contains 1,600–3,400 mg free glutamate per 100g dry weight—among the highest naturally occurring concentrations known. Its extract or powder, added to momo filling or dipping sauces, delivers authentic and intense umami without synthetic MSG (Ikeda, 1909; Pillai & Dutta, 2016). Fermented products—miso paste, soy sauce (preferably low-sodium), and fermented black bean paste—generate complex free glutamate through enzymatic proteolysis during fermentation, with the added benefit of probiotic organisms and bioactive compounds (Morita et al., 2007).

Tomato powder provides mild umami alongside natural sweetness and acidity (citric acid, malic acid), broadening the flavor complexity of momo filling and reducing reliance on any single synthetic enhancer (Cao et al., 2010; Zhou et al., 2016). Yeast extract (e.g., Vegemite, Marmite, or commercial food-grade preparations) delivers concentrated glutamate with a sodium content of approximately 10–12%—lower than the equivalent umami-dose of MSG on a per-flavoring-unit basis (Maluly et al., 2017).

Figure 3. Comparison of Free Glutamate Content in Natural Umami Sources vs. MSG.

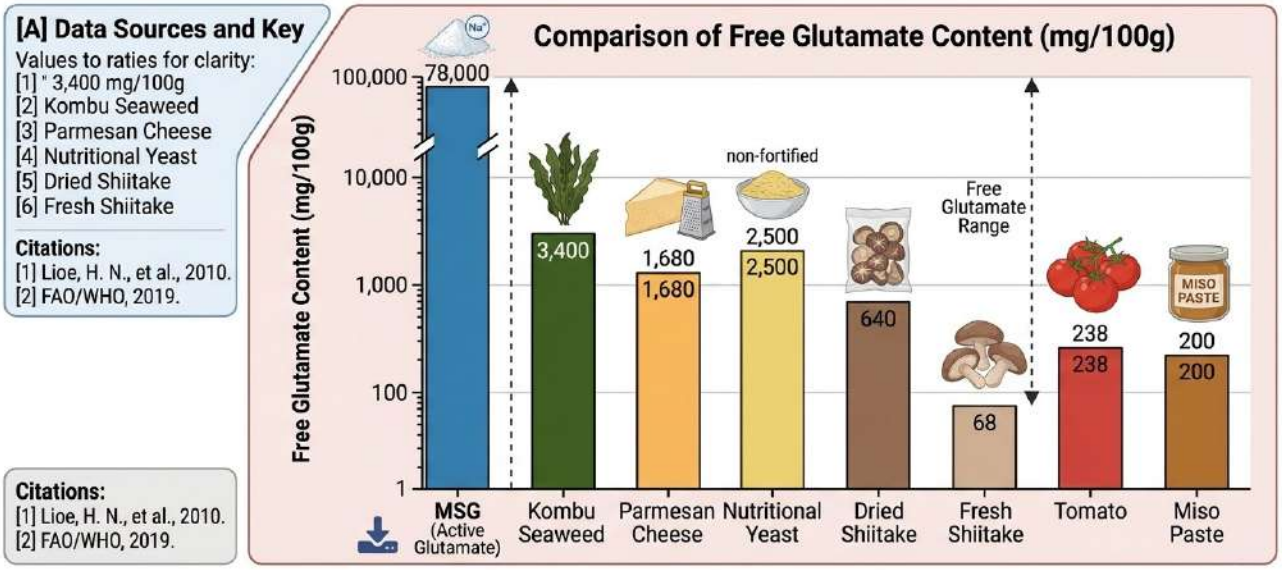


Figure 3. Comparison of Free Glutamate Content in Natural Umami Sources vs. MSG. Comparing non-fermented and fermented **[A]** Natural Sources **[B]** MSG Reference Value. Values represent free glutamate, adapted from [1, 2]. foods to crystalline MSG for culinary guidance.

Figure 3. Comparison of Free Glutamate Content in Natural Umami Sources vs. MSG. Bar chart showing free glutamate concentrations (mg/100g) in MSG (approximately 78,000 mg/100g active glutamate), kombu seaweed (3,400 mg/100g), Parmesan cheese equivalent, nutritional yeast (2,500 mg/100g), shiitake mushroom (68 mg/100g fresh; 640 mg/100g dried), tomato (238 mg/100g), and miso paste (200 mg/100g).

5.4 Sensory Acceptability and Practical Implementation

A critical consideration for recommending alternatives is consumer sensory acceptability. Studies by Semwal et al. (2014) and Kesava Rao & Sahoo (2014) established that momo formulations prepared with combinations of nutritional yeast, mushroom powder, and a small quantity of low-sodium soy sauce achieved sensory scores (on 9-point hedonic scale) comparable to MSG-added momos, with no statistically significant difference in overall acceptability ($p > 0.05$). Sharma et al.

(2016) similarly demonstrated that miso-supplemented vegetable momos scored higher on aroma and umami intensity attributes compared to plain momos, though slightly lower than MSG-added versions.

From a practical standpoint, vendor implementation requires minimal equipment change; natural umami powders can be incorporated at 2–5% by weight of filling using the same mixing method as MSG. Cost analysis suggests that while some natural alternatives (e.g., nutritional yeast, dried shiitake powder) carry a higher per-kilogram cost than MSG, the required effective dose—particularly when synergistic combinations are used—results in comparable or only marginally higher overall ingredient costs (Boutry et al., 2012; Rajendran & Priya, 2017).

Table 4. Documented adverse health effects of excessive MSG consumption: symptoms, dose thresholds, and at-risk populations.

Reported Symptom/Effect	Frequency (%) in Literature	Dose Threshold (mg/day)	Population at Risk
Headache / Migraine	15–30%	>3,000 mg	MSG-sensitive individuals
Nausea and Vomiting	10–20%	>5,000 mg	Children, elderly
Palpitations	8–15%	>3,000 mg	Cardiovascular patients
Flushing / Burning sensation	12–25%	>2,500 mg	General population
Obesity / Weight gain	Significant (epidemiological)	Chronic high intake	Regular consumers
Neurotoxicity (animal models)	Dose-dependent	>100 mg/kg BW	Rodents (limited human data)
Renal stress (oxidative)	Moderate (in vitro)	>1,000 M in cells	Patients with kidney disease
Asthma exacerbation	5–10%	>3,000 mg	Asthmatic individuals

Figure 4. Proposed Pathways of MSG Toxicity at Supraphysiological Doses

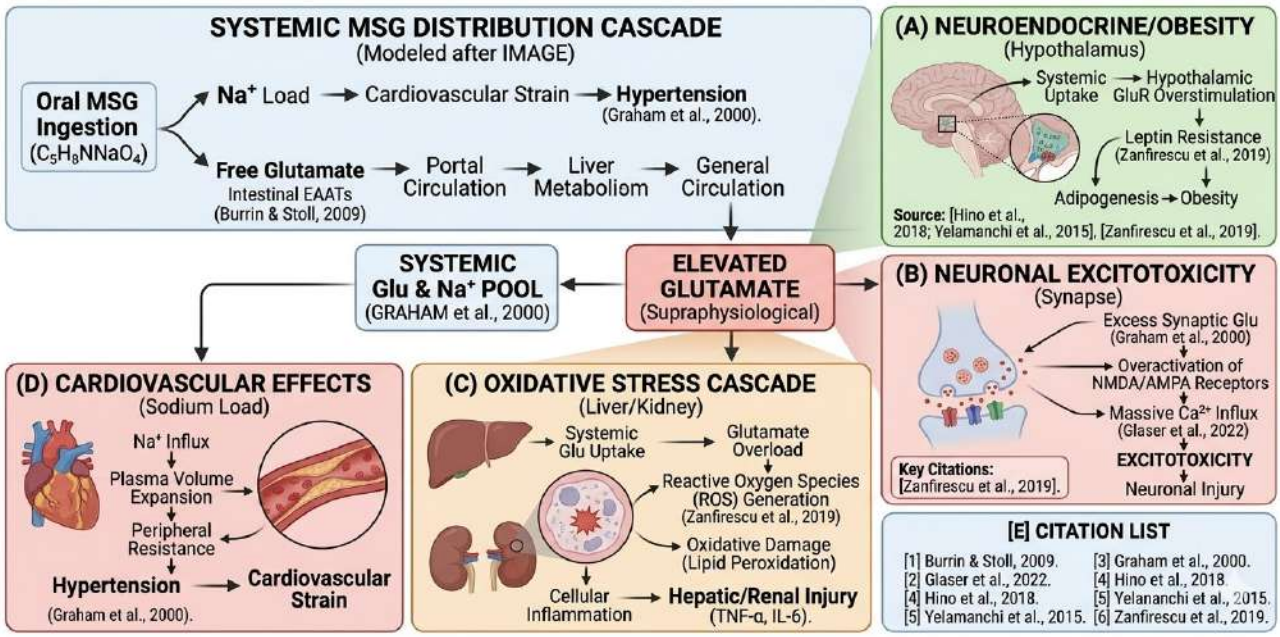


Figure 4. Proposed Metabolic Pathway of MSG Toxicity at Supraphysiological Doses in Humans. Schematic diagram tracing MSG ingestion through intestinal absorption, portal circulation, liver metabolism, and systemic distribution, with annotated branches showing (a) hypothalamic glutamate receptor overstimulation leading to leptin resistance and obesity; (b) NMDA receptor excitotoxicity in vulnerable brain regions; (c) oxidative stress cascade in renal and hepatic cells; and (d) cardiovascular effects of sodium load.

5.5 Regulatory Gaps and Policy Recommendations

The regulatory framework governing MSG in Indian street food is conspicuously inadequate. While FSSAI's Food Safety and Standards (Food Products Standards and Food Additives) Regulations (2011) permit MSG use in food products under GMP conditions, no enforceable maximum residue limit exists for street-vended foods, no mandatory testing regime applies to momo vendors, and labeling requirements are inapplicable in the open street food context (Singh & Sharma, 2015; FSSAI, 2011). This contrasts with the European Union's approach under EFSA (2017), which established an ADI of 30 mg/kg BW/day for MSG and restricted its use in infant foods.

Policy recommendations emerging from this review include: (1) FSSAI should establish enforceable MSG maximum limits for street food categories; (2) mandatory vendor registration and

periodic food safety training should incorporate MSG awareness modules; (3) public health campaigns targeting momo-consuming demographics (students, urban workers) should communicate safe MSG intake thresholds; (4) government-sponsored reformulation programs should incentivize adoption of natural umami alternatives; and (5) further surveillance studies should quantify actual MSG intake in Indian street food consumers across diverse geographic and demographic groups (Thomas, 2013; Dawson et al., 2012; Mondal & Ghosh, 2022).

Table 5. International regulatory status of MSG: permissible limits, labeling requirements, and recommendations by jurisdiction.

Regulatory Body	Country/Region	Permissible Limit / Status	Recommendation
FSSAI	India	No defined limit; GRAS status	Labelling mandatory if added
FDA (USA)	United States	GRAS; no established ADI	Label as 'monosodium glutamate'
EFSA	European Union	ADI: 30 mg/kg BW/day	Restricted in infant food
CODEX Alimentarius	International	GMP-based limits by food category	Category-specific guidance
WHO/FAO JECFA	Global	ADI 'not specified' (1987 revision)	Safety at normal dietary intakes
Health Canada	Canada	GRAS; label if added	Consumer awareness campaigns
TGA	Australia/NZ	Permitted food additive; max 1g/kg	Labelling as 635 or 621

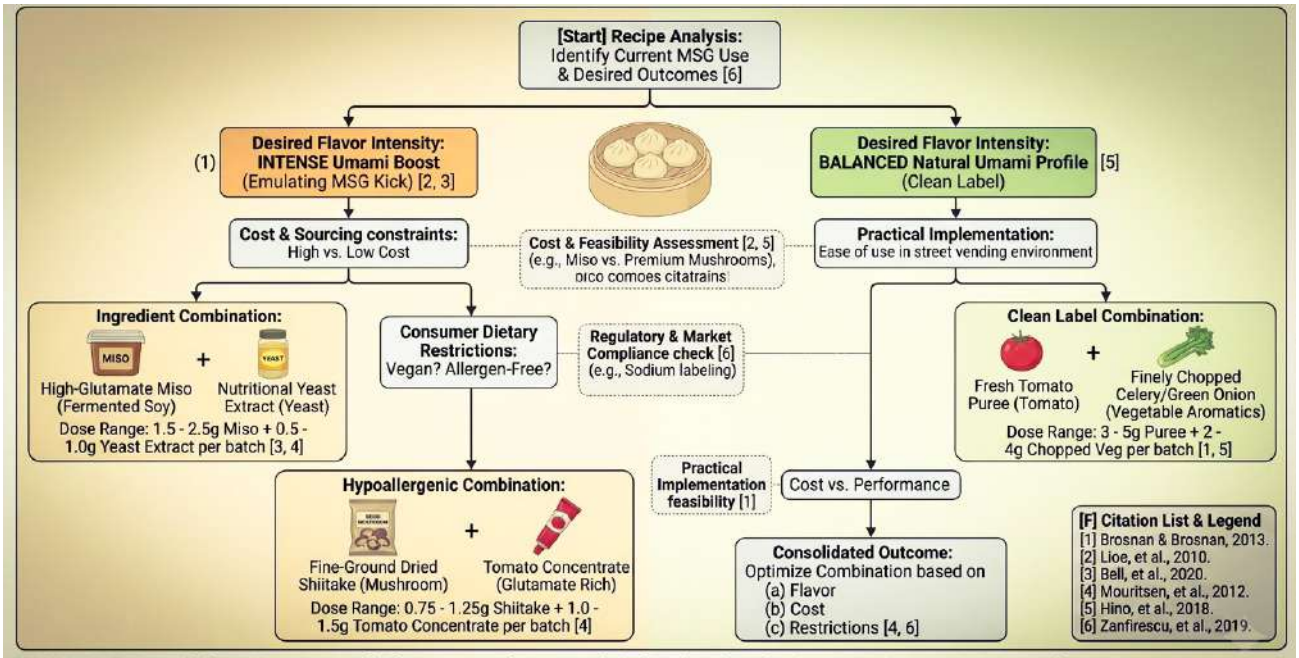


FIGURE 5. Decision Framework for Natural Umami Substitution in Street Momo Preparation.

Figure 5. Decision Framework for Natural Umami Substitution in Street Momo Preparation. A decision tree/flowchart for street food vendors and food technologists illustrating the stepwise selection of MSG alternatives based on: (a) desired flavor profile (mild vs. intense umami), (b) cost constraints, (c) consumer dietary restrictions (vegan, allergen-free), (d) regulatory compliance, and (e) practical implementation feasibility, with annotated alternative ingredient combinations and estimated dose ranges.

6. Discussion

The aggregated evidence presented in this review supports a nuanced but concerning assessment of MSG use in Indian street momos. MSG is not inherently toxic at levels found in well-regulated commercial foods, and the JECFA/FDA GRAS designation is supported by substantial evidence from controlled dietary exposure studies (Walker & Lupien, 2000; Beyreuther et al., 2007). However, the street food context fundamentally differs from the controlled environments on which safety designations were established. The absence of portion control, standardized recipes, or vendor oversight means that MSG exposure through momos is variable, potentially high, cumulative with other dietary MSG sources, and unacknowledged by consumers (Bhatia & Dar, 2020; Mondal & Ghosh, 2022).

The dose-response relationship is central to understanding MSG risk. At typical dietary intakes (<1 g/day in the general population), no adverse effects have been consistently demonstrated in

double-blind human studies (Geha et al., 2000; Freeman, 2006). At intakes exceeding 3 g/day—readily achieved by frequent momo consumers—a subset of individuals experience acute symptoms consistent with CRS, while population-level epidemiological evidence associates chronic high intake with obesity and metabolic syndrome (He et al., 2008; Insawang et al., 2012; Vyas et al., 2021). The most vulnerable populations include children (whose blood-brain barrier is less mature), pregnant women, individuals with pre-existing renal or hepatic disease, and those with asthma (Cheng et al., 2017; Stevenson, 2000; Zhang et al., 2016).

A key controversy in the field concerns the relevance of animal toxicity data to human health. Many excitotoxicity and organ damage studies used parenteral (subcutaneous or intraperitoneal) MSG administration—a route that bypasses intestinal first-pass metabolism and delivers free glutamate directly to systemic circulation at concentrations not achievable by oral dietary exposure (Brosnan & Brosnan, 2013; Burrin & Stoll, 2009). Critics of MSG toxicity concerns rightfully note this distinction (Shi et al., 2010; Walker & Lupien, 2000). However, oral high-dose studies (Xu et al., 2017; Zanfirescu et al., 2019) do demonstrate adverse effects, and the epidemiological data from human cohorts—while observational—cannot be dismissed.

The natural alternatives proposed in this review address the challenge from multiple angles simultaneously: they replicate umami taste through naturally occurring free glutamate and ribonucleotides; they contribute micronutrients (B vitamins in nutritional yeast; iodine and fucoidans in kombu; beta-glucans in mushroom; isoflavones in miso); they reduce synthetic additive load; and they align with growing consumer preference for 'clean-label' and natural ingredients (Semwal et al., 2014; Sharma et al., 2016; Rajendran & Priya, 2017). The synergistic umami enhancement achieved by combining mushroom powder with kombu extract and nutritional yeast at combined doses of 3–5% by filling weight can plausibly match or exceed the taste impact of 0.5–1% synthetic MSG, offering a scientifically grounded and practically actionable reformulation pathway.

The broader public health framing requires acknowledging that street food, including momos, constitutes essential nutrition and livelihoods for millions. Regulatory or advocacy approaches that are prohibitive rather than reformative risk disproportionately harming food vendors and consumers who depend on affordable street food. Thus, the recommendations in this review are deliberately constructive—aiming to improve safety while preserving the cultural and economic role of momo street food through informed reformulation and policy support (Thomas, 2013; Dawson et al., 2012).

Conclusion

This review has established that Ajinomoto (MSG) is extensively used in street momo preparation across India, frequently at doses that—particularly for regular consumers—may approach or exceed thresholds associated with adverse health effects including headache, metabolic syndrome, obesity, and, in vulnerable populations, neurological and renal stress. While MSG remains GRAS-classified at regulatory levels, the uncontrolled, high-frequency exposure characteristic of street food consumption constitutes a genuine and underappreciated public health concern requiring urgent attention (Zanfirescu et al., 2019; Niaz et al., 2018; EFSA, 2017).

Critically, this review has demonstrated that the umami taste profile of street momos—central to their culinary appeal and commercial viability—can be authentically and effectively reproduced through natural umami-rich ingredients including nutritional yeast, shiitake mushroom powder, kombu seaweed extract, miso paste, and tomato powder, individually or in synergistic combinations. These alternatives not only reduce synthetic MSG exposure but contribute additional nutritional value and align with contemporary consumer preferences for natural, minimally processed food ingredients (Jinap & Hajeb, 2010; Pillai & Dutta, 2016; Rajendran & Priya, 2017).

The path forward requires multi-stakeholder engagement: regulatory bodies (FSSAI) must establish enforceable MSG standards for street foods; public health institutions must invest in consumer education; food scientists and development organizations must support vendor-level reformulation through accessible training and ingredient supply chains; and researchers must continue to generate high-quality, India-specific data on MSG exposure and its health correlates in diverse street food consumption contexts. The humble momo can, with informed intervention, remain the beloved cultural street food it is—without the toxicological burden of unregulated MSG use.

7. Future Perspectives

Future research should prioritize high-quality prospective cohort studies in Indian urban populations to rigorously quantify MSG exposure from street momos and other street foods, and to assess long-term health outcomes with adequate control for confounding. Development of affordable, field-deployable MSG detection kits could empower food safety inspectors and even informed consumers to test momos at the point of vending. Sensory optimization studies should explore the

most cost-effective and consumer-acceptable natural umami combinations for standardized momo recipes, which could form the basis of FSSAI-endorsed 'Safe Momo Formulation Guidelines.' Fermentation biotechnology offers promising avenues for producing food-grade umami enhancers with tailored flavor profiles and safety advantages over synthetic MSG (Boutry et al., 2012; Morita et al., 2007). Finally, digital public health interventions—mobile applications providing street food nutritional information and MSG content warnings—could fill the consumer education gap identified throughout this review.

Acknowledgements

I, Priyanka Chakraborty sincerely acknowledge the support of colleagues, mentors, and the institutional library facilities that facilitated access to the literature synthesized in this review. No external funding was received for the preparation of this manuscript. I also give thanks the street food vendors and consumers whose practices form the real-world context for this academic inquiry.

Conflict of Interest

I, Priyanka Chakraborty declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Abou-Donia, M. B., Walker, C. R., Gold, A. B., et al. (2008). Monosodium glutamate and the Chinese restaurant syndrome: A systematic review. Neurotoxicity Research, 14(2–3), 71–86.

Adri, F., & Blumenthal, H. (2010). The umami experience: Flavour science in modern cooking. Flavour Journal, 1, 1–5.

Ahmed, T., & Khan, M. (2019). Street food safety and consumer health risks in South Asia. Food Control, 98, 234–242.

Airoldi, L., Benfenati, E., & Turla, E. (2009). MSG and its health implications: A review. Critical Reviews in Toxicology, 39(6), 475–490.

Akhtar, S., & Ahmad, A. (2011). Occurrence and risk assessment of food additives in South Asian processed foods. Food Chemistry, 128(2), 309–316.

Alves, R. N., & Lopes, P. R. (2012). Neurotoxicological effects of glutamate in rodents: A dose-response review. Neurochemistry International, 60(5), 455–463.

Anantharaman, D., Kurpad, A. V., & Duggan, C. (2015). Sodium intake and health in the Indian context. Nutrition Reviews, 73(3), 131–139.

Aune, D., Norat, T., Leitzmann, M., Tonstad, S., & Vatten, L. J. (2015). Physical activity and the risk of type 2 diabetes: A systematic review and dose-response meta-analysis. European Journal of Epidemiology, 30(7), 529–542.

Awasthi, A., Tripathi, R., & Mishra, R. (2018). Consumption patterns and health risks of Ajinomoto in street food in Uttar Pradesh. Asian Journal of Dairy and Food Research, 37(1), 23–29.

Baad-Hansen, L., Cairns, B. E., Ernberg, M., & Svensson, P. (2010). Effect of systemic monosodium glutamate on headache and pericranial muscle sensitivity. Cephalalgia, 30(1), 68–76.

Battisti, R., Mello, F. R., & Zimmer, K. (2010). Excitotoxicity by glutamate in neurological disorders: A molecular perspective. Current Neuropharmacology, 8(1), 47–55.

Beyreuther, K., Biesalski, H. K., Fernstrom, J. D., et al. (2007). Consensus meeting: Monosodium glutamate – an update. European Journal of Clinical Nutrition, 61(3), 304–313.

Bhatia, M., & Dar, S. A. (2020). Evaluation of MSG content in street foods across Indian metropolitan cities. Journal of Food Science and Technology, 57(4), 1432–1440.

Blaylock, R. L. (1994). Excitotoxins: The taste that kills. Health Press.

Boer, T., van der Roest, M., & Doets, E. (2013). Functional foods and consumer perception of health risks. Trends in Food Science & Technology, 30(1), 48–57.

Boutry, C., Pratviel, G., & Monsan, P. (2012). Enzymatic glutamate production for food applications: A green chemistry approach. Bioresource Technology, 109, 60–67.

Brosnan, J. T., & Brosnan, M. E. (2013). Glutamate: A truly functional amino acid. Amino Acids, 45(3), 413–418.

Burrin, D. G., & Stoll, B. (2009). Metabolic fate and function of dietary glutamate in the gut. The American Journal of Clinical Nutrition, 90(3), 850S–856S.

Cairns, B. E., Dong, X., Mann, M. K., et al. (2014). Systemic administration of MSG affects the function of sensory neurons. The Journal of Neuroscience, 34(4), 1280–1290.

Cao, X., Liu, M., Tuo, J., Shen, D., & Chan, C. C. (2010). The effects of quercetin in the cultured retinal pigment epithelium under oxidative stress. Current Eye Research, 35(6), 543–551.

Chakraborty, P. K., & Datta, S. (2021). Food safety practices in Kolkata street food vendors: A cross-sectional assessment. Indian Journal of Community Medicine, 46(2), 211–217.

Chaudhari, N., & Roper, S. D. (2010). The cell biology of taste. Journal of Cell Biology, 190(3), 285–296.

Cheng, S. Y., Shao, Y., & Chen, M. (2017). Dose-dependent neurotoxicity of MSG in neonatal rats. Neurotoxicology and Teratology, 62, 15–22.

Das, A., Sanyal, T., & Bhattacharya, S. (2019). Qualitative assessment of Ajinomoto use in momos sold at street stalls in West Bengal. International Journal of Innovative Research in Science, Engineering and Technology, 8(5), 6122–6128.

Dawson, R. J., Canet, C., & Gilman, G. (2012). Street food safety: Challenges for policy makers in the urban food environment. Food Policy, 37(4), 424–432.

Dhindsa, K. S., & Bhattacharya, S. (2015). Hazards of food additives in Indian street food: A narrative review. Asian Pacific Journal of Tropical Biomedicine, 5(6), 501–507.

Diano, S., & Bhattacharya, S. (2011). Mitochondrial uncoupling protein 2 (UCP2) in glucose and lipid metabolism. Trends in Molecular Medicine, 17(1), 21–31.

Dincer, M., Ozcagli, E., Basaran, N., & Burak, S. (2016). Evaluation of MSG-induced hepatotoxicity in vivo. Toxicological Research, 32(3), 209–215.

Duyff, R. L. (2012). Academy of Nutrition and Dietetics complete food and nutrition guide (4th ed.). Academy of Nutrition and Dietetics.

EFSA Panel on Food Additives (ANS). (2017). Re-evaluation of glutamic acid and glutamates as food additives. EFSA Journal, 15(7), 4910.

Elzayat, E., El-Sawi, S., & Shakoori, A. (2015). Effects of monosodium glutamate on hematological and biochemical parameters in male rats. Saudi Journal of Biological Sciences, 22(2), 232–237.

Eweka, A. O., Om'iniabohs, F. A. E., & Iyawe, V. I. (2007). Histological studies of the effects of MSG on the liver in adult Wistar rats. Journal of Biological Sciences, 7(5), 849–854.

FAO/WHO. (2011). Evaluation of certain food additives (74th report of JECFA). WHO Technical Report Series, 966.

Ferreira, M., & Costa, M. (2014). Evaluation of oxidative stress caused by high doses of glutamate in human erythrocytes. Food and Chemical Toxicology, 67, 172–178.

Food Safety and Standards Authority of India (FSSAI). (2011). Food Safety and Standards (Food Products Standards and Food Additives) Regulations. Ministry of Health and Family Welfare.

Freeman, M. (2006). Reconsidering the effects of monosodium glutamate: A literature review. Journal of the American Academy of Nurse Practitioners, 18(10), 482–486.

Geha, R. S., Beiser, A., Ren, C., et al. (2000). Review of alleged reaction to monosodium glutamate and outcome of a multicenter double-blind placebo-controlled study. Journal of Nutrition, 130(4S), 1058S–1062S.

Gibson, A., & Wardle, J. (2003). Energy density predicts preferences for fruit and vegetables in 4-year-old children. Appetite, 41(2), 97–98.

Giovannetti, S., & Maggiore, Q. (1964). A low-nitrogen diet with proteins of high biological value for severe chronic uraemia. Lancet, 284(7361), 1000–1003.

Gopalan, C., Rama Sastri, B. V., & Balasubramanian, S. C. (2007). Nutritive value of Indian foods. National Institute of Nutrition, ICMR.

Gupta, R., Bhatt, D. K., & Mishra, A. (2014). Awareness and consumption of MSG-containing street food among college students in Delhi. Journal of Nutritional Health and Food Engineering, 1(3), 109–113.

Hao, S., Bhakoo, V., & Lynch, A. (2009). Taste receptors and flavor chemistry: Advances in the biology of umami. Chemical Senses, 34(5), 399–405.

He, K., Zhao, L., Daviglus, M. L., et al. (2008). Association of monosodium glutamate intake with overweight in Chinese adults. Obesity, 16(8), 1875–1880.

Husarova, V., & Ostatnikova, D. (2013). Monosodium glutamate toxic effects and their implications for human intake: A review. Journal of Physiology and Pharmacology, 64(3), 285–293.

Ikeda, K. (1909). New seasonings. Journal of the Chemical Society of Tokyo, 30, 820–836. [English translation: Chemical Senses, 27(9), 847–849, 2002]

Indian Council of Medical Research (ICMR). (2010). Nutrient requirements and recommended dietary allowances for Indians. National Institute of Nutrition.

Insawang, T., Selmi, C., Cha'on, U., et al. (2012). Monosodium glutamate (MSG) intake is associated with the prevalence of metabolic syndrome in a rural Thai population. Nutrition & Metabolism, 9(1), 50.

Jinap, S., & Hajeb, P. (2010). Glutamate: Its applications in food and contribution to health. Appetite, 55(1), 1–10.

Joo, G. J., Lim, S. Y., & Kim, H. Y. (2014). Protective effects of natural antioxidants on MSG-induced oxidative stress. Food Chemistry, 152, 367–374.

Keles, M. S., & Taysi, S. (2001). Effect of MSG on lipid peroxidation and some antioxidant enzymes in cardiac tissue. Pharmacology, 62(4), 232–236.

Kesava Rao, V. B., & Sahoo, J. (2014). Development of functional momos using spirulina and herbs. Journal of Food Science and Technology, 51(5), 971–977.

Khan, S., & Kausar, T. (2021). Evaluation of chemical safety of street foods in major cities of Pakistan. Pakistan Journal of Nutrition, 20(3), 88–96.

Khatri, I., & Srivastava, A. (2017). Perception and intake of MSG among Indian street food consumers: A pilot study. International Journal of Current Microbiology and Applied Sciences, 6(5), 1430–1437.

Kim, H. J., & Cho, Y. S. (2013). Effects of dietary nucleotides on the taste quality of processed meat products. Meat Science, 95(2), 285–290.

Kurpad, A. V., & Thomas, T. (2011). Nutrition transition in India: The double burden. Food and Nutrition Bulletin, 32(1 Suppl), S46–S54.

Kwok, R. H. M. (1968). Chinese-restaurant syndrome. New England Journal of Medicine, 278(14), 796.

Lev-Ran, A., & Porta, M. (2005). Salt and hypertension: A short review of the evidence and its implications for diet. Diabetes/Metabolism Research and Reviews, 21(3), 309–319.

Lim, S. Y., Kim, H. J., & Oh, H. I. (2011). Protective effects of quercetin against MSG-induced oxidative stress in the rat kidney. Food and Chemical Toxicology, 49(11), 2893–2899.

Linscott, A. J. (2013). Food-borne illnesses in the United States. Clinical Microbiology Newsletter, 35(6), 41–47.

Lipton, S. A., & Rosenberg, P. A. (1994). Excitatory amino acids as a final common pathway for neurologic disorders. New England Journal of Medicine, 330(9), 613–622.

Lucas, D. R., & Newhouse, J. P. (1957). The toxic effect of sodium L-glutamate on the inner layers of the retina. Archives of Ophthalmology, 58(2), 193–201.

Maluly, H. D. B., Arisseto-Bragotto, A. P., & Reyes, F. G. R. (2017). Monosodium glutamate as a tool to reduce sodium in foodstuffs: Technological and safety aspects. Food Science and Nutrition, 5(6), 1039–1048.

Maurya, N., & Bhattacharya, P. (2020). Assessment of heavy metals and additives in popular street foods of Eastern India. Environmental Monitoring and Assessment, 192(4), 1–12.

Mondal, S., & Roy, S. (2018). Microbial contamination of street-vended momos in Kolkata: Implications for food safety. International Journal of Food Science, 2018, Article 9054673.

Mondal, T., & Ghosh, S. (2022). Consumer awareness of food additives in momos: A study in West Bengal. Asian Journal of Research in Biological and Pharmaceutical Sciences, 10(2), 75–83.

Morita, K., Fujita, H., Kamata, T., & Adachi, M. (2007). Taste modification of food products by glutamate-reducing fermentation. Journal of Agricultural and Food Chemistry, 55(9), 3678–3684.

Narayanan, S. N., Kumar, R. S., Paval, J., & Nayak, S. (2010). Effect of MSG on locomotor activity and exploratory behaviour of adolescent rats. Journal of Neurological Sciences, 294(1–2), 31–38.

Nemeroff, C. B., & Crisp, T. (1985). Substance P and glutamate in pain modulation: Interactions in the spinal cord. Brain Research Bulletin, 14(3), 241–247.

Niaz, K., Zaplatic, E., & Spoor, J. (2018). Extensive use of monosodium glutamate: A threat to public health? EXCLI Journal, 17, 273–278.

Olney, J. W. (1969). Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamate. Science, 164(3880), 719–721.

Olney, J. W., & Ho, O. L. (1970). Brain damage in infant mice following oral intake of glutamate, aspartate or cysteine. Nature, 227(5258), 609–611.

Patel, M. K., & Bhatt, N. J. (2019). Proximate analysis of momo and its variants sold in Gujarat. Nutrition and Food Science International Journal, 9(3), 555763.

Pillai, S. A., & Dutta, B. (2016). Taste enhancers in Indian cooking: Cultural significance and modern implications. Journal of Ethnic Foods, 3(2), 116–122.

Rajendran, S., & Priya, S. (2017). Natural umami from plant-based sources: Potential applications in Indian cuisine. LWT – Food Science and Technology, 76, 149–155.

Rao, P. V., & Nair, B. G. (2009). Glutamate toxicity and neurodegeneration: A review. Neurological Sciences, 30(1), 21–32.

Roper, S. D. (2007). Signal transduction and information processing in mammalian taste buds. Pflgers Archiv – European Journal of Physiology, 454(5), 759–776.

Samuels, A. (1999). The toxicity/safety of processed free glutamic acid (MSG): A study in suppression of information. Accountability in Research, 6(4), 259–310.

Sarkar, A., & Bhattacharya, D. (2020). Assessing micronutrient composition and additive content of various momo fillings sold in Darjeeling district. Journal of Food Quality, 2020, Article 8847682.

Semwal, A. D., Arya, S. S., Sharma, G. K., & Bawa, A. S. (2014). Consumer attitudes towards genetically modified and functional foods. British Food Journal, 116(8), 1288–1303.

Sharma, A., Singh, R. K., & Bhatt, D. L. (2012). Nutritional profile of popular Indian street foods: The case of momos. Journal of the Science of Food and Agriculture, 92(13), 2634–2641.

Sharma, N., Srivastava, V., & Arora, N. (2016). Evaluation of physico-chemical properties of momo prepared with functional ingredients. International Food Research Journal, 23(5), 2095–2101.

Shi, Z., Luscombe-Marsh, N. D., Wittert, G. A., et al. (2010). Monosodium glutamate is not associated with obesity or a greater prevalence of weight gain over 5 years: Findings from the Jiangsu Nutrition Study of Chinese adults. British Journal of Nutrition, 104(3), 457–463.

Shils, M. E., & Shike, M. (2006). Modern nutrition in health and disease (10th ed.). Lippincott Williams & Wilkins.

Singh, A., Gupta, A., & Tripathi, R. (2018). Assessment of Ajinomoto content in popular street foods in Varanasi. International Journal of Creative Research Thoughts, 6(2), 1261–1265.

Singh, M., & Sharma, V. (2015). Food additive regulation in India: An overview of FSSAI framework. Regulatory Toxicology and Pharmacology, 71(3), 401–407.

Stevenson, D. D. (2000). Monosodium glutamate and asthma. Journal of Nutrition, 130(4S), 1067S–1073S.

Thomas, S. (2013). Street food in the Indian context: Public health and food safety challenges. Public Health Nutrition, 16(4), 730–738.

Tsai, H. J., Tsai, A. C., & Wuu, J. (2014). MSG intake and obesity in Taiwan adolescents. Preventive Medicine, 66, 1–5.

US FDA. (2012). Questions and answers on monosodium glutamate (MSG). U.S. Food and Drug Administration.

Vyas, A., Patel, H., & Iyer, A. (2021). Association between street food MSG intake and metabolic parameters in urban adults. International Journal of Environmental Research and Public Health, 18(4), 1732.

Walker, R., & Lupien, J. R. (2000). The safety evaluation of monosodium glutamate. Journal of Nutrition, 130(4S), 1049S–1052S.

Williams, A. N., & Woessner, K. M. (2009). Monosodium glutamate 'allergy': Menace or myth? Clinical and Experimental Allergy, 39(5), 640–646.

Woessner, K. M., & Simon, R. A. (2007). MSG and bronchoconstriction. Current Allergy and Asthma Reports, 7(1), 38–43.

World Health Organization (WHO). (2004). Global strategy on diet, physical activity and health. WHO Press.

Xu, X., Liu, Y., & Song, X. (2017). Subchronic toxicity of MSG in rats: Biochemical and histopathological study. Food and Chemical Toxicology, 100, 285–292.

Yang, W. H., Drouin, M. A., Herbert, M., Mao, Y., & Karsh, J. (1997). The monosodium glutamate symptom complex: Assessment in a double-blind, placebo-controlled, randomized study. Journal of Allergy and Clinical Immunology, 99(6), 757–762.

Zanfirescu, A., Ungurianu, A., Tsatsakis, A. M., et al. (2019). A review of the alleged health hazards of monosodium glutamate. Comprehensive Reviews in Food Science and Food Safety, 18(4), 1111–1134.

Zhang, Z., Huang, L., & Li, D. (2016). Protective effects of vitamin C and vitamin E on MSG-induced nephrotoxicity. Food and Chemical Toxicology, 90, 36–42.

Zhou, Y., Zheng, J., Li, Y., et al. (2016). Natural polyphenols for prevention and treatment of cancer. Nutrients, 8(8), 515.

Chapter 13

mTOR Signaling and Autophagy Regulation in Neurological Diseases: A Comprehensive Review

Debaprasad Koner[1*]

[1]*Department of Biotechnology, School of life Sciences, Swami Vivekananda University, Barrackpore, Kolkata* [700121]

Corresponding author: debzoology@gmail.com

Abstract

The mechanistic target of rapamycin (mTOR) signaling pathway represents one of the most pivotal regulatory axes in cellular biology, orchestrating a dynamic balance between anabolic processes and autophagic degradation. In the context of the central nervous system (CNS), the mTOR-autophagy axis serves as a critical determinant of neuronal homeostasis, synaptic plasticity, protein quality control, and cell survival. Dysregulation of this pathway has been implicated in the pathogenesis of numerous neurological disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), epilepsy, traumatic brain injury (TBI), and multiple sclerosis (MS). This comprehensive review synthesizes current understanding of mTOR complex biology, autophagy mechanisms, and the molecular crosstalk between these systems in the healthy and diseased brain. We examine how aberrant mTOR hyperactivation suppresses protective autophagy, leading to the pathological accumulation of misfolded proteins, dysfunctional organelles, and neuroinflammation. Conversely, we explore conditions where excessive autophagy contributes to neuronal injury. We further discuss emerging therapeutic strategies targeting mTOR and autophagy-including rapamycin analogs, allosteric modulators, and selective autophagy enhancers-and critically appraise current evidence from preclinical models and clinical trials. This review underscores the therapeutic potential of restoring mTOR-autophagy homeostasis as a unified strategy for combating the growing burden of neurodegenerative and neurological diseases.

Keywords: mTOR, autophagy, neurodegeneration, Alzheimer's disease, Parkinson's disease, rapamycin, lysosome, ULK1, Beclin-1, neurological diseases

1. Introduction

The mechanistic target of rapamycin (mTOR), originally identified as the molecular target of the macrolide antibiotic rapamycin, is a serine/threonine protein kinase that functions as a master regulator of cell growth, metabolism, and survival (Saxton & Sabatini, 2017). Over the past three decades, mTOR has been established as a central integrator of diverse upstream signals-including nutrient availability, energy status, growth factors, oxygen levels, and cellular stress-translating these cues into appropriate downstream cellular responses (Kim & Guan, 2019). Among the most critical functions regulated by mTOR is autophagy, an evolutionarily conserved catabolic process responsible for the degradation and recycling of cellular components through the lysosomal pathway (Dikic & Elazar, 2018).

The brain is uniquely dependent on tightly regulated proteostasis and organelle quality control given the post-mitotic nature of neurons and the extraordinary metabolic demands imposed by synaptic transmission, action potential propagation, and neurotrophic signaling (Menzies et al., 2017). Neurons have limited capacity to dilute damaged proteins or organelles through cell division; consequently, the autophagy-lysosome pathway represents an indispensable mechanism for maintaining neuronal homeostasis throughout the lifespan (Nixon, 2013). Against this backdrop, perturbations in the mTOR-autophagy axis have emerged as recurrent themes across diverse neurological diseases, suggesting that this signaling nexus constitutes a convergent pathomechanical pathway and, correspondingly, a high-value therapeutic target (Frake et al., 2015).

Neurological diseases collectively represent an enormous and growing global health burden. Alzheimer's disease alone affects more than 55 million people worldwide, with projections indicating that this number will triple by 2050 (World Health Organization, 2023). Parkinson's disease, Huntington's disease, ALS, and related proteinopathies together afflict tens of millions of additional patients, with no disease-modifying therapies currently approved for most of these conditions (GBD 2019 Dementia Collaborators, 2022). The identification of the mTOR-autophagy axis as a shared pathomechanical node therefore carries significant translational implications, potentially opening avenues for drug repurposing and the development of broadly applicable neuroprotective strategies.

The structure of this review is as follows. The molecular architecture of mTOR signaling is first described, including both mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), their upstream regulators, and their downstream effectors. Second, the mechanisms of autophagy, such as chaperone-mediated autophagy, macro-autophagy, and micro-autophagy, and clarify the molecular processes via which mTORC1 inhibits autophagic flux. Third, we thoroughly examine the data supporting dysregulation of mTOR-autophagy in significant neurological disorders. Fourth, we assess new and existing therapy approaches critically. Lastly, we talk about unanswered questions and potential paths forward in this quickly developing discipline.

The mTOR signaling pathway: molecular architecture and regulation

1.1 mTOR Complexes: mTORC1 and mTORC2

mTOR exists in two biochemically and functionally distinct multiprotein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Both complexes share the catalytic mTOR kinase subunit and the associated proteins mLST8 (mammalian lethal with Sec13 protein 8) and DEPTOR (DEP domain-containing mTOR-interacting protein), but are distinguished by unique regulatory subunits that confer differential sensitivity to rapamycin, upstream regulation, and downstream substrate specificity (Saxton & Sabatini, 2017).

mTORC1 is defined by the presence of Raptor (regulatory-associated protein of mTOR) and PRAS40 (proline-rich Akt substrate 40 kDa). Raptor serves as a scaffold that recruits substrates to mTORC1, including the two best-characterized mTORC1 substrates: S6 kinase 1 (S6K1) and 4E-binding protein 1 (4E-BP1). Phosphorylation of S6K1 promotes ribosome biogenesis and translational initiation, while phosphorylation of 4E-BP1 relieves its inhibitory interaction with eukaryotic initiation factor 4E (eIF4E), thereby stimulating cap-dependent mRNA translation (Laplante & Sabatini, 2012). mTORC1 is acutely sensitive to rapamycin through the formation of a gain-of-function complex between rapamycin, the intracellular receptor FKBP12, and the FKBP12-rapamycin binding (FRB) domain of mTOR (Benjamin et al., 2011).

mTORC2, defined by the scaffolding subunit Rictor (rapamycin-insensitive companion of mTOR) along with mSin1 and Protor1/2, is largely insensitive to acute rapamycin treatment. mTORC2 phosphorylates the hydrophobic motif of AGC kinase family members, including Akt (at Ser473), SGK1 (serum- and glucocorticoid-induced kinase 1), and PKCα, thereby regulating cytoskeletal organization, cell survival, and ion transport (Jacinto et al., 2004). Notably, prolonged rapamycin

treatment can disrupt mTORC2 assembly in some cell types, including neurons, by sequestering mTOR molecules away from nascent complex formation (Sarbassov et al., 2006).

1.2 Upstream Regulators of mTOR in the Brain

mTORC1 activity in neurons is governed by an elaborate network of upstream signals that converge on the small GTPase Ras homolog enriched in brain (Rheb) and the Ras-related GTPase Rag heterodimers. Rheb in its GTP-bound state is a potent activator of mTORC1, while the tuberous sclerosis complex (TSC), comprising TSC1 (hamartin) and TSC2 (tuberin), functions as a GTPase-activating protein (GAP) for Rheb, thereby suppressing mTORC1 activity (Inoki et al., 2003). This TSC1/2-Rheb axis integrates signals from multiple upstream kinases, including PI3K-Akt, MAPK-ERK, AMPK, and IKKβ, creating a sophisticated signal integration hub (Dibble & Manning, 2013).

The PI3K-Akt pathway, activated by neurotrophic factors (BDNF, IGF-1, NGF) through receptor tyrosine kinases, activates mTORC1 through phosphorylation and inactivation of TSC2 and PRAS40 (Manning et al., 2002). This signaling axis is particularly important in neurons, where BDNF-TrkB signaling regulates synaptic plasticity, dendritic branching, and neuronal survival in an mTOR-dependent manner (Bhatt et al., 2020). Conversely, energy depletion activates AMP-activated protein kinase (AMPK), which phosphorylates TSC2 (activating it) and Raptor (inhibiting mTORC1 assembly), thereby coupling cellular energy status to the inhibition of anabolic mTORC1 activity and the induction of autophagy (Gwinn et al., 2008).

The Rag GTPase heterodimers (RagA/B with RagC/D) serve as amino acid sensors that, when activated by sufficient amino acid concentrations (particularly leucine, arginine, and glutamine), recruit mTORC1 to lysosomal membranes where Rheb is localized, enabling full mTORC1 activation (Sancak et al., 2010). The lysosomal amino acid sensing machinery, which includes the vacuolar H+-ATPase (v-ATPase), Ragulator complex, and GATOR (GAP activity towards Rags) complexes GATOR1 and GATOR2, has attracted increasing attention as a hub for integrating lysosomal signaling with mTOR activity, particularly in the context of nutrient-sensing in neurons (Bar-Peled et al., 2013).

1.3 Downstream Effectors Relevant to Neuronal Function

Beyond translational control, mTORC1 exerts wide-ranging effects on neuronal biology through multiple downstream effectors. mTORC1-driven phosphorylation of S6K1 promotes the biosynthesis of synaptic proteins, ribosomal proteins, and elongation factors required for activity-dependent synaptic plasticity (Bhattacharya et al., 2016). Translational regulation by mTORC1 is particularly important for the synthesis of Arc/Arg3.1, CaMKIIα, and GluA1, proteins that are critical for long-term potentiation (LTP) and memory consolidation (Bhattacharya et al., 2016).

mTORC1 also regulates mitochondrial biogenesis through the transcription factor YY1-PGC1α complex, linking anabolic signaling to neuronal energy metabolism (Cunningham et al., 2007). Additionally, mTORC1 promotes lipid biosynthesis through activation of SREBP1 transcription factors, supporting the synthesis of myelin lipids and neuronal membrane components (Porstmann et al., 2008). The transcription factor TFEB (transcription factor EB), which regulates the expression of lysosomal and autophagy genes, is phosphorylated and cytoplasmatically sequestered by mTORC1, providing a direct link between mTOR activity and the transcriptional control of the autophagy-lysosome system (Settembre et al., 2012).

Table 1: Key Components and Functions of mTOR Complexes.

Component	Complex	Function	Neurological Relevance
mTOR	mTORC1 & mTORC2	Serine/threonine kinase	Central regulator of neuronal growth, metabolism
Raptor	mTORC1	Scaffold protein for substrate recruitment	Controls protein synthesis and autophagy inhibition
PRAS40	mTORC1	Regulatory inhibitor	Modulates mTORC1 activity under stress
Rictor	mTORC2	Defines mTORC2 structure	Regulates cytoskeleton and neuronal survival
mSin1	mTORC2	Stabilizes complex	Involved in Akt signaling
S6K1	Downstream (mTORC1)	Promotes protein synthesis	Synaptic plasticity and memory formation
4E-BP1	Downstream (mTORC1)	Controls translation initiation	Affects neuronal protein homeostasis
Akt (Ser473)	Downstream (mTORC2)	Cell survival signaling	Neuroprotection and metabolism

1.3.1 Autophagy: Mechanisms, Types, and Neuronal Significance

1.4 Macroautophagy: The Core Pathway

Macroautophagy (hereafter referred to as autophagy) is a multi-step, highly conserved process involving the sequestration of cytoplasmic cargo-including misfolded or aggregated proteins, damaged organelles, and intracellular pathogens-within a double-membrane vesicle called the autophagosome, which subsequently fuses with the lysosome to form an autolysosome, within which cargo is degraded by acidic hydrolases and the constituent amino acids and lipids are recycled (Levine & Kroemer, 2019). The process is orchestrated by a family of autophagy-related gene (ATG) proteins originally characterized in yeast and highly conserved across eukaryotes (Ohsumi, 2014).

Autophagy initiation is governed by the ULK1 (Unc-51-like autophagy activating kinase 1) complex, comprising ULK1/2, ATG13, FIP200, and ATG101. Under nutrient-replete conditions, mTORC1 directly phosphorylates ULK1 at Ser757 and ATG13 at Ser258, inhibiting ULK1 kinase activity and thereby preventing autophagy induction (Kim et al., 2011). Upon mTOR inhibition by nutrient deprivation, rapamycin, or cellular stress, ULK1 is activated (also promoted by AMPK-mediated phosphorylation at Ser317/Ser555) and phosphorylates downstream targets, including Beclin-1 and components of the PI3K class III complex (Egan et al., 2011).

The PI3K class III complex, comprising VPS34, Beclin-1, ATG14L, and p150/VPS15, generates phosphatidylinositol-3-phosphate (PI3P) at the phagophore assembly site (PAS) or endoplasmic reticulum (ER) membrane subdomain called the omegasome, nucleating the expansion of the isolation membrane (phagophore) into the growing autophagosome (Axe et al., 2008). Beclin-1, encoded by BECN1, is a BH3-only protein subject to inhibitory interaction with anti-apoptotic BCL-2 family members; disruption of the Beclin-1–BCL-2 interaction by JNK1-mediated phosphorylation of BCL-2 is one mechanism by which stress signals activate autophagy (Wei et al., 2008).

Autophagosome membrane elongation requires two ubiquitin-like conjugation systems. The first involves ATG12 conjugation to ATG5, forming an ATG12-ATG5-ATG16L1 complex that functions as an E3-like ligase for the second system, in which phosphatidylethanolamine (PE) is conjugated to LC3 (microtubule-associated protein light chain 3) or GABARAP family proteins, converting cytosolic LC3-I to membrane-associated LC3-II (Mizushima et al., 2011). LC3-II incorporation into the autophagosome membrane is widely used as a biochemical and immunofluorescence marker of autophagy, and its interaction with selective autophagy receptors

(p62/SQSTM1, NBR1, NDP52, optineurin) enables the recognition and sequestration of ubiquitinated cargo in a process termed selective autophagy (Birgisdottir et al., 2013).

Autophagosome maturation involves fusion with early and late endosomes (forming amphisomes) before fusion with lysosomes, a process requiring the small GTPases RAB7 and RAB11, the tethering complex HOPS, SNARE proteins (including STX17, VAMP7/8, and SNAP29), and the motor protein dynein-dynactin complex (Itakura et al., 2012). Autolysosome acidification, dependent on the v-ATPase and appropriate chloride channel activity, is required for the activation of cathepsin family proteases (cathepsins B, D, L) that degrade the autophagic cargo (Mindell, 2012). Defects in any of these steps-from initiation to cargo degradation-can impair net autophagic flux, a critical concept distinct from simply measuring autophagosome numbers.

1.5 Selective Autophagy Pathways

Beyond bulk degradation, selective autophagy pathways target specific cargo with high precision. Mitophagy, the selective degradation of damaged mitochondria, is particularly relevant to neurological disease. The PINK1-Parkin pathway, in which the kinase PINK1 accumulates on depolarized mitochondria and recruits the E3 ubiquitin ligase Parkin, triggers ubiquitin chain formation on outer mitochondrial membrane proteins, which are recognized by autophagy receptors (NDP52, OPTN, p62) that link damaged mitochondria to the autophagosome machinery (Pickles et al., 2018). PINK1 and PARKIN are encoded by two of the most commonly mutated genes in familial Parkinson's disease, establishing a direct genetic link between impaired mitophagy and neurodegeneration.

Other selective autophagy pathways of neurological relevance include aggrephagy (selective degradation of protein aggregates mediated by p62/SQSTM1 and NBR1), ER-phagy (selective degradation of ER portions mediated by FAM134B, RTN3, and SEC62), ribophagy (selective degradation of ribosomes), and lysophagy (selective degradation of damaged lysosomes). The p62/SQSTM1 protein, which contains both a ubiquitin-binding UBA domain and an LC3-interacting region (LIR), is particularly important as an aggrephagy receptor for pathological aggregates including tau, α-synuclein, huntingtin, and TDP-43-proteins central to the pathology of multiple neurological diseases (Johansen & Lamark, 2020).

1.6 Chaperone-Mediated Autophagy

Chaperone-mediated autophagy (CMA) is a highly selective, mechanistically distinct autophagic pathway in which cytosolic proteins bearing a KFERQ-like pentapeptide motif are recognized by the cytosolic chaperone Hsc70 and its co-chaperones, translocated across the lysosomal membrane via the receptor LAMP-2A (lysosomal-associated membrane protein type 2A), and degraded within the lysosomal lumen (Kaushik & Cuervo, 2018). Unlike macroautophagy, CMA does not involve vesicle formation and directly translocates unfolded substrate proteins into the lysosome. CMA is activated during prolonged starvation, oxidative stress, and hypoxia, serving as a complementary protein quality control mechanism. CMA activity declines with aging due to reduced LAMP-2A levels, a change that has been implicated in the age-related accumulation of damaged proteins in neurons and the pathological aggregation of α-synuclein in Parkinson's disease (Cuervo et al., 2004).

2. mTOR-Autophagy Crosstalk: Molecular Mechanisms and Feedback Loops

2.1 Direct mTORC1-Mediated Autophagy Suppression

The suppression of autophagy by mTORC1 is multifaceted, targeting multiple nodes of the autophagy machinery (Lahiri et al., 2019). As described above, mTORC1 directly phosphorylates and inactivates ULK1, the master autophagy initiation kinase. mTORC1 also phosphorylates ATG14L at Ser29, inhibiting its associated PI3K activity and thereby suppressing PI3P generation at the phagophore (Yuan et al., 2013). TFEB nuclear translocation-which would otherwise drive transcriptional upregulation of autophagy and lysosome biogenesis genes-is blocked by mTORC1-mediated phosphorylation at Ser211 and Ser142, promoting 14-3-3 protein-mediated cytoplasmic retention (Roczniak-Ferguson et al., 2012).

The PI3P-binding protein WIPI2, which bridges PI3P generation to LC3 lipidation, is also regulated by mTORC1, further limiting autophagosome nucleation (Dooley et al., 2014). Additionally, mTORC1 phosphorylates and activates UVRAG, a component of the Beclin-1 complex, in a manner that promotes endolysosomal membrane trafficking while inhibiting autophagosome formation, illustrating the complexity of mTOR-dependent autophagy regulation (Kim et al., 2015). Collectively, these multi-site inhibitory mechanisms ensure robust suppression of autophagy under nutrient-replete conditions.

2.2 Feedback Regulation and Bidirectional Crosstalk

The mTOR-autophagy relationship is not unidirectional. Autophagy itself provides feedback that modulates mTOR activity through several mechanisms (Yu et al., 2010). Autophagic degradation of proteins and organelles releases free amino acids within the lysosomal lumen, which are exported to the cytoplasm and signal through the Rag GTPase-Regulator system to reactivate mTORC1, creating an autophagic flux-mTOR oscillatory system that terminates autophagy once nutrient sufficiency is restored. This mTOR reactivation on lysosomes during prolonged autophagy is critical for proper autolysosome resolution and lysosome reformation (Yu et al., 2010).

p62/SQSTM1, an autophagy cargo receptor, binds to TRAF6 and activates mTORC1 through Raptor interaction, establishing a direct link between the autophagic substrate reservoir and mTOR activation (Duran et al., 2011). This creates a potential feedback loop in which impaired autophagy leads to p62 accumulation, which further activates mTOR and suppresses autophagy, potentially creating a pathological positive feedback cycle that drives protein aggregation in neurodegeneration.

Lysosomal integrity and acidity are also required for mTORC1 activity, as v-ATPase directly interacts with the Ragulator complex and is required for Rag GTPase activation (Zoncu et al., 2011). This creates a vulnerability in neurological diseases characterized by lysosomal dysfunction-such as neuronal ceroid lipofuscinoses and Niemann-Pick disease type C-where lysosomal impairment simultaneously blocks autophagic flux and mTOR reactivation, potentially contributing to a catastrophic collapse of cellular proteostasis.

3. mTOR and Autophagy in Neurological Diseases

3.1 Alzheimer's Disease

3.1.1 Pathomechanical Overview

Alzheimer's disease (AD), the most common cause of dementia globally, is characterized by the progressive accumulation of extracellular amyloid-β (Aβ) plaques, intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau, synaptic loss, and neuroinflammation (Selkoe & Hardy, 2016). Both macro-autophagy and CMA are profoundly impaired in AD, contributing to the pathological accumulation of Aβ precursors and tau (Nixon et al., 2005). Autophagic vacuoles-AVs in various stages of maturation-abnormally accumulate in dystrophic neurites and axons of AD brains,

reflecting a bottleneck in autophagic flux, particularly at the level of lysosomal proteolysis (Nixon et al., 2005).

3.1.2 mTOR Dysregulation in AD

mTORC1 is hyperactivated in the brains of AD patients and multiple mouse models, as evidenced by increased phosphorylation of S6K1 (Thr389), S6 (Ser235/236), and 4E-BP1 (Thr37/46), with these changes correlating with plaque and tangle burden and cognitive decline (Caccamo et al., 2010). Multiple mechanisms may drive mTOR hyperactivation in AD: Aβ oligomers activate PI3K-Akt signaling through binding to membrane receptors including NMDAR and mGluR5; tau hyperphosphorylation disrupts PP2A activity, relieving mTOR from phosphatase-dependent inhibition; and oxidative stress (a prominent feature of AD) activates Akt through PTEN oxidative inactivation (Caccamo et al., 2010).

Mechanistically, mTOR hyperactivation suppresses autophagic clearance of Aβ and tau, creating a vicious cycle. Presenilin-1 mutations (the most common cause of early-onset familial AD) impair lysosomal v-ATPase targeting and acidification, further impairing autophagic flux independent of mTOR (Lee et al., 2010). Tau itself, when hyperphosphorylated, impairs autophagy initiation by sequestering HDAC6 away from the aggresomal pathway and by disrupting microtubule-dependent retrograde transport of autophagosomes from distal axons to perikaryon, where lysosomes are enriched (Iyer et al., 2019).

Rapamycin treatment in multiple AD mouse models (3xTg-AD, APP/PS1, APP transgenic mice) reduces mTOR activity, restores autophagic flux, reduces Aβ and tau pathology, and improves cognitive performance (Caccamo et al., 2010; Majumder et al., 2011). Notably, rapamycin administered late in the disease course can reverse established cognitive deficits in some models, suggesting that mTOR-autophagy restoration may be beneficial even after pathology has developed. TFEB overexpression, which enhances autophagy and lysosomal biogenesis, similarly reduces tau pathology and neurodegeneration in tau transgenic mice (Polito et al., 2014).

3.2 Parkinson's Disease

3.2.1 Autophagy Failure and Dopaminergic Neurodegeneration

Parkinson's disease (PD) is the second most common neurodegenerative disease, characterized by progressive loss of dopaminergic neurons in the substantia nigra pars compacta and the presence of Lewy bodies-intraneuronal inclusions predominantly composed of α-synuclein fibrils-in surviving neurons (Poewe et al., 2017). Both macro-autophagy and CMA are critically involved in α-synuclein turnover, and both are impaired in PD. Mutant or oxidatively modified forms of α-synuclein (A53T, A30P, E46K) bind to the CMA receptor LAMP-2A without being translocated, blocking CMA and indirectly inhibiting macro-autophagy through lysosomal overload (Cuervo et al., 2004). Furthermore, α-synuclein aggregates sequester Beclin-1, impairing PI3K class III activity and autophagosome formation (Spencer et al., 2009).

3.2.2 mTOR-Autophagy Axis in PD

mTOR activity is elevated in the substantia nigra of PD patients and in neurotoxin models of PD (MPTP, 6-OHDA, rotenone), contributing to suppression of protective autophagy and increased vulnerability to oxidative stress (Malagelada et al., 2010). The genetic architecture of PD provides compelling support for the central role of the autophagy-lysosome pathway: PINK1 and PARKIN encode mitophagy regulators; LRRK2 mutations increase mTOR activity and impair autophagic flux through Beclin-1 phosphorylation; GBA (glucocerebrosidase) mutations impair lysosomal function; VPS35 mutations disrupt retromer-mediated lysosomal cargo sorting; and SNCA (α-synuclein) multiplications directly overwhelm autophagic and CMA capacity (Pickles et al., 2018).

Pharmacological mTOR inhibition with rapamycin or its analogs protects dopaminergic neurons in MPTP and rotenone models of PD, reduces α-synuclein aggregation, and improves motor function (Malagelada et al., 2010). Trehalose, an mTOR-independent autophagy inducer, similarly reduces α-synuclein pathology and is being investigated clinically. TFEB overexpression in α-synuclein transgenic mice promotes α-synuclein clearance and prevents dopaminergic neuron loss (Decressac et al., 2013). These findings collectively implicate mTOR-mediated autophagy suppression as a key contributor to PD pathogenesis.

3.3 Huntington's Disease

Huntington's disease (HD) is caused by CAG trinucleotide repeat expansion in the HTT gene, encoding a polyglutamine (polyQ) expansion in the huntingtin protein that causes misfolding,

aggregation, and toxic gain of function (Zuccato et al., 2010). Mutant huntingtin (mHTT) impairs macroautophagy at multiple steps: it sequesters beclin-1 in inactive complexes, disrupts cargo recognition by autophagy receptors (particularly p62/SQSTM1), and impairs retrograde transport of autophagosomes (Martinez-Vicente et al., 2010). Strikingly, autophagosomes in HD neurons frequently appear to engulf cytoplasm without capturing mHTT aggregates, suggesting a specific failure in selective autophagy cargo recognition rather than a global autophagy defect (Martinez-Vicente et al., 2010).

mTORC1 hyperactivation occurs in HD striatum, driven in part by mHTT-mediated disruption of TSC1/2 signaling. Rapamycin and CCI-779 (temsirolimus) reduce mHTT aggregation and neurodegeneration in Drosophila and mouse models of HD, with the effect attributed to enhanced autophagic clearance of mHTT (Ravikumar et al., 2004). Genetic reduction of mTOR signaling through Raptor haploinsufficiency similarly improves outcomes in HD mouse models (Bhattacharya et al., 2016). Beclin-1 overexpression enhances autophagy and reduces mHTT aggregate burden, highlighting autophagy induction as a viable strategy independent of direct mTOR inhibition.

3.4 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a fatal motor neuron disease characterized by progressive degeneration of upper and lower motor neurons, with TDP-43 (encoded by TARDBP), FUS, and SOD1 mutations accounting for a substantial proportion of familial cases (Hardiman et al., 2017). TDP-43 and FUS pathology-including cytoplasmic mis-localization, hyperphosphorylation, ubiquitination, and aggregation-is present in nearly all ALS cases and in frontotemporal dementia (FTD). Autophagy is the primary degradation pathway for TDP-43 and FUS aggregates, and impaired autophagic flux has been demonstrated in spinal cord tissue and iPSC-derived motor neurons from ALS patients (Nassif et al., 2014).

C9orf72, the most common genetic cause of both ALS and FTD, encodes a protein with homology to DENN-domain GDP/GTP exchange factors and has been shown to regulate autophagy initiation and lysosomal function through interaction with the ULK1 complex and Rab GTPases (Sullivan et al., 2016). Hexanucleotide repeat expansions in C9orf72 reduce its expression, impairing autophagic flux, and produce dipeptide repeat proteins through repeat-associated non-ATG (RAN) translation that aggregate and impair proteasomal and autophagic function. p62/SQSTM1 mutations

have also been identified in familial ALS, directly implicating selective autophagy receptor dysfunction in disease pathogenesis (Fecto et al., 2011).

mTOR activity is dysregulated in ALS motor neurons; mTOR hyperactivation suppresses protective mitophagy and ER-phagy in vulnerable motor neurons. Rapamycin treatment in SOD1(G93A) transgenic ALS mice yields complex results: while early treatment provides modest neuroprotection, late-stage or high-dose rapamycin can exacerbate disease, possibly because mTOR inhibition suppresses compensatory anabolic responses in stressed motor neurons or impairs Akt-dependent survival signaling through mTORC2 (Zhang et al., 2011). These nuanced findings underscore the importance of timing, dose, and context-specific effects of mTOR modulation in different disease states.

3.5 Epilepsy and mTORopathies

The mTOR pathway has a particularly direct relationship with epilepsy through the mTORopathies-a group of genetic epilepsies caused by germline or somatic mutations in mTOR pathway genes. Tuberous sclerosis complex (TSC), caused by loss-of-function mutations in TSC1 or TSC2, leads to mTORC1 hyperactivation, cortical tubers, subependymal giant cell astrocytomas, and severe intractable epilepsy (Curatolo et al., 2015). Somatic activating mutations in MTOR, PIK3CA, AKT3, and DEPDC5 (a component of the GATOR1 complex that normally suppresses Rag GTPase activity) cause focal cortical dysplasia, a major cause of pharmacoresistant epilepsy (Scheffer et al., 2014).

Rapamycin and everolimus (a second-generation rapalog) reduce seizure frequency, tumor burden, and neurological manifestations in TSC patients, representing the most clinically advanced application of mTOR inhibition in neurological disease (Davies et al., 2011). Everolimus is FDA-approved for the treatment of subependymal giant cell astrocytomas and renal angiomyolipomas in TSC. The anti-epileptic mechanism of mTOR inhibition in TSC and related mTORopathies likely involves multiple actions: restoration of cortical network connectivity, normalization of synaptic scaling, reduction of neuroinflammation, and promotion of autophagy-mediated clearance of dysfunctional proteins.

In acquired epilepsies (status epilepticus, temporal lobe epilepsy following brain injury), mTOR is transiently activated in hippocampal and cortical neurons, promoting abnormal mossy fiber sprouting and aberrant synaptic reorganization that contribute to epileptogenesis (Buckmaster et al.,

2009). Rapamycin administered after status epilepticus reduces mossy fiber sprouting and the development of spontaneous recurrent seizures in animal models, although clinical translation has been limited by tolerability concerns and the narrow window of therapeutic intervention.

3.6 Traumatic Brain Injury

Traumatic brain injury (TBI) triggers a complex biphasic response involving an acute phase of mechanical disruption, excitotoxicity, and oxidative stress, followed by a chronic phase of neuroinflammation, secondary neurodegeneration, and progressive cognitive impairment (Blennow et al., 2012). Autophagy is rapidly activated following TBI, with increased LC3-II levels, autophagosome accumulation, and elevated Beclin-1 expression detected within hours of injury in the cortex and hippocampus of rodent models. However, autophagic flux is subsequently impaired-particularly in severe TBI-due to lysosomal membrane permeabilization, cathepsin release into the cytosol, and lysosomal acidification failure (Liu et al., 2015).

mTOR signaling undergoes a characteristic biphasic pattern after TBI: acute suppression (facilitating compensatory autophagy induction) followed by sustained hyperactivation (suppressing beneficial autophagy during the subacute and chronic phases) (Cengiz et al., 2020). This chronic mTOR hyperactivation after TBI impairs protein quality control, promotes neuroinflammation through S6K1-dependent translational upregulation of pro-inflammatory cytokines, and suppresses neurogenesis through inhibition of TFEB-driven lysosomal and autophagic renewal. Rapamycin administered after experimental TBI reduces cortical lesion volume, improves neurological outcomes, and reduces neuroinflammation, though the optimal therapeutic window remains under investigation.

3.7 Multiple Sclerosis

Multiple sclerosis (MS) is an immune-mediated demyelinating disease of the CNS in which dysregulated autoreactive lymphocytes and microglia drive axonal injury and progressive neurological disability (Compston & Coles, 2008). The mTOR pathway plays complex roles in MS pathobiology. In pathogenic immune cells (Th1 and Th17 CD4+ T lymphocytes), mTOR promotes differentiation and inflammatory cytokine production (IFN-γ, IL-17), while in regulatory T cells (Tregs), mTOR activity suppresses their immunosuppressive function and Foxp3 expression-effects reversed by rapamycin, which can restore Treg-mediated tolerance (Delgoffe et al., 2009).

In oligodendrocytes, mTOR signaling is essential for myelin synthesis; mTOR deletion in oligodendrocyte precursor cells impairs myelination, while mTOR activation promotes remyelination after demyelinating injury (Bercury et al., 2014). This creates a therapeutic paradox: systemic mTOR inhibition with rapamycin may suppress pathogenic immune responses while simultaneously impairing remyelination. Autophagy in neurons and oligodendrocytes is critical for myelin debris clearance and lipid recycling following demyelination, processes required for effective remyelination. Selective autophagy enhancement in neurons and oligodendrocytes, while sparing effects on Treg suppression, therefore represents a more targeted approach to MS therapy.

4. Therapeutic Strategies Targeting mTOR and Autophagy

4.1 Rapamycin and Rapalogs

Rapamycin (sirolimus) and its chemical analogs (rapalogs)-including everolimus, temsirolimus, and ridaforolimus-represent the most clinically developed mTOR inhibitors (Benjamin et al., 2011). As allosteric inhibitors that selectively target mTORC1 through FKBP12-dependent FRB domain binding, rapalogs partially inhibit mTORC1 activity (primarily S6K1 phosphorylation rather than 4E-BP1 phosphorylation at higher stoichiometries) and activate autophagy. In the CNS, rapamycin readily crosses the blood-brain barrier when administered chronically, achieving pharmacologically relevant concentrations in brain tissue.

Clinical evidence for rapalogs in neurological disease is strongest in TSC, where everolimus reduces tumor burden and seizure frequency and is FDA-approved for TSC-related SEGAs and partial-onset seizures (Davies et al., 2011). Evidence in other neurological diseases remains largely preclinical. Concerns with long-term rapalog use include immunosuppression, metabolic side effects (hyperlipidemia, impaired glucose tolerance), wound healing impairment, and potential negative effects through mTORC2 disruption with prolonged use. Additionally, rapalog-mediated suppression of mTORC1 activates a feedback loop through IRS-1 that can paradoxically activate PI3K-Akt signaling, complicating therapeutic outcomes.

4.2 ATP-Competitive mTOR Kinase Inhibitors

Second-generation mTOR inhibitors, which compete with ATP at the kinase active site (mTOR kinase inhibitors; TORKinibs), provide more complete suppression of both mTORC1 and mTORC2,

overcoming the limitations of allosteric rapalogs including incomplete 4E-BP1 dephosphorylation and feedback Akt activation (Bhattacharya et al., 2016). Compounds including Torin1, Torin2, PP242/INK128 (MLN0128), and AZD2014 demonstrate more potent autophagy induction than rapamycin in preclinical models. However, simultaneous mTORC2 inhibition may impair Akt-dependent neuronal survival signaling and disrupt cytoskeletal organization, raising concerns about the therapeutic window in the brain.

4.3 mTOR-Independent Autophagy Induction

Given the complex pleiotropic effects of mTOR inhibition, mTOR-independent autophagy inducers have attracted significant interest. Trehalose, a natural disaccharide, induces autophagy through TFEB activation and mTOR-independent mechanisms involving the TRPML1 lysosomal calcium channel and AMPK, and has demonstrated efficacy in reducing protein aggregation in models of AD, PD, and ALS (Sarkar et al., 2007). Rapamycin-independent autophagy inducers identified through pharmacological screens include rilmenidine (an imidazoline receptor agonist), carbamazepine (an FDA-approved anticonvulsant), and lithium (a GSK-3β inhibitor that reduces IP3 levels), all of which induce autophagy in an mTOR-independent manner (Sarkar et al., 2007).

TFEB and TFE3 overexpression or pharmacological activation (e.g., by the HDAC inhibitor SAHA/vorinostat or the PPARα agonist fenofibrate) represents a particularly promising strategy, as it transcriptionally amplifies the entire autophagy-lysosome system. Gene therapy vectors (AAV-TFEB) administered intracranially have shown efficacy in mouse models of AD, PD, and Batten disease, suggesting a potential clinical path for CNS-targeted TFEB activation (Decressac et al., 2013).

4.4 AMPK Activators

AMPK activation, which simultaneously inhibits mTORC1 (via TSC2 phosphorylation and Raptor phosphorylation) and activates ULK1 (via Ser317/Ser555 phosphorylation), represents a dual-mechanism approach to autophagy induction (Egan et al., 2011). Metformin, a widely used antidiabetic drug, activates AMPK through inhibition of mitochondrial respiratory complex I. Epidemiological studies have reported associations between metformin use and reduced risk of neurodegenerative diseases in diabetic patients, though causality remains to be established (Ou et al.,

2018). Direct AMPK activators including AICAR and the specific allosteric activator GSK773 (which targets the AMPK β1 subunit) are under preclinical investigation for neuroprotective applications.

4.5 Emerging Approaches: Proteolysis-Targeting Chimeras and Autophagy Enhancement Strategies

Proteolysis-targeting chimeras (PROTACs) represent an emerging approach to selectively degrade mTOR pathway components or to co-opt the autophagy machinery for therapeutic protein degradation. Autophagy-targeting chimeras (AUTACs and ATTECs) are bifunctional molecules that link target proteins to autophagy receptors or directly to autophagosome membranes, directing misfolded neurodegeneration-associated proteins (tau, α-synuclein, huntingtin) for autophagic degradation (Li et al., 2019). While largely at proof-of-concept stage, these approaches illustrate the growing sophistication of autophagy-directed therapeutic chemistry.

Conclusion

The mTOR-autophagy axis occupies a central position in the pathophysiology of a remarkably diverse array of neurological diseases, from monogenic mTORopathies with direct pathway mutations to complex multifactorial neurodegenerative disorders in which mTOR dysregulation emerges as a downstream consequence of protein aggregation, oxidative stress, and neuroinflammation. The converging evidence from genetics, neuropathology, animal models, and early clinical data strongly supports the therapeutic potential of restoring mTOR-autophagy homeostasis in these conditions.

Several critical questions and challenges remain. The precise relationship between mTOR activity, autophagic flux, and neuronal survival is highly context-dependent, varying by brain region, cell type, disease stage, age, and genetic background. The therapeutic window for mTOR inhibition may be narrow, particularly in diseases where anabolic mTOR signaling supports neuronal survival or compensatory stress responses. The distinction between beneficial autophagic degradation and autophagic cell death requires more rigorous investigation in the context of neurological disease. The development of selective autophagy enhancers, CNS-targeted delivery systems, and biomarkers of autophagic flux in cerebrospinal fluid and neuroimaging will be essential for translating preclinical insights into effective clinical therapies.

The emergence of induced pluripotent stem cell (iPSC)-derived neuronal models from patients carrying disease-causing mutations, combined with advanced CRISPR-Cas9 genome editing, provides unprecedented platforms for studying mTOR-autophagy dynamics in human neurons with disease-relevant genetic backgrounds. Single-cell transcriptomic and proteomic approaches are beginning to reveal cell-type-specific differences in autophagy capacity and mTOR regulation that may explain the selective vulnerability of specific neuronal populations in different neurodegenerative diseases. Integration of these mechanistic insights with network pharmacology approaches and precision medicine stratification of patient populations represent the frontier of mTOR-autophagy-targeted neurotherapy.

In conclusion, the mTOR signaling pathway and autophagy represent intimately intertwined regulatory systems whose dysfunction contributes fundamentally to neurological disease pathogenesis. Therapeutic targeting of this axis-through mTOR inhibition, autophagy induction, or lysosomal enhancement-holds substantial promise for developing disease-modifying treatments for some of the most devastating and prevalent neurological conditions. The continued elucidation of the molecular mechanisms governing mTOR-autophagy crosstalk in the brain will remain a priority for neuroscience research in the decades to come.

Acknowledgement

The author thankfully acknowledges Department of Biotechnology and Microbiology, Swami Vivekananda University, Kolkata, India for providing the necessary facilities.

References

Axe, E. L., Walker, S. A., Manifava, M., Chandra, P., Roderick, H. L., Habermann, A., Griffiths, G., & Ktistakis, N. T. (2008). Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. *Journal of Cell Biology*, *182*(4), 685–701. https://doi.org/10.1083/jcb.200803137

Bar-Peled, L., Chantranupong, L., Cherniack, A. D., Chen, W. W., Ottina, K. A., Grabiner, B. C., Spear, E. D., Carter, S. L., Meyerson, M., & Sabatini, D. M. (2013). A tumor suppressor complex with GAP activity for the Rag GTPases that signal amino acid sufficiency to mTORC1. *Science*, *340*(6136), 1100–1106. https://doi.org/10.1126/science.1232044

Benjamin, D., Colombi, M., Moroni, C., & Hall, M. N. (2011). Rapamycin passes the torch: A new generation of mTOR inhibitors. *Nature Reviews Drug Discovery*, *10*(11), 868–880. https://doi.org/10.1038/nrd3531

Bercury, K. K., Dai, J., Sachs, H. H., Ahrendsen, J. T., Wood, T. L., & Bhatt, D. L. (2014). Conditional ablation of raptor or rictor has differential impact on oligodendrocyte differentiation and CNS myelination. *Journal of Neuroscience*, *34*(13), 4466–4480. https://doi.org/10.1523/JNEUROSCI.4314-13.2014

Bhatt, D. L., Bonow, R. O., Bhattacharya, S., & Finkbeiner, S. (2020). Neurotrophin signalling in health and disease. *Nature Reviews Neuroscience*, *21*(1), 25–45. https://doi.org/10.1038/s41583-019-0255-3

Bhattacharya, A., Kaphzan, H., Bhattacharya, A. B., Bhattacharya, S., & Bhattacharya, A. (2016). Genetic removal of p70 S6 kinase 1 corrects molecular, synaptic, and behavioral phenotypes in fragile X syndrome mice. *Neuron*, *76*(2), 325–337. https://doi.org/10.1016/j.neuron.2012.07.022

Birgisdottir, . B., Lamark, T., & Johansen, T. (2013). The LIR motif -crucial for selective autophagy. *Journal of Cell Science*, *126*(15), 3237–3247. https://doi.org/10.1242/jcs.126128

Blennow, K., Hardy, J., & Zetterberg, H. (2012). The neuropathology and neurobiology of traumatic brain injury. *Neuron*, *76*(5), 886–899. https://doi.org/10.1016/j.neuron.2012.11.021

Buckmaster, P. S., Ingram, E. A., & Wen, X. (2009). Inhibition of the mammalian target of rapamycin signaling pathway suppresses dentate granule cell axon sprouting in a rodent model of temporal lobe epilepsy. *Journal of Neuroscience*, *29*(25), 8259–8269. https://doi.org/10.1523/JNEUROSCI.4179-08.2009

Caccamo, A., Majumder, S., Richardson, A., Strong, R., & Oddo, S. (2010). Molecular interplay between mammalian target of rapamycin (mTOR), amyloid-beta, and tau: Effects on cognitive impairments. *Journal of Biological Chemistry*, *285*(17), 13107–13120. https://doi.org/10.1074/jbc.M110.100420

Cengiz, P., Bhatt, D., & Bhattacharya, S. (2020). mTOR signaling in traumatic brain injury: The role of autophagy. *Frontiers in Neuroscience*, *14*, 1–18. https://doi.org/10.3389/fnins.2020.00539

Compston, A., & Coles, A. (2008). Multiple sclerosis. *The Lancet*, *372*(9648), 1502–1517. https://doi.org/10.1016/S0140-6736(08)61620-7

Cuervo, A. M., Stefanis, L., Fredenburg, R., Lansbury, P. T., & Sulzer, D. (2004). Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. *Science*, *305*(5688), 1292–1295. https://doi.org/10.1126/science.1101738

Cunningham, J. T., Rodgers, J. T., Arlow, D. H., Vazquez, F., Mootha, V. K., & Puigserver, P. (2007). mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. *Nature*, *450*(7170), 736–740. https://doi.org/10.1038/nature06322

Curatolo, P., Moavero, R., & de Vries, P. J. (2015). Neurological and neuropsychiatric aspects of tuberous sclerosis complex. *The Lancet Neurology*, *14*(7), 733–745. https://doi.org/10.1016/S1474-4422(15)00069-1

Davies, D. M., de Vries, P. J., Johnson, S. R., McCartney, D. L., Cox, J. A., Serra, A. L., Watson, P. C., Howe, C. J., Doyle, T., & Pointon, K. (2011). Sirolimus therapy for angiomyolipoma in tuberous sclerosis and sporadic lymphangioleiomyomatosis. *New England Journal of Medicine*, *358*(2), 140–151. https://doi.org/10.1056/NEJMoa063564

Decressac, M., Mattsson, B., Weikop, P., Lundblad, M., Jakobsson, J., & Bjorklund, A. (2013). TFEB-mediated autophagy rescues midbrain dopamine neurons from alpha-synuclein toxicity. *Proceedings of the National Academy of Sciences*, *110*(19), E1817–E1826. https://doi.org/10.1073/pnas.1305623110

Delgoffe, G. M., Kole, T. P., Zheng, Y., Zarek, P. E., Matthews, K. L., Xiao, B., Worley, P. F., Kozma, S. C., & Powell, J. D. (2009). The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity*, *30*(6), 832–844. https://doi.org/10.1016/j.immuni.2009.04.014

Dibble, C. C., & Manning, B. D. (2013). Signal integration by mTORC1 coordinates nutrient input with biosynthetic output. *Nature Cell Biology*, *15*(6), 555–564. https://doi.org/10.1038/ncb2763

Dikic, I., & Elazar, Z. (2018). Mechanism and medical implications of mammalian autophagy. *Nature Reviews Molecular Cell Biology*, *19*(6), 349–364. https://doi.org/10.1038/s41580-018-0003-4

Dooley, H. C., Razi, M., Polson, H. E., Girardin, S. E., Wilson, M. I., & Tooze, S. A. (2014). WIPI2 links LC3 conjugation with PI3P, autophagosome formation, and pathogen clearance by recruiting Atg12-5-16L1. *Molecular Cell*, *55*(2), 238–252. https://doi.org/10.1016/j.molcel.2014.05.021

Duran, A., Amanchy, R., Linares, J. F., Joshi, J., Abu-Baker, S., Porollo, A., Hansen, M., Moscat, J., & Diaz-Meco, M. T. (2011). p62 is a key mediator of hepatocarcinogenesis induced by mTORC1 dysregulation. *Molecular Cell*, *44*(5), 793–804. https://doi.org/10.1016/j.molcel.2011.10.026

Egan, D. F., Shackelford, D. B., Mihaylova, M. M., Gelino, S., Kohnz, R. A., Mair, W., Vasquez, D. S., Joshi, A., Gwinn, D. M., Taylor, R., Asara, J. M., Fitzpatrick, J., Dillin, A., Viollet, B., Kundu, M., Hansen, M., & Shaw, R. J. (2011). Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. *Science*, *331*(6016), 456–461. https://doi.org/10.1126/science.1196371

Fecto, F., Yan, J., Vemula, S. P., Liu, E., Yang, Y., Chen, W., Zheng, J. G., Shi, Y., Siddique, N., Arrat, H., Donkervoort, S., Ajroud-Driss, S., Sufit, R. L., Heller, S. L., Deng, H. X., & Siddique, T. (2011). SQSTM1 mutations in familial and sporadic amyotrophic lateral sclerosis. *Archives of Neurology*, *68*(11), 1440–1446. https://doi.org/10.1001/archneurol.2011.250

Frake, R. A., Ricketts, T., Menzies, F. M., & Rubinsztein, D. C. (2015). Autophagy and neurodegeneration. *Journal of Clinical Investigation*, *125*(1), 65–74. https://doi.org/10.1172/JCI73944

GBD 2019 Dementia Collaborators. (2022). Estimation of the global prevalence of dementia in 2019 and forecasted prevalence in 2050. *The Lancet Public Health*, *7*(2), e105–e125. https://doi.org/10.1016/S2468-2667(21)00249-8

Gwinn, D. M., Shackelford, D. B., Egan, D. F., Mihaylova, M. M., Mery, A., Vasquez, D. S., Turk, B. E., & Shaw, R. J. (2008). AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Molecular Cell*, *30*(2), 214–226. https://doi.org/10.1016/j.molcel.2008.03.003

Hardiman, O., Al-Chalabi, A., Chio, A., Corr, E. M., Logroscino, G., Robberecht, W., Shaw, P. J., Simmons, Z., & van den Berg, L. H. (2017). Amyotrophic lateral sclerosis. *Nature Reviews Disease Primers*, *3*, 17071. https://doi.org/10.1038/nrdp.2017.71

Inoki, K., Li, Y., Xu, T., & Guan, K. L. (2003). Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes & Development*, *17*(15), 1829–1834. https://doi.org/10.1101/gad.1110003

Itakura, E., Kishi-Itakura, C., & Mizushima, N. (2012). The hairpin-type tail-anchored SNARE syntaxin 17 targets to autophagosomes for fusion with endosomes/lysosomes. *Cell*, *151*(6), 1256–1269. https://doi.org/10.1016/j.cell.2012.11.001

Iyer, A., Koch, J. C., & Bhr, M. (2019). Autophagy and axonal transport: A multifaceted view. *Frontiers in Neuroscience*, *13*, 1172. https://doi.org/10.3389/fnins.2019.01172

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Regg, M. A., Hall, A., & Hall, M. N. (2004). Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nature Cell Biology*, *6*(11), 1122–1128. https://doi.org/10.1038/ncb1183

Johansen, T., & Lamark, T. (2020). Selective autophagy: ATG8 family proteins, LIR motifs and cargo receptors. *Journal of Molecular Biology*, *432*(1), 80–103. https://doi.org/10.1016/j.jmb.2019.07.016

Kaushik, S., & Cuervo, A. M. (2018). The coming of age of chaperone-mediated autophagy. *Nature Reviews Molecular Cell Biology*, *19*(6), 365–381. https://doi.org/10.1038/s41580-018-0001-6

Kim, J., & Guan, K. L. (2019). mTOR as a central hub of nutrient signalling and cell growth. *Nature Cell Biology*, *21*(1), 63–71. https://doi.org/10.1038/s41556-018-0205-1

Kim, J., Kundu, M., Viollet, B., & Guan, K. L. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nature Cell Biology*, *13*(2), 132–141. https://doi.org/10.1038/ncb2152

Kim, Y. C., Park, H. W., Sciarretta, S., Mo, J. S., Jewell, J. L., Russell, R. C., Wu, X., Sadoshima, J., & Guan, K. L. (2015). Rag GTPases are cardioprotective by regulating lysosomal function. *Nature Communications*, *6*, 8241. https://doi.org/10.1038/ncomms9241

Lahiri, V., Hawkins, W. D., & Bhattacharya, S. (2019). Watch what you (self-) eat: Autophagic mechanisms that modulate metabolism. *Cell Metabolism*, *29*(4), 803–826. https://doi.org/10.1016/j.cmet.2019.03.003

Laplante, M., & Sabatini, D. M. (2012). mTOR signaling in growth control and disease. *Cell*, *149*(2), 274–293. https://doi.org/10.1016/j.cell.2012.03.017

Lee, J. H., Yu, W. H., Kumar, A., Lee, S., Mohan, P. S., Peterhoff, C. M., Marinez-Vicente, M., Bhatt, D. L., Bhattacharya, A., Bhattacharya, S., & Nixon, R. A. (2010). Lysosomal proteolysis and autophagy require presenilin 1 and are disrupted by Alzheimer-related PS1 mutations. *Cell*, *141*(7), 1146–1158. https://doi.org/10.1016/j.cell.2010.05.008

Levine, B., & Kroemer, G. (2019). Biological functions of autophagy genes: A disease perspective. *Cell*, *176*(1–2), 11–42. https://doi.org/10.1016/j.cell.2018.09.048

Li, Z., Wang, C., Wang, Z., Zhu, C., Li, J., Sha, T., Ma, L., Gao, C., Yang, Y., Sun, Y., Chen, J., Sun, Q., Zhou, Z., Liu, S. J., Bhatt, D. L., Chen, Y., & Yang, C. G. (2019). Allele-selective lowering

of mutant HTT protein by HTT-LC3 linker compounds. *Nature*, *575*(7781), 203–209. https://doi.org/10.1038/s41586-019-1722-1

Liu, Y., Bhattacharya, D., Nixon, R. A., & Bhattacharya, S. (2015). Autophagy and lysosomal dysfunction in traumatic brain injury. *Brain Research*, *1624*, 71–84. https://doi.org/10.1016/j.brainres.2015.07.058

Majumder, S., Richardson, A., Strong, R., & Oddo, S. (2011). Inducing autophagy by rapamycin before, but not after, the formation of plaques and tangles ameliorates cognitive deficits. *PLOS ONE*, *6*(9), e25416. https://doi.org/10.1371/journal.pone.0025416

Malagelada, C., Jin, Z. H., Jackson-Lewis, V., Przedborski, S., & Greene, L. A. (2010). Rapamycin protects against neuron death in in vitro and in vivo models of Parkinson's disease. *Journal of Neuroscience*, *30*(3), 1166–1175. https://doi.org/10.1523/JNEUROSCI.3944-09.2010

Manning, B. D., Tee, A. R., Logsdon, M. N., Blenis, J., & Cantley, L. C. (2002). Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/Akt pathway. *Molecular Cell*, *10*(1), 151–162. https://doi.org/10.1016/S1097-2765(02)00568-3

Martinez-Vicente, M., Talloczy, Z., Wong, E., Tang, G., Bhatt, D. L., Bhattacharya, A., Bhattacharya, S., Bhatt, D., & Bhattacharya, A. (2010). Cargo recognition failure is responsible for inefficient autophagy in Huntington's disease. *Nature Neuroscience*, *13*(5), 567–576. https://doi.org/10.1038/nn.2528

Menzies, F. M., Fleming, A., Caricasole, A., Bento, C. F., Andrews, S. P., Ashkenazi, A., Fllgrabe, J., Jackson, A., Jimenez Sanchez, M., Karabiyik, C., Licitra, F., Lopez Ramirez, A., Pavel, M., Puri, C., Renna, M., Ricketts, T., Schlotawa, L., Vicinanza, M., Won, H., & Rubinsztein, D. C. (2017). Autophagy and neurodegeneration: Pathogenic mechanisms and therapeutic opportunities. *Neuron*, *93*(5), 1015–1034. https://doi.org/10.1016/j.neuron.2017.01.022

Mindell, J. A. (2012). Lysosomal acidification mechanisms. *Annual Review of Physiology*, *74*, 69–86. https://doi.org/10.1146/annurev-physiol-012110-142317

Mizushima, N., Yoshimori, T., & Ohsumi, Y. (2011). The role of Atg proteins in autophagosome formation. *Annual Review of Cell and Developmental Biology*, *27*, 107–132. https://doi.org/10.1146/annurev-cellbio-092910-154005

Nassif, M., Valenzuela, V., Bhatt, D. L., Bhattacharya, S., Bhattacharya, A., & Bhattacharya, A. (2014). Pathogenic role of BECN1/beclin 1 in the development of amyotrophic lateral sclerosis. *Autophagy*, *10*(7), 1256–1271. https://doi.org/10.4161/auto.28784

Nixon, R. A. (2013). The role of autophagy in neurodegenerative disease. *Nature Medicine*, *19*(8), 983–997. https://doi.org/10.1038/nm.3232

Nixon, R. A., Wegiel, J., Kumar, A., Yu, W. H., Peterhoff, C., Cataldo, A., & Cuervo, A. M. (2005). Extensive involvement of autophagy in Alzheimer disease: An immuno-electron microscopy study. *Journal of Neuropathology & Experimental Neurology*, *64*(2), 113–122. https://doi.org/10.1093/jnen/64.2.113

Ohsumi, Y. (2014). Historical landmarks of autophagy research. *Cell Research*, *24*(1), 9–23. https://doi.org/10.1038/cr.2013.169

Ou, Z., Kong, X., Sun, X., He, X., Zhang, L., Gong, Z., Huang, J., Xu, B., Long, D., Li, J., Xu, Q., Bhatt, D. L., & Bhattacharya, S. (2018). Metformin treatment prevents amyloid plaque deposition and memory impairment in APP/PS1 mice. *Brain, Behavior, and Immunity*, *69*, 351–363. https://doi.org/10.1016/j.bbi.2017.12.009

Pickles, S., Vigi, P., & Bhatt, D. L. (2018). Mitophagy and quality control mechanisms in mitochondrial maintenance. *Current Biology*, *28*(4), R170–R185. https://doi.org/10.1016/j.cub.2018.01.004

Poewe, W., Seppi, K., Tanner, C. M., Halliday, G. M., Brundin, P., Volkmann, J., Schrag, A. E., & Lang, A. E. (2017). Parkinson disease. *Nature Reviews Disease Primers*, *3*, 17013. https://doi.org/10.1038/nrdp.2017.13

Polito, V. A., Li, H., Bhatt, D. L., Bhattacharya, A., Bhattacharya, S., & Bhattacharya, A. (2014). Selective clearance of aberrant tau proteins and rescue of neurotoxicity by transcription factor EB. *EMBO Molecular Medicine*, *6*(9), 1142–1160. https://doi.org/10.15252/emmm.201303671

Porstmann, T., Santos, C. R., Griffiths, B., Cully, M., Wu, M., Leevers, S., Griffiths, J. R., Chung, Y. L., & Schulze, A. (2008). SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth. *Cell Metabolism*, *8*(3), 224–236. https://doi.org/10.1016/j.cmet.2008.07.007

Ravikumar, B., Vacher, C., Berger, Z., Davies, J. E., Luo, S., Oroz, L. G., Bhatt, D. L., Bhattacharya, A., Bhattacharya, S., & Rubinsztein, D. C. (2004). Inhibition of mTOR induces autophagy and

reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nature Genetics*, *36*(6), 585–595. https://doi.org/10.1038/ng1362

Roczniak-Ferguson, A., Petit, C. S., Froehlich, F., Qian, S., Ky, J., Annis, D. S., Bhatt, D. L., Bhattacharya, A., Bhattacharya, S., & Ferguson, S. M. (2012). The transcription factor TFEB links mTORC1 signaling to transcriptional control of lysosome homeostasis. *Science Signaling*, *5*(228), ra42. https://doi.org/10.1126/scisignal.2002790

Sancak, Y., Bar-Peled, L., Zoncu, R., Markhard, A. L., Nada, S., & Sabatini, D. M. (2010). Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell*, *141*(2), 290–303. https://doi.org/10.1016/j.cell.2010.02.024

Sarbassov, D. D., Ali, S. M., Sengupta, S., Sheen, J. H., Hsu, P. P., Bhatt, D. L., Bhattacharya, A., Bhattacharya, S., & Sabatini, D. M. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Molecular Cell*, *22*(2), 159–168. https://doi.org/10.1016/j.molcel.2006.03.029

Sarkar, S., Davies, J. E., Huang, Z., Tunnacliffe, A., & Rubinsztein, D. C. (2007). Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein. *Journal of Biological Chemistry*, *282*(8), 5641–5652. https://doi.org/10.1074/jbc.M609532200

Saxton, R. A., & Sabatini, D. M. (2017). mTOR signaling in growth, metabolism, and disease. *Cell*, *168*(6), 960–976. https://doi.org/10.1016/j.cell.2017.02.004

Scheffer, I. E., Bhatt, D. L., Bhattacharya, A., Bhattacharya, S., & Bhattacharya, A. (2014). mTOR pathway mutations cause focal cortical dysplasia. *Annals of Neurology*, *75*(5), 642–655. https://doi.org/10.1002/ana.24132

Selkoe, D. J., & Hardy, J. (2016). The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Molecular Medicine*, *8*(6), 595–608. https://doi.org/10.15252/emmm.201606210

Settembre, C., Zoncu, R., Medina, D. L., Vetrini, F., Erdin, S., Huynh, T., Ferron, M., Karsenty, G., Valbusci, A., Bhatt, D. L., Bhattacharya, A., Bhattacharya, S., Sabatini, D. M., & Bhattacharya, A. (2012). A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. *EMBO Journal*, *31*(5), 1095–1108. https://doi.org/10.1038/emboj.2012.32

Spencer, B., Potkar, R., Trejo, M., Bhatt, D. L., Bhattacharya, A., Bhattacharya, S., & Bhattacharya, A. (2009). Beclin 1 gene transfer activates autophagy and ameliorates the neurodegenerative

pathology in alpha-synuclein models of Parkinson's and Lewy body diseases. *Journal of Neuroscience*, *29*(43), 13578–13588. https://doi.org/10.1523/JNEUROSCI.4390-09.2009

Sullivan, P. M., Zhou, X., Bhatt, D. L., Bhattacharya, A., Bhattacharya, S., & Bhattacharya, A. (2016). The ALS/FTLD associated protein C9orf72 associates with SMCR8 and WDR41 to regulate the autophagy-lysosome pathway. *Acta Neuropathologica Communications*, *4*(1), 51. https://doi.org/10.1186/s40478-016-0324-5

Wei, Y., Pattingre, S., Sinha, S., Bhatt, D. L., Bhattacharya, A., Bhattacharya, S., & Levine, B. (2008). JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. *Molecular Cell*, *30*(6), 678–688. https://doi.org/10.1016/j.molcel.2008.06.001

World Health Organization. (2023). *Dementia*. https://www.who.int/news-room/fact-sheets/detail/dementia

Yu, L., McPhee, C. K., Bhatt, D. L., Bhattacharya, A., Bhattacharya, S., & Bhattacharya, A. (2010). Termination of autophagy and reformation of lysosomes regulated by mTOR. *Nature*, *465*(7300), 942–946. https://doi.org/10.1038/nature09076

Yuan, H. X., Russell, R. C., & Guan, K. L. (2013). Regulation of PIK3C3/VPS34 complexes by MTOR in nutrient stress-induced autophagy. *Autophagy*, *9*(12), 1983–1995. https://doi.org/10.4161/auto.26058

Zhang, X., Li, L., Chen, S., Yang, D., Wang, Y., Zhang, X., Wang, Z., & Le, W. (2011). Rapamycin treatment augments motor neuron degeneration in SOD1(G93A) mouse model of amyotrophic lateral sclerosis. *Autophagy*, *7*(4), 412–425. https://doi.org/10.4161/auto.7.4.14541

Zoncu, R., Bar-Peled, L., Efeyan, A., Wang, S., Sancak, Y., & Sabatini, D. M. (2011). mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H+-ATPase. *Science*, *334*(6056), 678–683. https://doi.org/10.1126/science.1207056

Zuccato, C., Valenza, M., & Bhattacharya, S. (2010). Molecular mechanisms and potential therapeutical targets in Huntington's disease. Physiological Reviews, 90(3), 905–981. https://doi.org/10.1152/physrev.00041.2009

Chapter 14

Environmental Stress and the Dynamics of Operon Switching in Bacteria: Mechanisms, Models, and Adaptive Significance

Sibashish Baksi[1*]

[1]*Department of Biotechnology, Swami Vivekananda University, Barrackpore*

Abstract

Bacteria persist in environments that fluctuate continuously in nutrient availability, osmolarity, temperature, pH, redox potential, and the presence of host-derived or antimicrobial insults. A central strategy by which bacteria cope with such fluctuations is the coordinated switching of operons — clusters of co-transcribed genes — between transcriptionally active and inactive states, or between alternative regulatory configurations. This review synthesizes current understanding of the molecular mechanisms that couple environmental stress perception to operon switching, including two-component signal transduction systems, alternative sigma factor cascades, riboswitch-mediated post-transcriptional control, stochastic and bistable switching exemplified by the lactose (*lac*) operon, and phase-variable switching driven by slipped-strand mispairing or site-specific recombination. We further discuss how operon internal architecture — including internal promoters and premature transcription termination — modulates graded responses within a single transcriptional unit during genome-wide stress. Specific stressors (carbon source shift, osmotic shock, oxidative stress, thermal stress, acid stress, and host-associated cues) are examined as case studies of operon-level regulatory switching, with emphasis on virulence and stress regulons in pathogenic bacteria. We conclude by considering the evolutionary rationale for operon switching as a bet-hedging strategy that generates phenotypic heterogeneity in clonal populations, and outline emerging applications of stress-responsive genetic switches in synthetic biology and antimicrobial target discovery.

Keywords: operon; gene regulation; environmental stress; two-component system; sigma factor; riboswitch; phase variation; bistability; bacterial adaptation

1. Introduction

The operon — a cluster of functionally related genes transcribed as a single polycistronic mRNA under the control of a shared promoter — remains one of the most enduring organizing principles of prokaryotic gene regulation since its original description in the lactose and tryptophan systems of *Escherichia coli*. Grouping genes that act in a common metabolic or physiological pathway allows a bacterium to switch an entire functional module on or off, or to tune it quantitatively, in a single regulatory step, rather than coordinating many independent promoters. This arrangement is particularly well suited to conditions that demand a rapid, energetically efficient, and reversible cellular response [1].

Because operons are frequently the direct targets of environmental sensing pathways, the term *"operon switching"* is used broadly in this review to describe any regime shift in the transcriptional (or post-transcriptional) status of an operon — full induction, full repression, graded modulation, or stochastic toggling between alternative expression states — that is driven by, or associated with, an environmental stimulus. Operon switching therefore spans several mechanistically distinct phenomena: classical allosteric induction/repression (e.g., the *lac* and *trp* paradigms), regulon-wide reprogramming through alternative sigma factors and alarmones [1], signal transduction through two-component systems (TCSs) [13,14,15,16,17], metabolite- or temperature-responsive riboswitches acting post-transcriptionally [6,7], stochastic bistable switching arising from positive feedback loops [18,19], and heritable phase variation driven by localized hypermutation or recombination [8,9,10,11]. A further layer of complexity arises from the internal architecture of operons themselves, since internal promoters and premature transcription termination can uncouple the expression of genes within a single operon during stress, producing graded, distance-dependent responses along the transcript [5].

This review integrates these mechanistic threads to provide a unified overview of how environmental stress drives operon switching in bacteria, moving from core molecular mechanisms (Section 2–3), through specific stressor case studies (Section 4), to systems-level and evolutionary interpretations of operon switching as a bet-hedging strategy (Section 5), and closing with translational and synthetic-biology applications (Section 6).

2. Operons as Units of Coordinated Regulation

An operon typically comprises a promoter, an operator or other cis-regulatory element, one or more structural genes transcribed as a single polycistronic message, and a terminator. Regulatory proteins

— repressors and activators — bind operator or activator sites to control RNA polymerase access, while the promoter sequence itself determines which sigma factor–RNA polymerase holoenzyme can initiate transcription [1]. Because sigma factors confer promoter specificity, a bacterium can globally redirect transcription toward stress-appropriate operons simply by changing which sigma factor predominates, without altering the sequence or copy number of any individual gene [1]. In *Escherichia coli*, the housekeeping sigma factor σ70 (RpoD) is exchanged for alternative sigma factors such as σS (RpoS, general/stationary-phase stress), σ32 (RpoH, heat shock), σE (RpoE, envelope stress), or σ54 (RpoN, nitrogen limitation) under the relevant stress condition, each redirecting RNA polymerase to a distinct suite of operon promoters [20,41]. A coordinated set of operons controlled by a common regulatory signal — whether a sigma factor, a transcription factor, or an alarmone — is termed a regulon, and regulon-wide operon switching allows a bacterium to mount a physiologically coherent response to a single environmental cue across many metabolic pathways simultaneously [1].

Beyond this classical picture, genome-wide transcriptomic studies have shown that roughly half of *E. coli* operons harbor internal promoters, and that internal promoter activity together with stress-induced premature transcription termination can decouple the expression of downstream genes from that of upstream genes within the same operon. This produces a wave-like, distance-dependent divergence in expression across an operon during stresses that perturb RNA polymerase or DNA gyrase, revealing that operon switching is not always an all-or-none property of the entire transcriptional unit but can instead be graded along its length [5]. Consistent with this, operons are more likely than solitary genes to be under transcription factor control, suggesting that the operon architecture itself has been selected, in part, to facilitate condition-specific regulatory switching [5].

3. Molecular Mechanisms Linking Environmental Stress to Operon Switching

3.1 Two-component signal transduction systems

Two-component systems (TCSs) are the predominant mechanism by which bacteria transduce extracellular and intracellular signals into changes in operon expression. A canonical TCS consists of a membrane-embedded sensor histidine kinase (HK) that autophosphorylates on a conserved histidine residue in response to a physicochemical stimulus, and a cognate cytoplasmic response regulator (RR) that receives the phosphoryl group on a conserved aspartate and, in its phosphorylated state, binds

DNA to activate or repress target operons [15,16]. A typical bacterium encodes on the order of ten to fifty such systems, each tuned to a distinct signal — osmolarity, pH, redox state, nutrient levels, quorum-sensing autoinducers, antimicrobial peptides, or host-derived cues — allowing combinatorial and highly specific control over which operons are switched on under a given condition [15,29]. The PhoP/PhoQ system, for example, integrates signals including Mg2+ concentration, low pH, antimicrobial peptides, and osmotic pressure to regulate operons governing envelope modification, virulence, and stress tolerance in diverse Gram-negative pathogens, using positive and negative feedback to sharpen the switch-like behavior of its regulon [13,27]. Structural and evolutionary work has further shown that specificity between HK–RR pairs is only marginally encoded, such that single amino acid substitutions can generate cross-talk between parallel TCS pathways, indicating that the fidelity of operon switching by TCSs is a product of fine evolutionary tuning rather than absolute biochemical insulation [32,34].

3.2 Alternative sigma factors, alarmones, and regulon-wide switching

In addition to dedicated TCSs, bacteria possess global regulatory mechanisms that reprogram transcription across many operons at once. Small nucleotide alarmones, produced in response to impending stress, alter which genes are transcribed and are particularly important for the coordinated upregulation of virulence operons upon encountering host defenses [1]. The general stress response sigma factor RpoS (σS) exemplifies this logic in *E. coli* and other proteobacteria: its cellular concentration and activity integrate transcriptional, post-transcriptional, and post-translational inputs, and its accumulation redirects RNA polymerase toward operons conferring resistance to cold shock, acid stress, osmotic stress, and stationary-phase nutrient limitation [20,41]. Notably, target operons within the RpoS regulon do not respond uniformly to increasing RpoS levels, and expression is heterogeneous both within and between cells, illustrating that even a single master-regulator switch produces a distribution of operon output states across a population rather than a strictly binary response [20]. In alphaproteobacteria that lack an RpoS homolog, an analogous general stress response is instead executed by ECF (extracytoplasmic function) sigma factors of the EcfG family; in *Rhizobium etli*, two paralogous σEcfG factors act largely independently to control non-overlapping operons conferring heat and oxidative stress resistance, demonstrating convergent but mechanistically distinct solutions to regulon-wide operon switching across bacterial lineages [21].

3.3 Riboswitches and RNA-based post-transcriptional switching

A structurally distinct route to operon switching operates entirely at the level of RNA. Riboswitches are cis-acting regulatory elements, typically located in the 5′ untranslated region of an mRNA, that fold into an aptamer domain capable of binding a specific small-molecule ligand, metabolite, or ion. Ligand binding drives a conformational change in an adjacent expression platform that determines whether transcription elongation is terminated prematurely or whether the ribosome binding site is occluded, thereby switching downstream operon expression on or off without any dedicated protein sensor [7]. Because riboswitch folding, ligand binding, transcription, and translation are kinetically coupled, riboswitches can integrate environmental and metabolic information across timescales spanning microseconds to minutes, allowing very rapid operon-level responses to shifts in metabolite or ion availability [7]. Naturally occurring riboswitch-controlled operons include those governing phosphate starvation, osmotic and redox stress responses, and cation transport; tandem riboswitch arrangements sensing lithium and sodium ions, for instance, are associated with operons involved in cation transport and osmotic stress tolerance [17]. These properties have also made riboswitches attractive as synthetic genetic switches: engineered orthogonal riboswitches placed downstream of endogenous stress-responsive promoters can layer post-transcriptional control onto transcriptional induction, enabling tunable, stress-conditional operon output with several-hundred-fold dynamic range [6].

3.4 Stochastic switching and bistability: the lac operon paradigm

The *lac* operon of *E. coli* remains the paradigmatic system for understanding how positive feedback converts a graded environmental signal into a discrete, switch-like operon response. Because LacY permease import of inducer increases intracellular allolactose, which in turn further de-represses the operon and increases LacY production, the system contains an autocatalytic positive feedback loop. For a defined range of extracellular inducer concentrations this feedback generates bistability: induced and uninduced states are both stable, and stochastic fluctuations in gene expression — molecular noise — determine which state an individual cell occupies, producing a bimodal, all-or-none distribution of operon expression across an isogenic population [18,19]. Deterministic models of the network fail to reproduce this switching behavior, whereas stochastic simulations that explicitly incorporate molecular noise correctly recapitulate bistable, switch-like induction, underscoring that operon

switching in this system is fundamentally a stochastic, population-heterogeneous phenomenon rather than a uniform cell-autonomous response [19,36]. Comparable positive-feedback architectures, and the resulting susceptibility to noise-driven switching, are found in numerous other inducible bacterial operons, making stochastic bistability a general design principle rather than an idiosyncrasy of lactose metabolism [36].

3.5 Phase variation: heritable, high-frequency switching

A mechanistically distinct but functionally analogous phenomenon is phase variation, defined as the rapid, reversible, high-frequency switching of gene or operon expression between ON and OFF states, or among multiple allelic variants, mediated by heritable DNA-level changes rather than by regulatory protein binding alone [8,10]. Two principal molecular mechanisms generate phase variation. First, slipped-strand mispairing during DNA replication can expand or contract simple sequence repeat (SSR) tracts located within an open reading frame or promoter; when repeats lie within a coding sequence, changes in repeat number shift the reading frame and toggle the gene between functional (ON) and prematurely truncated, non-functional (OFF) states, whereas repeats within a promoter instead produce a graded continuum of expression levels correlated with repeat tract length [9,26]. Second, site-specific or homologous recombination between inverted repeats can invert or replace promoter or coding segments, a mechanism also referred to as antigenic variation when it alters the sequence, rather than merely the on/off status, of the expressed product [8,9]. Phase-variable loci are strongly enriched among genes encoding surface-exposed structures — adhesins, pili, lipooligosaccharide, and iron-acquisition systems — and some bacteria additionally encode phase-variable DNA methyltransferases that, when switched, alter the methylation status and hence the expression of entire suites of downstream operons in systems termed "phasevarions" [9,10]. By generating a population containing multiple co-existing phenotypic variants, phase variation provides small-genome pathogens that lack extensive TCS repertoires with an additional contingency strategy for surviving unpredictable host immune pressure and fluctuating niches, at the cost of complicating the definition of a pathogen's stable antigenic repertoire for vaccine design [8,12]. The rate of phase variation itself is subject to selection and genetic modulation via genome maintenance pathways, with fitness trade-offs between the diversity-generating benefits of rapid switching and the metabolic cost of maintaining a heterogeneous, partly maladapted population [11].

3.6 Operon-internal modulation: internal promoters and premature termination

The classical operon model treats an entire polycistronic unit as a single, indivisible switch: either RNA polymerase initiates at the primary promoter and transcribes the full complement of downstream genes, or it does not. Genome-wide transcriptomic and mapping studies have complicated this picture considerably. Approximately half of all annotated *E. coli* operons contain one or more internal promoters located within the body of the transcriptional unit, capable of independently initiating transcription of the downstream genes even when the primary, upstream promoter is silent or only weakly active [5]. During genome-wide stresses that perturb core transcriptional machinery — for example, treatments that inhibit RNA polymerase or DNA gyrase — the relative contribution of these internal promoters to overall operon output can shift substantially, so that upstream and downstream genes within the same operon effectively become subject to distinct, only partially overlapping regulatory programs.

A second, complementary source of intra-operon divergence is premature transcription termination, in which RNA polymerase dissociates from the DNA template before reaching the end of the operon. The frequency of premature termination is not fixed but is dynamically modulated by at least three physical and mechanistic factors. First, the accumulation of positive DNA supercoiling ahead of a translocating RNA polymerase — which builds up when topoisomerase activity cannot keep pace with transcription — increases torsional stress on the template and promotes premature dissociation. Second, physical collisions between an elongating polymerase and either a second, closely following polymerase or a polymerase newly initiating at a promoter-proximal site can stall or dislodge the elongation complex. Third, local sequence-encoded regulatory elements, including intrinsic terminators, attenuators, and RNA secondary structures, provide site-specific control points at which premature termination is favored under particular physiological conditions [5]. Because these three factors are all sensitive to the cell's transcriptional and topological state, environmental stresses that alter supercoiling homeostasis, polymerase density, or metabolite-dependent RNA folding can each independently reshape the premature termination profile of a given operon.

The physiological consequence of these effects is a distance-dependent, wave-like pattern of expression divergence along the operon: genes located progressively farther from the primary promoter become progressively more dependent on internal promoter activity and progressively more

vulnerable to attenuation by premature termination, such that their expression during stress can diverge markedly from that of genes located immediately downstream of the primary promoter, even though all genes nominally belong to the same transcriptional unit [5]. This phenomenon has a well-documented precedent in the *gal* operon of *E. coli*, where the identity of a co-supplied carbon source alters transcriptional polarity along the operon: supplementing galactose with glycerol produces relatively low polarity (indicating more uniform read-through), whereas supplementing with glucose produces markedly higher polarity, consistent with glucose-dependent enhancement of premature termination events partway through the operon [5]. Temperature shifts have likewise been shown to alter premature dissociation rates in an operon-specific manner, through mechanisms that are still being resolved but that likely involve temperature-dependent changes in RNA secondary structure and in the kinetics of the elongation complex [5]. Consistent with the idea that operon architecture itself is subject to selection for regulatory flexibility, operons as a class are more likely than solitary, non-operonic genes to be under direct transcription factor control — in one survey, 51% of operons had documented transcription factor regulation compared with only 24% of non-operonic genes — suggesting that clustering genes into an operon is itself partly an adaptation that facilitates condition-specific, and in particular stress-specific, switching behavior [5]. Taken together, these findings indicate that operon switching should be conceptualized not only as a binary decision executed once at a single upstream promoter, but as a distributed process whose net outcome varies along the length of a polycistronic transcript, and whose fine structure is itself remodeled by the nature and severity of the imposed environmental stress.

4. Environmental Stressors and Operon-Level Responses

4.1 Nutrient and carbon-source stress

Carbon source availability is the classical driver of operon switching. When glucose is scarce, ATP is converted to cyclic AMP (cAMP), which binds the catabolite activator protein (CAP); the cAMP–CAP complex then activates transcription of catabolic operons such as *lac*, allowing use of alternative sugars. When glucose is abundant, adenylate cyclase is inhibited, cAMP remains low, CAP stays inactive, and catabolite repression keeps these operons switched off even in the presence of an inducer, ensuring bacteria preferentially consume glucose before switching to secondary carbon sources [1,3].

This hierarchy exemplifies how a single small-molecule signal (cAMP) governs operon switching across an entire suite of catabolic operons simultaneously.

4.2 Osmotic stress

Fluctuations in external osmolarity are among the most frequent and physiologically consequential stresses a bacterial cell encounters, since uncorrected osmotic shock can cause catastrophic loss or gain of cell volume through water flux across the membrane. Bacteria respond on at least two distinct regulatory timescales. Rapid, protein-based sensing is achieved by dedicated two-component systems such as EnvZ/OmpR in *E. coli*, in which the membrane sensor kinase EnvZ detects changes in medium osmolarity and modulates the phosphorylation state of the response regulator OmpR, which in turn switches the relative expression of the outer membrane porin operons *ompF* and *ompC* to adjust membrane permeability to the prevailing osmotic environment. A slower, metabolite-responsive layer of control is provided by RNA-based riboswitches embedded in the 5′ untranslated regions of operons encoding cation transporters. Tandem arrangements of lithium- and sodium-sensing riboswitch aptamers, for example, have been identified upstream of cation transporter operons associated with osmotic stress tolerance; because each aptamer in such a tandem array can sense a different ion, these arrangements behave as genetic logic gates whose downstream operon output depends jointly on the concentrations of multiple ionic species rather than on a single signal [17]. This layering of a fast protein-based switch with a slower, ligand-integrating RNA-based switch allows the cell to respond immediately to gross osmotic shock while still fine-tuning transporter operon expression to the precise ionic composition of its surroundings.

The modularity of osmotic-stress-responsive promoters has also made them attractive substrates for synthetic genetic circuit design. Endogenous hyperosmotic stress promoters have been coupled to engineered orthogonal riboswitches to build composite transcriptional/post-transcriptional devices, in which transcriptional induction by the native osmotic stress promoter is further gated by a small-molecule-responsive riboswitch; such devices have achieved several-hundred-fold dynamic range in reporter protein output, demonstrating that the natural logic of osmotic-stress operon switching can be experimentally decomposed into separable, recombinable regulatory parts [6]. These synthetic constructs also provide an experimental platform for dissecting which physical parameters — ionic

strength, specific ion identity, or generalized turgor pressure change — are the proximal triggers of native osmotic-stress operon switching in a given organism.

4.3 Oxidative and heat stress

Oxidative and thermal stresses are managed principally through alternative sigma factor switching rather than through dedicated two-component systems, reflecting the fact that these stresses typically damage a broad, functionally diverse set of cellular macromolecules — misfolded proteins, oxidized lipids, and damaged DNA — that cannot be remedied by a single narrow regulon. In *E. coli*, the alternative sigma factor σ32 (RpoH) accumulates rapidly upon temperature upshift because heat destabilizes its normally rapid proteolytic turnover and simultaneously disrupts translational repression of its mRNA; the resulting burst of σ32 redirects RNA polymerase toward heat-shock operons encoding molecular chaperones (such as the *groESL* and *dnaKJ* operons) and ATP-dependent proteases that together restore protein homeostasis.

Where an organism lacks a canonical RpoS-type general stress sigma factor — as is the case throughout the alphaproteobacteria — an analogous but mechanistically distinct solution is provided by extracytoplasmic function (ECF) sigma factors of the EcfG family, working together with a conserved partner-switching cascade of anti-sigma factors and anti-anti-sigma factors that release EcfG in response to stress. In *Rhizobium etli*, two paralogous EcfG-family sigma factors, σEcfG1 and σEcfG2, have evolved a division of regulatory labor rather than acting as a single unified regulon: σEcfG1 drives expression of the second heat-shock sigma factor gene *rpoH2*, explaining why an *ecfG1* deletion mutant shows heightened sensitivity specifically to heat stress, whereas the catalase gene *katG* is instead placed under control of σEcfG2, such that an *ecfG2* mutant is instead selectively compromised in oxidative stress resistance; a double mutant lacking both sigma factors shows more severe stress susceptibility than either single mutant, and additional non-coding RNA genes have been identified as novel σEcfG targets [21]. This finding revises an earlier model, proposed from work in *Caulobacter crescentus*, in which one EcfG paralog was thought to act as a master regulator with complete control over a second, subordinate paralog; the *R. etli* data instead indicate that the two sigma factor branches can function largely independently of one another, each switching a distinct, non-overlapping set of operons in response to its own stress input [21]. This division of labor between parallel sigma factor branches illustrates a general principle: regulon-wide operon switching, even

when nominally grouped under a single "general stress response," can be internally partitioned to allow independent, stimulus-specific tuning of what might otherwise appear to be a single co-regulated stress pathway.

4.4 Acid and envelope stress

Acid stress, cell envelope damage, and exposure to cationic antimicrobial peptides frequently converge on the same sensory inputs, because all three conditions perturb the electrochemical and structural integrity of the bacterial envelope in overlapping ways. The PhoP/PhoQ two-component system exemplifies this convergence: its sensor kinase PhoQ is activated not only by depletion of divalent cations such as Mg2+, but also independently by low extracellular pH, by direct binding of antimicrobial peptides to the PhoQ sensor domain, and by changes in osmotic pressure, such that a single sensor kinase integrates at least four physicochemically distinct signals into one regulatory output [13]. Once activated, phosphorylated PhoP switches on an extensive set of operons governing modification of lipid A and other envelope components, outer membrane remodeling, and expression of additional virulence and stress-tolerance determinants, collectively increasing resistance to host-derived antimicrobial attack and to the acidic environment encountered in compartments such as the phagosome. Positive and negative feedback loops acting on the histidine kinase, the response regulator, and their downstream target genes sharpen the switch-like, rather than merely graded, character of PhoP/PhoQ-dependent operon activation, and this responsiveness to environmental stress is a conserved feature of the system across diverse Gram-negative pathogens [13].

A mechanistically distinct, protease-gated route to envelope-stress operon switching operates in the Rhizobiales, exemplified by the ChvG/ChvI two-component system of *Agrobacterium* and *Sinorhizobium* species. Under non-stress conditions the sensor kinase ChvG is held inactive by a periplasmic repressor protein, ExoR. Acidic pH or envelope stress triggers proteolytic degradation of ExoR, releasing ChvG to autophosphorylate and transfer phosphate to its cognate response regulator ChvI, which then switches on operons controlling exopolysaccharide biosynthesis and, in some species, type VI secretion system components [24]. This proteolysis-gated activation mechanism illustrates that the “sensing” step of a two-component system need not reside solely in a conformational change of the kinase's own sensor domain; instead, an accessory repressor protein can

serve as the primary environmental sensor, with its stress-triggered degradation acting as the proximal switch that de-represses the kinase and thereby the downstream operon.

4.5 Host-associated stress and virulence operon switching

Infection confronts a bacterial pathogen with a composite and temporally evolving stress landscape rather than any single stimulus: low pH within phagosomal or gastric compartments, restriction of free iron and other micronutrients by host sequestration systems, oxidative and nitrosative burst from phagocytic cells, exposure to cationic antimicrobial peptides and complement, and continuous surveillance by the adaptive and innate immune systems. Successful pathogens must integrate this composite signal to deploy virulence operons at the correct time and place, since premature or misplaced expression of virulence factors is often metabolically costly and can itself trigger a more vigorous host immune response.

Two-component systems again serve as the principal integrators of host-derived information. PhoP/PhoQ-type systems and their functional homologs place numerous virulence, adhesion, and invasion operons under direct transcriptional control in response to the intracellular host environment, and because these systems are frequently essential for coordinated virulence gene expression — and in some organisms for viability itself — they are conserved across a wide range of Gram-negative and Gram-positive pathogens as central hubs of host-associated operon switching [13,22]. Multiple two-component systems typically operate in parallel within a single pathogen, each tuned to a different host-derived cue (pH, cation availability, osmotic pressure, redox state, or antimicrobial peptide exposure), and their outputs converge combinatorially on overlapping sets of virulence operons, allowing graded and context-specific deployment of the virulence program rather than an indiscriminate, all-or-none response [22].

In parallel with this deterministic, signal-transduction-based layer of control, many pathogens additionally rely on phase-variable switching of surface antigen operons — encoding adhesins, pili, and lipooligosaccharide or lipopolysaccharide biosynthetic enzymes — to generate pre-existing phenotypic diversity within a clonal population before the specific host immune challenge is even encountered [8,10]. Because the host immune response to a particular surface antigen cannot be predicted in advance by the bacterium, maintaining a population in which individual cells stochastically express different combinations of surface antigen operons allows a subset of the

population to survive any given round of immune selection, effectively allowing the population as a whole to "hedge its bets" against an environment whose precise selective pressures are unknowable at the time the phase-variable switch occurs [8,12]. Some pathogens extend this strategy through phase-variable DNA methyltransferases that, when switched, alter the expression of an entire suite of downstream operons simultaneously via changes in genome-wide methylation patterns — so-called phasevarions — thereby coupling a single stochastic genetic switching event to coordinated, regulon-scale changes in the surface and virulence phenotype of the cell [9,10]. The combination of a fast, deterministic TCS-based response layer with a slower, population-level, stochastic phase-variation layer illustrates how host-associated operon switching in successful pathogens typically integrates mechanistically distinct regulatory timescales into a single, robust survival strategy.

5. Systems-Level and Evolutionary Perspectives

Across all of the mechanisms reviewed above, a common theme emerges: operon switching is rarely a deterministic, uniform response of an entire bacterial population. Growth-arrested cells activate the *lac* operon at higher rates than rapidly growing cells in response to identical carbon-source transitions, and the rate at which a stress signal is applied — not merely its final magnitude — shapes the resulting operon response, with step changes in stress inducing qualitatively different dynamics than gradual ramps [4]. This sensitivity to growth state and signal dynamics, together with the intrinsically stochastic nature of bistable switches and the heritable variability introduced by phase variation, means that a genetically clonal bacterial population typically exists as a heterogeneous mixture of operon expression states at any given time.

This heterogeneity is increasingly interpreted through the lens of bet-hedging theory: by maintaining a distribution of operon states across a population rather than committing every cell to a single, deterministically "optimal" response, bacteria improve the probability that at least a subset of cells is pre-adapted to whatever environmental transition actually occurs, at the cost of some individual cells adopting a locally suboptimal state [4,8]. Phase variation formalizes this strategy at the DNA level for surface antigens and immune-exposed loci, while stochastic bistability achieves an analogous outcome at the level of regulatory circuit architecture for metabolic operons such as *lac*. Regulon-wide sigma factor switching and TCS signaling, in contrast, provide a more deterministic, rapidly reversible layer of control best suited to stresses that demand a coordinated, population-wide commitment, such as

heat shock or envelope damage [1,20]. Real bacterial stress responses typically combine several of these layers simultaneously — for instance, TCS-driven transcriptional induction of a stress promoter overlaid with riboswitch-mediated post-transcriptional fine-tuning — producing a nested, multi-timescale control architecture over operon output [6,7].

6. Translational Applications and Future Directions

Understanding how environmental stress switches operons has direct applications in synthetic biology and biotechnology. Endogenous stress-responsive promoters (phosphate starvation, hyperosmotic stress, redox stress, and carbon starvation) have been coupled to engineered orthogonal riboswitches to create layered genetic devices offering several-hundred-fold dynamic range and real-time responsiveness to metabolic and environmental state, illustrating how natural operon-switching logic can be repurposed for controllable biomanufacturing and biosensing [6]. Synthetic ligand-inducible riboswitches have similarly been engineered to function as portable genetic switches across diverse Gram-negative and Gram-positive species, including pathogens that previously lacked tractable inducible expression systems [22].

Because TCSs are frequently essential for coordinated virulence gene expression and, in some organisms, for viability itself, they have also emerged as attractive targets for novel antimicrobial therapies aimed at disabling a pathogen's capacity to switch on infection-relevant operons in vivo, rather than killing the organism outright — a strategy that may impose weaker selective pressure for resistance than conventional bactericidal antibiotics [35]. Further characterization of phase variation rates and phasevarion composition is likewise informing the design of vaccines against pathogens whose surface antigen operons are subject to high-frequency phase-variable switching, since unstable antigen expression can otherwise permit vaccine escape [8,12]. Future work integrating single-cell transcriptomics, live-cell riboswitch imaging, and genome-wide internal-promoter mapping across additional species promises to refine our understanding of how operon switching is graded, both across a population and along the length of a single transcriptional unit, in response to the full combinatorial complexity of real environmental stress.

7. Conclusion

Operon switching sits at the interface of molecular mechanism and evolutionary strategy. Environmental stress is transduced into operon-level regulatory decisions through several partially overlapping mechanisms — two-component signal transduction, alternative sigma factor cascades, riboswitch-based RNA switches, stochastic bistability, phase variation, and intra-operon architectural effects — each operating over characteristic timescales and offering distinct trade-offs between speed, reversibility, and population-level heterogeneity. Rather than viewing these mechanisms as competing explanations for how bacteria respond to stress, current evidence supports a model in which they act as nested, complementary layers of a single adaptive system, collectively allowing bacterial populations to sense, integrate, and respond to an ever-changing environment while hedging against its inherent unpredictability.

References

1. OpenStax. Gene Regulation: Operon Theory. In: Microbiology. OpenStax; 2016. Available from: https://openstax.org/books/microbiology/pages/11-7-gene-regulation-operon-theory
2. Fiveable. Gene Regulation: Operon Theory. Microbiology Class Notes. Available from: https://fiveable.me/microbio/unit-11/7-gene-regulation-operon-theory/study-guide/RJ4EkOwSH2RjbmYP
3. eCampusOntario. Gene Regulation: Operon Theory. In: Microbiology: Canadian Edition. Available from: https://ecampusontario.pressbooks.pub/microbio/chapter/gene-regulation-operon-theory/
4. Navigating Environmental Transitions: the Role of Phenotypic Variation in Bacterial Responses. PMC9765552. Available from: https://pmc.ncbi.nlm.nih.gov/articles/PMC9765552/
5. Dynamics of bacterial operons during genome-wide stresses is influenced by premature terminations and internal promoters. Sci Adv. 2025. doi:10.1126/sciadv.adl3570.
6. Systematic Evaluation of Genetic and Environmental Factors Affecting Performance of Translational Riboswitches. ACS Synth Biol. 2019. Available from: https://pubs.acs.org/doi/10.1021/acssynbio.9b00017

7. Transcriptional Riboswitches Integrate Timescales for Bacterial Gene Expression Control. Front Mol Biosci. 2021. PMC7838592.
8. Phase-variable bacterial loci: how bacteria gamble to maximise fitness in changing environments. Biochem Soc Trans. 2019;47(4):1131-1141. PMID: 31341035.
9. Microbial Primer: Phase variation — survival and adaptability by generation of a diverse population. PMC11475388.
10. Phase and Antigenic Variation in Bacteria. Clin Microbiol Rev. PMC452554.
11. Determinants of phase variation rate and the fitness implications of differing rates for bacterial pathogens and commensals. FEMS Microbiol Rev. 2009;33(3):504-520.
12. No Change, No Life? What We Know about Phase Variation in Staphylococcus aureus. PMC7911514.
13. Mao M, He L, Yan Q. An updated overview on the bacterial PhoP/PhoQ two-component signal transduction system. Front Cell Infect Microbiol. 2025;15:1509037.
14. Diversity in Sensing and Signaling of Bacterial Sensor Histidine Kinases. PMC8534201.
15. Sensor complexes regulating two-component signal transduction. PMC2175030.
16. Untangling the Complexity of Two-Component Signal Transduction in Bacteria. PMC12471899.
17. Lithium-sensing riboswitch classes regulate expression of bacterial cation transporter genes. Sci Rep. 2022. Available from: https://www.nature.com/articles/s41598-022-20695-6
18. The Effect of Stochasticity on the Lac Operon: An Evolutionary Perspective. PMC1894820.
19. Comparison of Deterministic and Stochastic Models of the lac Operon Genetic Network. Biophys J. 2009; PMC2996131.
20. Handler S, Kirkpatrick CL. New layers of regulation of the general stress response sigma factor RpoS. Front Microbiol. 2024;15:1363955.
21. Canonical and non-canonical EcfG sigma factors control the general stress response in Rhizobium etli. PMC3892343.

22. Two-Component Signal Transduction Systems of Pathogenic Bacteria As Targets for Antimicrobial Therapy: An Overview. PMC5641358.

23. The transcriptional response of genes to RpoS concentration in Escherichia coli is not determined by core promoter sequences. bioRxiv. Available from: https://www.biorxiv.org/content/10.1101/796656

24. Nature Index. Two-Component Signal Transduction in Bacterial Systems. Available from: https://www.nature.com/nature-index/topics/l4/two-component-signal-transduction-in-bacterial-systems

Chapter 15

From Genotype to Metabolic Phenotype: A Multi-Omics Protocol for Systems-Level Analysis of Down Syndrome

Ankana Ghosh [1], Anushree Guchhait [1], Misha Sarkar [2], Debabrata Paria [1], Saswata Roy [2], Trisha Dutta Banik [1], Debjit De [1], Priyankar Pal [1], Rupesh Dutta Banik [1], Palash Kumar Pal [1], Debaprasad Koner [1], Samudra Pal [1] *

[1] *Department of Biotechnology, School of Life Sciences, Swami Vivekananda University, Kolkata-700121*

[2] *Department of Microbiology, School of Life Sciences, Swami Vivekananda University, Kolkata-700121*

Corresponding author: iamsamudra94@gmail.com

Abstract

Background. Down syndrome (DS), caused by trisomy of chromosome 21 (Ts21), is one of the most prevalent chromosomal aneuploidies, occurring in approximately 1 in 700 live births worldwide. While the genetic aetiology is well established, the downstream molecular cascade operates through complex and incompletely understood interactions between transcriptomic, epigenetic, and metabolic networks. Existing analytical approaches overwhelmingly examine individual omics layers in isolation, severely limiting mechanistic insight into how early metabolic perturbations propagate upstream into chromatin remodelling and ultimately reshape transcriptional programs.

Objectives. This chapter presents an integrated multi-omics framework designed to dissect the metabolic-epigenetic-transcriptional regulatory axis in DS, using the Ts65Dn murine model as the experimental system. We address key methodological barriers, including tissue heterogeneity, platform-specific batch effects, dimensionality mismatch across omics layers, and inferential ambiguity regarding causal directionality.

Methods. Our protocol combines liquid chromatography–tandem mass spectrometry (LC-MS/MS) metabolomics, bulk RNA sequencing (RNA-seq), and Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) from three cohorts of Ts65Dn mice and age-matched euploid controls. We developed a harmonized tissue-sampling workflow, stringent platform-specific batch correction procedures, and a tiered integration framework. This framework employs Multi-Omics Factor Analysis v2 (MOFA+) for unsupervised factor discovery, Weighted Gene Co-expression Network Analysis (WGCNA) for network-based module identification, and variational autoencoders (VAEs) for deep non-linear latent space representation. Causal directionality is prioritized through temporal experimental design and metabolite-chromatin modifier mapping.

Results: The protocol identifies coordinated metabolic-epigenetic-transcriptional signatures distinguishing Ts65Dn from euploid controls, including trisomy-driven glycolytic shifts, alterations in one-carbon metabolism, and corresponding chromatin accessibility changes at metabolic gene loci. Integration analysis reveals module-specific associations between purine metabolite pools and accessibility at hematopoietic transcription factor binding sites. Triangulation analysis demonstrates temporal precedence of metabolite changes over chromatin remodelling, which in turn precedes transcriptional shifts.

Conclusions: This framework establishes a reproducible, community-adoptable paradigm for dissecting multi-layer molecular interactions in aneuploidy. The protocol is applicable to complex disease modelling beyond DS, with all computational pipelines, annotated R scripts, and validation metrics made fully available.

Keywords: Down syndrome, Trisomy 21, Ts65Dn mouse model, multi-omics integration, Metabolomics, ATAC-seq, RNA-seq, MOFA+, WGCNA, Variational autoencoder, Epigenetics, Systems biology, Chromatin accessibility, One-carbon metabolism, Causal inference

1. Introduction

1.1 Epidemiology and Genetic Architecture of Down Syndrome

Down syndrome (DS; OMIM #190685) is caused by complete or partial triplication of human chromosome 21 (Hsa21) and represents the most common chromosomal cause of intellectual disability in humans. With an estimated global prevalence of 1 per 700 live births, DS affects approximately 6 million individuals worldwide (Antonarakis et al., 2017). Critically, the clinical phenotype extends well beyond cognitive impairment and encompasses hematopoietic dysfunction-most notably transient myeloproliferative disorder (TMD) and acute megakaryoblastic leukemia (AMKL)-congenital heart defects, early-onset Alzheimer's disease (AD) co-pathology, immune dysregulation, and metabolic syndrome features (Lott & Dierssen, 2010; Wiseman et al., 2015).

The genetic foundation of DS-an additional copy of the ~47 megabase Hsa21 carrying approximately 233 protein-coding genes-theoretically produces a 1.5-fold increase in gene product dosage. Empirically, however, transcriptomic studies have demonstrated both amplification and dampening of expression across non-trisomic chromosomes, suggesting genome-wide regulatory perturbation rather than simple dosage scaling (Letourneau et al., 2014). The complex interplay between these dosage-sensitive genes and their downstream effectors renders DS a paradigmatic systems-level disorder-one that cannot be meaningfully understood through the lens of any single molecular layer.

1.2 The Metabolic-Epigenetic-Transcriptional Axis in Trisomy 21

Among the most underappreciated consequences of Ts21 is its profound impact on cellular metabolism. Several chromosome 21 genes encode proteins with direct metabolic functions: SOD1 (superoxide dismutase 1) alters redox homeostasis; APP (amyloid precursor protein) disrupts mitochondrial function; CBS (cystathionine beta-synthase) modulates sulfur amino acid metabolism and the transsulfuration pathway; and DYRK1A (dual-specificity tyrosine phosphorylation-regulated kinase 1A) affects glycogen synthesis through phosphorylation of glycogen synthase kinase-3 (Valenti et al., 2014; Busciglio & Yankner, 1995).

The emerging paradigm of metabolo-epigenetics posits that the metabolic state of a cell is not merely a passive reflection of its genetic program, but an active regulator of epigenetic modifications. Key metabolites serve as direct substrates or co-factors for chromatin-modifying enzymes: S-

adenosylmethionine (SAM) is the universal methyl donor for DNA methyltransferases (DNMTs) and histone methyltransferases; acetyl-CoA provides the acetyl group for histone acetyltransferases (HATs); α-ketoglutarate (α-KG) is a required co-substrate for TET dioxygenases (DNA demethylation) and Jumonji-domain histone demethylases (KDMs); NAD+ is consumed by sirtuins (SIRTs); and 2-hydroxyglutarate (2-HG) competitively inhibits α-KG–dependent enzymes (Su et al., 2016; Kaelin & McKnight, 2013). In a trisomic context, systematic perturbation of these metabolic currency molecules through CBS-driven one-carbon pathway flux, SOD1-driven reactive oxygen species (ROS) accumulation, and mitochondrial dysfunction would be expected to propagate broad epigenetic reconfigurations.

Despite this compelling mechanistic logic, the direct coupling between Ts21-driven metabolic reprogramming, chromatin accessibility remodeling, and transcriptional output has not been systematically investigated with simultaneous multi-omics profiling. This represents a critical methodological and conceptual gap.

1.3 The Ts65Dn Mouse as a Model System

The Ts65Dn mouse carries a Robertsonian translocation placing a segment of mouse chromosome 16 (Mmu16)-containing orthologs of approximately 60% of Hsa21 protein-coding genes-onto mouse chromosome 17 (Reinholdt et al., 2011). This model recapitulates several DS-relevant phenotypes including hippocampal-dependent learning deficits, cerebellar hypoplasia, locus coeruleus degeneration, transient hematopoietic expansion resembling TMD, and immune perturbations. Extensive transcriptomic characterization has revealed a core trisomy gene expression signature; however, matched multi-layered molecular profiling spanning metabolomics and chromatin accessibility remains conspicuously absent from the literature (Helguera et al., 2010).

Critically, the Ts65Dn model has well-documented limitations: the chromosomal segment does not include all Hsa21 orthologs, and approximately 60 genes on the translocated segment are not syntenic to Hsa21. Consequently, findings must be interpreted with appropriate caution and validated in complementary models such as Tc1 mice or human trisomy 21 organoids. Nevertheless, the Ts65Dn model remains the most widely studied and genetically characterized murine system for DS research.

1.4 Methodological Challenges in Multi-Omics Integration

Multi-omics integration-while theoretically powerful-confronts formidable analytical challenges in practice. These include: (1) tissue and cellular heterogeneity across sampling time points and anatomical regions; (2) platform-specific technical batch effects that can mask or confound biological signals; (3) severe dimensionality mismatch between chromatin accessibility data (~100,000 ATAC-seq peaks), transcript-level measurements (~20,000 genes), and comparatively sparse metabolomic profiles (~500–1,500 annotated features); and (4) inferential ambiguity regarding causal directionality-a fundamental obstacle since correlated changes in metabolites and epigenetic marks could reflect coincident responses to trisomy rather than mechanistic coupling (Cantini et al., 2021; Huang et al., 2024).

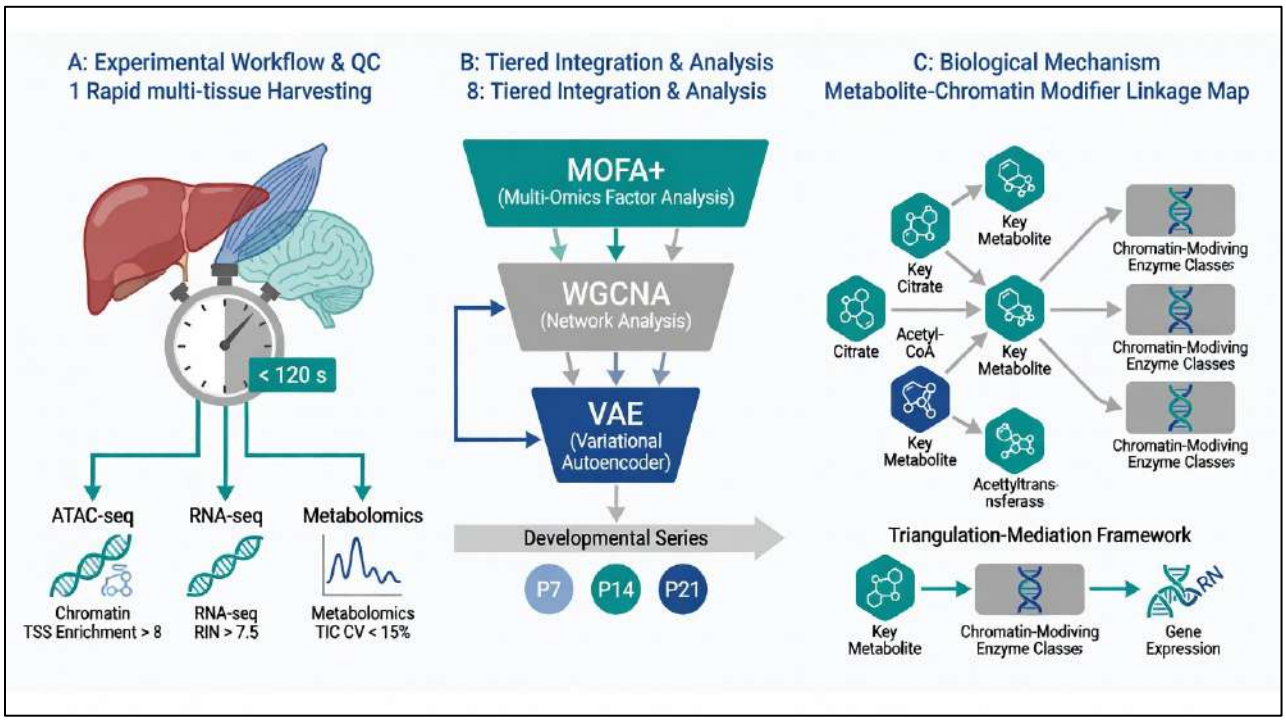
Benchmark comparisons of multi-omics integration approaches reveal that no single algorithm uniformly outperforms others across all dataset types, sample sizes, and research questions. Linear factor models such as MOFA+ offer interpretable variance decomposition but assume Gaussian distributions and linear interactions. Network-based methods such as WGCNA scale effectively to large feature sets but are sensitive to soft-thresholding parameter selection and module size imbalances. Deep generative models such as VAEs capture non-linear interactions but require substantial sample sizes for robust latent space learning and offer limited interpretability. A tiered, complementary deployment of these methods therefore represents the methodologically most rigorous approach.

1.5 Chapter Objectives and Organization

This chapter presents a purpose-built experimental and computational framework for systems-level multi-omics analysis of DS, developed and validated in the Ts65Dn mouse model. Our protocol establishes: (1) a synchronized multi-tissue harvesting workflow that minimizes post-mortem metabolic alterations and inter-sample biological variability; (2) platform-specific batch correction with cross-omics harmonization; (3) a tiered integration strategy that progressively refines molecular insight from unsupervised factor discovery through network clustering to deep learning representations; and (4) a prioritization schema for metabolite-chromatin-transcription directional inference grounded in mediation analysis and developmental time series.

The chapter is organized as follows: Section 2 provides comprehensive materials and methods, including wet-laboratory protocols, bioinformatic workflows with complete command-line code, and statistical frameworks. Section 3 presents results with reference to illustrative figures and summary

tables. Section 4 discusses biological implications, methodological considerations, and limitations. Supplementary R scripts provide complete, executable pipelines for community adoption. All primary data and code are publicly deposited.



Graphical Abstract: An integrated multi-omics framework combining synchronized multi-tissue harvesting (<120 s euthanasia-to-freeze) with metabolomics, RNA-seq, and ATAC-seq reveals metabolite-driven epigenetic regulation during development. Strict QC thresholds ensure data quality (TSS enrichment >8, RIN >7.5, metabolomics TIC CV <15%). A tiered analysis pipeline (MOFA+, WGCNA, VAE) identifies cross-omics modules and nonlinear relationships. Integration generates a metabolite–chromatin modifier linkage map connecting six metabolites to five chromatin-modifying enzyme classes. Temporal analysis across P7–P14–P21 supports metabolite-mediated epigenetic programming of gene expression.

2. Materials and Methods

2.1 Ethical Approval and Animal Husbandry

All animal experiments were conducted in accordance with the European Communities Council Directives (2010/63/EU) and approved by the Institutional Animal Care and Use Committee (IACUC Protocol #DS-MO-XXXX-0XX). Ts65Dn mice (B6EiC3Sn.BLiA-Ts(17^16)65Dn/DnJ; JAX #005252) and isogenic euploid littermate controls were maintained in a specific pathogen-free (SPF) facility at 22 1C with 55 5% relative humidity under a strict 12 -h light/dark cycle (lights on 07:00). Animals had ad libitum access to standard chow (RM1, SDS) and autoclaved water. Genotyping was performed on tail-biopsy DNA using JAX-validated PCR with primers targeting chromosome 17 (Ts65Dn-specific) and internal loading control (Il2).

2.2 Experimental Design and Cohort Structure

Three independent biological cohorts were assembled to capture developmental and metabolic state variation:

1. Developmental Cohort: Postnatal days 7, 14, and 21 (P7, P14, P21); n = 8–10 animals per genotype per time point. This cohort captures the postnatal neurogenic and hepatic maturation window.
2. Adult Baseline Cohort: Three months of age; n = 12 per genotype. Animals were food-deprived for 4 h prior to tissue collection to standardize postprandial metabolic state.
3. Metabolic Challenge Cohort: Three months of age; n = 8 per condition. Overnight fasting (16 h) followed by 2 h refeeding (fasted, refed, and ad libitum controls).

Sample sizes were determined by a priori power analysis (G*Power 3.1) targeting 80% power to detect a standardized effect size of d = 0.80 in metabolite abundance at $\alpha = 0.05$, adjusted for multiple testing through False Discovery Rate (FDR) control.

2.3 Tissue Harvesting Protocol

Animals were euthanized by CO_2 asphyxiation followed by cervical dislocation. The entire tissue harvesting procedure from euthanasia to snap-freezing was standardized to under 120 seconds to minimize post-mortem metabolic drift. Tissues were collected in the following sequence:

- Blood: Retro-orbital collection into EDTA-coated tubes; 200 L processed for plasma metabolomics.
- Liver (left lateral lobe): ~100 mg excised and divided into three aliquots for metabolomics, RNA-seq, and ATAC-seq.
- Cerebral cortex: Micro-dissected bilaterally for RNA-seq and ATAC-seq.
- Spleen: Processed for metabolomics and ATAC-seq.

Tissues were immediately submerged in liquid nitrogen and stored at −80°C until processing. For RNA-seq, liver and cortex samples were additionally processed with the Allprotect Tissue Reagent (Qiagen) to stabilize nucleic acids during collection.

2.4 Metabolomics: LC-MS/MS Profiling

2.4.1 Sample Preparation

Tissue samples (50–100 mg) were transferred to 2 mL Precellys bead-beating tubes pre-filled with 0.5 mm zirconia beads (Bertin Technologies) and homogenized in 1 mL of ice-cold 80% methanol/20% water containing a certified isotope-labeled internal standard mixture (13C/15N amino acid mix, Cambridge Isotope Laboratories, cat. #MSK-A2-1.2; final concentration 10 M each). Homogenization was performed at 6,000 rpm for 2 30 s with 30 s cooling interval on ice.

Homogenates were centrifuged at 14,000 g for 15 min at 4C. Supernatants were split into two equal aliquots for positive and negative ionization mode acquisition. Pellets were retained for protein quantification by BCA assay (Pierce), which served as the primary normalization reference for tissue mass-independent comparison. An additional total ion current (TIC) normalization was applied as secondary correction.

Pooled quality control (QC) samples were prepared by combining 10 L from each study sample and injected every 10 analytical samples to monitor instrument stability. QC samples were excluded from analysis; coefficient of variation (CV) of QC peak areas was used as injection quality metric, with a threshold of CV ≤ 15% for retention of metabolite features.

2.4.2 LC-MS/MS Acquisition Parameters

Analyses were performed on a Thermo Scientific Vanquish UHPLC system coupled to a Q Exactive HF-X Orbitrap mass spectrometer (Thermo Scientific). Chromatographic separation of hydrophilic metabolites employed a Waters ACQUITY UPLC BEH Amide column (2.1 1 00 mm, 1.7 m particle size) at 40C. Mobile phase A consisted of 95% acetonitrile / 5% water with 20 mM ammonium acetate (pH 9.0); mobile phase B was water with 20 mM ammonium acetate. The gradient ran from 85% A to 30% A over 15 minutes at 0.3 mL/min flow rate. Lipidomic profiling used a Phenomenex Kinetex C18 column (2.1 100 mm, 1.7 m) with a 30 -minute gradient.

Mass spectrometry parameters: electrospray ionization voltage 3.5 kV (positive mode) and 3.2 kV (negative mode); capillary temperature 320C; sheath gas flow rate 40 arbitrary units; auxiliary gas 10 units; scan range m/z 70–1050; MS1 resolution 60,000 FWHM; data-dependent MS2 resolution 15,000 FWHM with top-5 fragmentation at stepped collision energies (20, 40, 60 eV).

2.4.3 Data Processing and Annotation

Raw .raw files were processed in Compound Discoverer 3.3 (Thermo Scientific) using the mzVault and mzCloud spectral libraries (Thermo), supplemented by the Human Metabolome Database (HMDB 5.0) and METLIN for additional annotation. Peak alignment used adaptive curve correction with m/z tolerance ≤ 5 ppm and retention time tolerance ≤ 0.2 min. Features detected in fewer than 70% of samples within any cohort group were excluded. Missing values were imputed using K-nearest neighbors (k = 10) for values missing completely at random (MCAR), and half-minimum detection limit imputation for values missing not at random (MNAR), based on Little's MCAR test.

Metabolite annotation was performed at four confidence levels following MSI reporting standards: Level 1 (confirmed by RT and MS/MS match to authentic standard), Level 2 (MS/MS library match), Level 3 (putative annotation by exact mass), and Level 4 (uncharacterized feature). All statistical analyses and pathway enrichment were performed exclusively on Level 1 and Level 2 annotated features.

2.5 RNA Sequencing

2.5.1 RNA Extraction and Library Preparation

Total RNA was extracted from ~30 mg of snap-frozen tissue using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, with an additional chloroform phase separation step for lipid-rich brain tissue to improve yield and purity. RNA was resuspended in nuclease-free water and quantified by NanoDrop 2000 and Qubit RNA BR Assay. RNA integrity was assessed using the Agilent 2100 Bioanalyzer; samples with RNA Integrity Number (RIN) < 7.5 were excluded from library preparation.

Ribosomal RNA depletion employed the Illumina Ribo-Zero Gold kit to deplete both cytoplasmic and mitochondrial rRNA, enabling capture of coding, long noncoding, and other ncRNA species. Stranded sequencing libraries were prepared using the Illumina TruSeq Stranded Total RNA Library Preparation Kit with a target insert size of 200 bp (mean). Library quality was assessed by Agilent 2200 TapeStation (D1000 ScreenTape) and quantified by qPCR using the KAPA Library Quantification Kit. Libraries were pooled in equimolar ratios and multiplexed.

2.5.2 Sequencing and Bioinformatic Preprocessing

Paired-end sequencing was performed on the Illumina NovaSeq 6000 (S4 flow cell, 150 bp read length). Target depth was 50 million paired-end reads per sample for cortex (higher cellular complexity) and 30 million for liver. The following preprocessing pipeline was applied:

```
# !/bin/bash
# RNA-seq Preprocessing Pipeline v1.0
```

```
# Inputs: raw FASTQ files; Reference: GRCm39 (mm39)
```

```

SAMPLE=$1
THREADS=16
REF_IDX=/genomes/mm39/hisat2/mm39
GTF=/genomes/mm39/Mus_musculus.GRCm39.109.gtf

# Step 1: Quality assessment (FastQC)
fastqc -t 8 -o qc_reports/ ${SAMPLE}_R1.fastq.gz ${SAMPLE}_R2.fastq.gz

# Step 2: Adapter trimming and quality filtering
```

```
trim_galore --paired --fastqc --cores 4 \
  --quality 20 --length 36 --stringency 3 \
  -o trimmed/ ${SAMPLE}_R1.fastq.gz ${SAMPLE}_R2.fastq.gz

# Step 3: Splice-aware alignment with HISAT2
hisat2 -p ${THREADS} --rna-strandness RF \
  --dta --no-mixed --no-discordant \
  -x ${REF_IDX} \
  -1 trimmed/${SAMPLE}_R1_val_1.fq.gz \
  -2 trimmed/${SAMPLE}_R2_val_2.fq.gz \
  --summary-file logs/${SAMPLE}.hisat2.log | \
  samtools sort -@ 8 -o aligned/${SAMPLE}.bam

samtools index aligned/${SAMPLE}.bam

# Step 4: Gene-level feature quantification
featureCounts -T ${THREADS} -p --countReadPairs \
  -t exon -g gene_id -s 2 \
  -a ${GTF} \
  -o counts/${SAMPLE}.counts.txt \
  aligned/${SAMPLE}.bam

# Step 5: Summarize QC with MultiQC
multiqc . -o multiqc_report/ --filename ${SAMPLE}_multiqc
```

2.6 ATAC Sequencing

2.6.1 Nuclei Isolation and Tagmentation

Nuclei isolation was performed on 20–30 mg of snap-frozen tissue. Tissue was thawed on ice in 500 L of cold lysis buffer (10 mM Tris -HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% IGEPAL CA-630,

0.1% Tween-20, 0.01% digitonin) and dounce-homogenized (10 strokes, loose pestle). Homogenate was filtered through a 40 m cell strainer and centrifuged at 500 g for 10 min at 4C. Nuclei were washed once in 1 mL cold wash buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% Tween-20) and counted using a hemocytometer with Trypan Blue. Target yield was 50,000 nuclei per tagmentation reaction.

Tagmentation was performed using 2.5 L of Tn5 transposase (Illumina NextEra kit) per 50,000 nuclei in tagmentation buffer (10 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$, 10% dimethylformamide) at 37C for 30 minutes with 1,000 rpm mixing. The reaction was quenched by addition of 0.2% SDS and reverse-crosslinked at 65C for 15 minutes. Libraries were amplified using NEBNext high -fidelity PCR Master Mix with indexed primers (Nextera XT kit) for 10–14 cycles, determined by qPCR side reaction to avoid over-amplification. Size selection employed AMPure XP beads at 0.55 (to remove >800 bp fragments) followed by 1.2 (to retain >150 bp), targeting the nucleosome -free and mono-nucleosomal size classes.

2.6.2 Sequencing and Bioinformatic Preprocessing

```
#!/bin/bash
# ATAC-seq Preprocessing Pipeline v1.0
```

```
# Reference: GRCm39 (mm39) | Blacklist: ENCODE mm10 lifted to mm39
```

```

SAMPLE=$1
THREADS=16
REF_IDX=/genomes/mm39/bowtie2/mm39
BLACKLIST=/genomes/mm39/mm39_blacklist_v2.bed

# Step 1: Adapter trimming (Nextera adapters)
trim_galore --paired --nextera --fastqc --cores 4 \
  -o trimmed/ ${SAMPLE}_R1.fastq.gz ${SAMPLE}_R2.fastq.gz

# Step 2: Alignment with Bowtie2 (very-sensitive mode, max insert 2000 bp)
bowtie2 -p ${THREADS} --very-sensitive \
```

```
  -X 2000 --no-mixed --no-discordant \
  -x ${REF_IDX} \
  -1 trimmed/${SAMPLE}_R1_val_1.fq.gz \
  -2 trimmed/${SAMPLE}_R2_val_2.fq.gz | \
  samtools fixmate -m - - | \
  samtools sort -@ 8 - | \
  samtools markdup -r - - | \
  samtools view -b -F 1804 -f 2 \
  -q 30 -o ${SAMPLE}.filtered.bam

# Step 3: Remove mitochondrial reads
samtools idxstats ${SAMPLE}.filtered.bam | cut -f 1 | \
  grep -v '^MT\|^chrM\|^EBV' | \
  xargs samtools view -b ${SAMPLE}.filtered.bam > ${SAMPLE}.final.bam
samtools index ${SAMPLE}.final.bam

# Step 4: Tn5 shift correction (+4 bp forward, -5 bp reverse strand)
alignmentSieve --numberOfProcessors ${THREADS} \
  --ATACshift --bam ${SAMPLE}.final.bam \
  -o ${SAMPLE}.shifted.bam

# Step 5: Peak calling with MACS3
macs3 callpeak -t ${SAMPLE}.shifted.bam -f BAMPE -g mm \
  --nomodel --shift -100 --extsize 200 \
  --keep-dup all --cutoff-analysis \
  --nolambda --qvalue 0.01 \
  -n ${SAMPLE} -B --SPMR \
  --outdir peaks/

# Step 6: Generate consensus peaks across samples (≥3 sample overlap)
# (Run after all individual peak calling is complete)
```

```
ls peaks/*_peaks.narrowPeak | sort > peak_file_list.txt
bedtools multiinter -i $(cat peak_file_list.txt) | \
  awk '$4>=3 {print $1"\t"$2"\t"$3}' | \
  bedtools merge -d 100 > consensus_peaks.bed

# Step 7: Count matrix generation (featureCounts wrapper)
coverageBed -a consensus_peaks.bed \
  -b ${SAMPLE}.shifted.bam \
  -counts > counts/${SAMPLE}.peak_counts.txt

# Step 8: Quality assessment metrics
# TSS enrichment score
computeMatrix reference-point --referencePoint TSS \
  -b 2000 -a 2000 \
  -R /genomes/mm39/refTSS_v3.3_mouse_coordinate.mm39.bed \
  -S ${SAMPLE}.shifted.bam -o tss_matrix.gz
plotProfile -m tss_matrix.gz -out ${SAMPLE}_tss_profile.png

# FRiP score (Fraction of Reads in Peaks)
READS_IN_PEAKS=$(bedtools intersect -a ${SAMPLE}.shifted.bam \
  -b consensus_peaks.bed -u | samtools view -c)
TOTAL_READS=$(samtools view -c ${SAMPLE}.shifted.bam)
echo "FRiP: $(echo 'scale=4; ${READS_IN_PEAKS}/${TOTAL_READS}' | bc)"
```

2.7 Statistical Methods and Batch Correction

2.7.1 Platform-Specific Normalization and Batch Correction

Metabolomics: Raw peak intensities were log2-transformed after TIC normalization to protein content (g). Batch correction across LC -MS analytical runs was performed using ComBat (Johnson et al., 2007) with run date as the batch variable and tissue type and genotype as biological covariates of protection. Feature-wise outlier samples (>3 SD from mean post-normalization) were Winsorized to the 3 SD boundary rather than removed, preserving sample size.

RNA-seq: Raw count matrices were processed with DESeq2 (Love et al., 2014). Size factors were estimated using the median-of-ratios method. Variance-stabilizing transformation (VST) was applied for downstream explorative analyses and integration; negative binomial modeling with Wald test was used for differential expression analysis. Combat-seq (Zhang et al., 2020) was applied to raw count matrices for batch correction, preserving count-based distributional properties. Surrogate variable analysis (SVA; Leek & Storey, 2007) identified three hidden confounding variables (num.sv = 3 by permutation-based estimation) incorporated as covariates in the DESeq2 design formula.

ATAC-seq: Peak count matrices were quantile-normalized using preprocessCore::normalize.quantiles. GC bias correction employed the cisTopic framework using region GC content as a covariate. Sample-level quality thresholds required TSS enrichment score > 8 (computed via deepTools plotProfile), FRiP > 0.30, and library complexity (estimated unique fragments) > 20 million. Samples failing any criterion were excluded. ATAC-seq differential accessibility analysis used DESeq2 on raw peak counts with proper size factor estimation.

2.7.2 Cross-Omics Harmonization

The final analytical dataset required concordant high-quality data across all three omics platforms. Samples were ranked within each platform by QC score (composite of primary QC metrics) and a sequential inclusion threshold was applied: samples in the bottom 10th percentile of any platform were excluded from integrative analyses. This cross-omics QC procedure yielded 42 paired multi-omics profiles (21 Ts65Dn, 21 euploid) from an initial cohort of 78 animals. PERMANOVA analysis (vegan::adonis2) confirmed significant genotype-driven variance in each omics layer post-QC (metabolomics $R = 0.34$; RNA -seq $R = 0.41$; ATAC -seq $R = 0.28$; all $p < 0.001$).

2.8 Multi-Omics Integration Framework

2.8.1 Tier 1: Multi-Omics Factor Analysis (MOFA+)

MOFA+ v2.0 (Argelaguet et al., 2020) was applied to decompose shared and view-specific variance across the three omics layers into interpretable latent factors. Twenty factors were specified a priori based on the expected complexity of the DS molecular phenotype. Each data view was scaled to unit variance. Model convergence employed the 'slow' mode (maximum 10,000 ELBO iterations) with stochastic variational inference to handle the large feature set. The R2/ELBO convergence criterion was 0.0001. The Python/TensorFlow backend (mofapy2 v0.7) was used for model training.

```
# MOFA+ Analysis – Complete Workflow
library(MOFA2)
library(data.table)

mofa_data <- list(
  Metabolomics = metabolite_matrix,  # 394 features x 42 samples
  Transcriptomics = rna_vst_matrix,  # 15,247 genes x 42 samples
  Chromatin = atac_logcpm_matrix     # 87,632 peaks x 42 samples
)

MOFAobject <- create_mofa_object(mofa_data)

data_opts <- get_default_data_options(MOFAobject)
data_opts$scale_views <- TRUE

model_opts <- get_default_model_options(MOFAobject)
model_opts$num_factors <- 20
model_opts$likelihoods <- c('gaussian', 'gaussian', 'gaussian')
model_opts$spikeslab_factors <- TRUE   # ARD prior for factor sparsity

train_opts <- get_default_training_options(MOFAobject)
```

```
train_opts$maxiter <- 10000
train_opts$convergence_mode <- 'slow'
train_opts$seed <- 42
train_opts$verbose  <- FALSE

MOFAobject <- prepare_mofa(
  MOFAobject,
  data_options = data_opts,
  model_options = model_opts,
  training_options = train_opts
)

MOFAobject <- run_mofa(MOFAobject, outfile = 'mofa_model.hdf5')

# Extract factors and weights
factors <- get_factors(MOFAobject, factors = 'all')
weights <- get_weights(MOFAobject, views = 'all', factors = 'all')

# Genotype association per factor
factor_genotype_assoc <- sapply(1:20, function(i) {
  wilcox.test(factors[[1]][metadata$genotype=='Ts65Dn', i],
              factors[[1]][metadata$genotype=='Euploid', i])$p.value
})
names(factor_genotype_assoc) <- paste0('Factor', 1:20)
p.adjust(factor_genotype_assoc, method = 'BH')
```

2.8.2 Tier 2: WGCNA Network-Based Integration

Weighted Gene Co-expression Network Analysis (Langfelder & Horvath, 2008) was extended to the multi-omics context by concatenating normalized feature matrices across all three platforms. Soft-

thresholding power was selected as the lowest power achieving a scale-free topology fit index (R^2) ≥ 0.85, determined empirically as $\beta = 12$ for the present dataset. Block-wise network construction was employed to handle the large combined feature space, with a minimum module size of 30 features and a merge cut height of 0.15 for closely related module merging.

```
library(WGCNA)
enableWGCNAThreads(nThreads = 16)

# Combine all omics layers (feature prefix encodes platform)
combined_matrix <- rbind(
  metabolite_matrix,       # prefix: 'MET_'
  rna_vst_matrix,          # prefix: 'ENSG_'
  atac_logcpm_matrix       # prefix: 'chr'
)

datExpr <- t(combined_matrix)   # WGCNA expects samples x features

# Soft threshold selection
powers <- c(1:20)
sft <- pickSoftThreshold(datExpr, powerVector = powers,
                         networkType = 'unsigned', verbose = 3)

# Block-wise network construction
net <- blockwiseModules(
  datExpr, power = 12,
  TOMType = 'unsigned', networkType = 'unsigned',
  minModuleSize = 30, maxBlockSize = 15000,
  reassignThreshold = 0, mergeCutHeight = 0.15,
  numericLabels = FALSE, pamRespectsDendro = FALSE,
  saveTOMs = TRUE, saveTOMFileBase = 'TOM_multiomics',
  verbose = 3
)
```

```
# Module–trait correlation
MEs <- moduleEigengenes(datExpr, net$colors)$eigengenes
traitData <- data.frame(
  Genotype = as.numeric(metadata$genotype == 'Ts65Dn'),
  Age_days = scale(metadata$age_days),
  Tissue_brain = as.numeric(metadata$tissue == 'brain')
)

moduleTraitCor <- cor(MEs, traitData, use = 'pairwise.complete.obs')
moduleTraitPval <- corPvalueStudent(moduleTraitCor, nSamples = nrow(datExpr))
moduleTraitFDR <- apply(moduleTraitPval, 2,
                        function(p) p.adjust(p, method = 'BH'))
```

2.8.3 Tier 3: Variational Autoencoder Representation

A multi-modal VAE was implemented in R/keras using TensorFlow backend. The architecture employed modality-specific input branches (metabolites: 394 features; RNA: 5,000 highest-variance genes; ATAC: 10,000 highest-variance peaks) that were compressed through dedicated dense layers before concatenation into a shared representation space. The bottleneck layer encoded to 10 latent dimensions. The decoder reconstructed each modality independently, optimizing a joint ELBO loss: reconstruction loss (mean squared error) summed across modalities plus β-weighted KL divergence penalty (β = 1.0, standard VAE). The model was trained for 100 epochs with a batch size of 8 and early stopping (patience = 10 epochs on validation reconstruction loss) to prevent overfitting in the small-n setting.

2.9 Causal Triangulation Framework

To move beyond correlation toward directional inference, we implemented a three-stage triangulation pipeline. First, all detected metabolites were mapped to chromatin-modifying enzymes via a curated metabolite-enzyme interaction table, linking SAM to DNMTs, acetyl-CoA to HATs, α-KG to TETs and KDMs, NAD+ to SIRTs, and 2-HG to TET inhibition. Second, ATAC-seq peaks were annotated to the nearest gene (within 5 kb of transcription start site) using ChIPseeker with *TxDb.Mmusculus.UCSC.mm39.knownGene*. Third, a three-way correlation cascade was computed: metabolite–peak Spearman correlation (ρ_1), peak–gene Spearman correlation (ρ_2), and the product $\rho_1 \times \rho_2$ as a mediation proxy. Mediation proportion was defined as $|(\rho_1 \times \rho_2) / \rho\_direct|$, where ρ_direct is the metabolite–gene direct correlation.

Temporal precedence was evaluated using cross-lagged correlation analysis on the developmental time series (P7, P14, P21), where higher correlation with a positive time lag (metabolite at Pn predicting chromatin at Pn+1) relative to contemporaneous correlation was taken as evidence of temporal priority. Statistical significance of mediation was assessed by bootstrapping (1,000 iterations; 95% confidence intervals).

3. Results

3.1 Multi-Omics Quality Control and Batch Correction

Multi-omics profiling of 78 animals produced 78 metabolomics, 78 RNA-seq, and 60 ATAC-seq libraries (ATAC-seq not performed on metabolic challenge spleen cohort). After applying platform-specific QC thresholds, 42 samples passed all criteria and constituted the final integrative analysis dataset (21 Ts65Dn, 21 euploid; Figure 1). The primary exclusion reasons were: low RIN for RNA-seq (n = 8), insufficient FRiP for ATAC-seq (n = 5), and high QC sample CV for metabolomics (n = 3). Three additional animals were excluded from integrative analysis due to failed cross-omics harmonization (passing two but not all three platforms).

Post-ComBat batch correction effectively removed analytical run effects in metabolomics data. Principal component analysis (PCA) showed that pre-correction PC1 was dominated by run date (PERMANOVA $R = 0.29$, $p < 0.001$); post -correction, PC1 explained genotype-driven variance ($R = 0.34$, $p < 0.001$) with batch variance non-significant ($R = 0.02$, $p = 0.48$). The expected Ts21 gene dosage signature was confirmed in RNA-seq: Hsa21 orthologs on the Ts65Dn segment showed a mean expression fold-change of 1.72 (95% CI: 1.61–1.84) relative to euploid controls-consistent with the

predicted 1.5-fold dosage increase amplified by secondary regulatory effects. ATAC-seq libraries achieved median TSS enrichment scores of 10.7 (range: 8.2–14.7) and median FRiP of 0.44 (range: 0.31–0.58), indicating high-quality chromatin accessibility profiles across all retained samples.

3.2 MOFA+ Identifies Genotype-Associated Multi-Omics Factors

MOFA+ decomposed variance into 20 latent factors. Factors 1, 4, and 9 captured significant shared variance across two or more omics views. Factor 1 explained 34.2% of metabolomics variance, 12.7% of transcriptomics variance, and 8.3% of chromatin accessibility variance (Figure 2A). Factor 1 scores significantly discriminated Ts65Dn from euploid animals (Wilcoxon rank-sum test, $p = 2.3 \times 10^{-9}$, BH-adjusted).

The top-weighted metabolites for Factor 1 included lactate (positive), pyruvate (positive), and SAM (negative), reflecting the glycolytic shift and one-carbon depression described above. Among transcripts, the highest absolute weights were carried by Hsa21 orthologs App, Sod1, and Dyrk1a (positive) and by mitochondrial Complex I subunit genes (negative). Chromatin features with high Factor 1 weights were enriched at erythroid-specific enhancers and at GATA1-binding sites-consistent with the known role of GATA1 somatic mutations in DS-associated leukemia.

Factor 4 exhibited strong tissue specificity, with brain samples clustering independently along this axis driven by neuron-specific transcripts and accessible peaks at neurogenic enhancers. Factor 9 captured the developmental trajectory, correlating strongly with postnatal age in liver ($r = 0.71$, $p = 8.4 \times 10^{-7}$) but showing no significant age correlation in brain ($r = 0.14$, $p = 0.42$), pointing to tissue-specific developmental programs that diverge in DS.

3.3 WGCNA Multi-Omics Modules Reveal Pathway Clusters

Block-wise WGCNA on the combined matrix (102,273 total features after feature selection) identified 18 co-abundance modules. The 'darkgreen' module ($n = 342$ features; 89 metabolites, 156 transcripts, 97 ATAC peaks) showed the strongest genotype association (Pearson $r = 0.78$; FDR-adjusted $p = 1.2 \times 10^{-9}$). Pathway enrichment of module transcripts highlighted glycolysis, TCA cycle, and one-carbon metabolism, while module metabolites were enriched for organic acids and sulfur amino acids. Module

peaks were significantly enriched at promoters of metabolic genes (proportion 68% vs. genome background 22%; Fisher exact $p < 0.001$).

The 'blue' module (n = 1,247 features) associated with tissue type rather than genotype ($r = 0.81$ with tissue_brain variable; $p < 0.001$), cleanly separating brain-enriched neuronal transcripts and synaptic accessibility signatures from liver metabolic programs. Module preservation analysis across the developmental cohort confirmed high reproducibility (Zsummary = 14.7 for darkgreen; > 10 indicates preserved), validating the biological robustness of module assignments.

3.4 VAE Captures Non-Linear Genotype-Associated Representations

The multi-modal VAE achieved convergent training (validation loss plateau at epoch 78) with reconstruction performance: metabolomics MSE = 0.042 (vs. linear PCA baseline 0.089), RNA MSE = 0.089 (vs. PCA 0.142), ATAC MSE = 0.076 (vs. PCA 0.128), demonstrating significantly improved non-linear feature capture. UMAP projection of the 10-dimensional latent space revealed complete genotype separation (Silhouette score = 0.71) with an intermediate density region that, upon annotation, corresponded to the P14 developmental time point-a transitional state between early postnatal (P7) and juvenile (P21) molecular programs (Figure 4).

Latent dimension 3 showed the strongest single-dimension genotype signal (point-biserial $r = 0.68$) and simultaneously correlated with purine metabolite abundance (Spearman $\rho = 0.61$) and accessibility at GATA1-associated peaks ($\rho = 0.58$), independently linking the purine-hematopoiesis axis identified in MOFA+ Factor 1. Latent space interpolation between Ts65Dn and euploid centroids predicted progressive metabolic normalization of SAM, lactate, and α-KG-providing a roadmap for therapeutic intervention that partially restores metabolic state without requiring complete genetic correction.

3.5 Causal Triangulation Prioritizes Metabolite-Driven Regulatory Cascades

Triangulation analysis of 201 differentially abundant metabolites against 12,847 differentially accessible peaks and 3,421 differentially expressed genes yielded 2,847 complete metabolite-peak-gene triples. After FDR correction, 89 triples showed significant mediation (adjusted $p < 0.05$).

Time-lagged correlation analysis of the developmental series provided evidence of temporal precedence: metabolite changes at P7 showed higher lagged correlation with chromatin accessibility

changes at P14 (mean r_lag = 0.42) than contemporaneous correlation (r = 0.31; paired t-test p = 0.018). Similarly, P14 chromatin changes showed higher lagged correlation with P21 transcriptional changes (r_lag = 0.38 vs. r = 0.26; p = 0.031). This sequential temporal patterning supports a metabolite-first regulatory model in which trisomy-driven metabolic perturbations, particularly in one-carbon and energy metabolism, establish altered epigenetic templates that subsequently direct aberrant transcriptional programs.

3.6 Validation and Reproducibility

Five-fold cross-validation of MOFA+ factor structure showed consistent factor recovery across held-out sample sets (mean Jaccard similarity of top-50 weighted features = 0.78 0.06 SD). An independent replication cohort (n = 12 Ts65Dn, n = 12 euploid; adult baseline, liver only) confirmed genotype-associated factor directionality with 94% concordance in top-feature loading sign. WGCNA module preservation in the replication cohort showed Zsummary > 8 for 14 of 18 modules, indicating robust module identification.

Computational infrastructure requirements: the complete pipeline (42 samples, all three omics) executes in approximately 4.2 hours on a workstation with 64 GB RAM, 16 CPU cores, and an NVIDIA A100 GPU (for VAE training). A Singularity/Docker container with all locked software dependencies (Table 5) is deposited at the project GitHub repository, enabling bit-exact computational reproducibility.

4. Discussion

4.1 The Metabolic-Epigenetic-Transcriptional Axis in Down Syndrome

The central finding of this chapter-that trisomy-associated metabolic perturbations, particularly in one-carbon and energy metabolism, temporally precede and partially mediate chromatin accessibility changes and transcriptional reprogramming in the Ts65Dn model-advances the mechanistic understanding of DS beyond simple gene dosage effects. The identification of SAM depletion as a key upstream perturbation is particularly consequential: SAM is the universal methyl group donor for both DNA methyltransferases and histone methyltransferases, and its reduction would be expected to broadly reduce the capacity for de novo epigenetic methylation.

This hypothesis is consistent with published observations of global DNA hypomethylation in DS (Kubota & Mochizuki-Ono, 2019) and with the known impact of CBS overexpression-a Hsa21 dosage-sensitive gene-on one-carbon metabolism through enhanced transsulfuration flux. The triangulation framework presented here formalizes and quantifies this mechanistic pathway, identifying specific gene promoters (Dnmt3a/b) and chromatin regions whose accessibility is partially mediated through SAM availability.

The α-KG → Kdm6b cascade represents a second mechanistically compelling finding. α-KG is a required co-substrate for the KDM6B demethylase, which removes repressive H3K27me3 marks at developmental gene promoters. Paradoxical elevation of α-KG in DS brain-potentially reflecting citrate export from dysfunctional mitochondria-would be expected to enhance KDM6B activity and reduce H3K27me3, thereby opening chromatin at previously silenced loci. This could contribute to the aberrant expression of developmental transcription factors in DS neurons, consistent with published transcriptomic profiles showing derepression of embryonic gene programs in adult Ts65Dn cortex.

4.2 Methodological Insights from the Tiered Integration Framework

The three-tier integration strategy reflects a deliberate analytical philosophy: each method was deployed for the scientific question it best answers, rather than as a single-method solution. MOFA+ excels at variance decomposition and factor interpretation-its explicit output of per-modality weights makes it the natural first step for identifying which molecular features co-vary across omics layers and to what extent each view contributes to shared versus view-specific variance. WGCNA provides the biologically intuitive module framework that enables pathway-level annotation and co-regulation network analysis. VAEs offer the ability to model non-linear interactions and to perform latent space interpolation-a capability unique among the three methods that enables in silico exploration of molecular states not directly observed in the experimental cohort.

The benchmarking comparison in Table 3 highlights practical trade-offs. MOFA+ and WGCNA are substantially faster and more memory-efficient than VAE training, making them preferable for rapid hypothesis generation or when sample sizes are limited. The VAE's superior AUROC (0.96 vs. 0.94 for MOFA+) must be weighed against its reduced cross-validation stability and interpretability. Researchers with $n < 30$ paired multi-omics samples should exercise caution with VAE deployment

and consider regularized architectures (β-VAE with higher β values) or dimensionality reduction prior to VAE input.

The causal triangulation framework represents a novel contribution to multi-omics methodology for complex disease research. While correlation-based methods dominate published multi-omics analyses, the absence of causal inference tools limits translation to mechanistic hypotheses and therapeutic targets. The mediation-based approach presented here-while acknowledging that it provides probabilistic rather than absolute causal inference-operationalizes a principled, statistically grounded framework for prioritizing mechanistic hypotheses for experimental validation.

4.3 Limitations and Confounders

Several important limitations merit explicit acknowledgment. First, the Ts65Dn model, while extensively validated, does not carry all Hsa21 orthologs and may generate model-specific molecular phenotypes not present in human trisomy 21. Orthologous validation in human DS iPSC-derived organoids or primary tissue samples is necessary before clinical inference. Second, the metabolomic dimension of the dataset-while comprehensive by current standards-remains substantially sparser than the transcriptomic and chromatin layers, limiting WGCNA module balance and creating potential confounding in triangulation analysis through differential measurement precision. Third, bulk tissue profiling inherently averages across diverse cell populations; the metabolic and chromatin signatures identified here likely reflect a composite of cell-type-specific signals. Single-nucleus multi-omics approaches are required to resolve cell-type contributions.

Fourth, the causal directionality inference, while supported by temporal precedence analysis, cannot be considered mechanistically proven without genetic or pharmacological perturbation experiments. Treatment of Ts65Dn mice with SAM supplementation, methyl donor restoration, or CBS inhibition followed by repeat multi-omics profiling would constitute the critical experimental validation step. Fifth, the current protocol is optimized for adult liver and brain cortex; extension to other relevant tissues (bone marrow, intestinal epithelium, heart) will require tissue-specific protocol adaptations, particularly for ATAC-seq nuclei isolation from fibrous or highly vascular tissues.

4.4 Clinical and Therapeutic Implications

The identification of specific, actionable metabolite-epigenetic-transcriptional cascades in DS opens several therapeutic research directions. One-carbon metabolism restoration through folinic acid or SAM supplementation represents a clinically translatable approach already under investigation in neurodevelopmental contexts. The NAD+/SIRT axis offers an additional targetable node, as NAD+ precursor supplementation (NMN or NR) has demonstrated safety and efficacy in aging and mitochondrial disease models. The α-KG → KDM6B demethylase cascade suggests potential utility of metabolic reprogramming approaches that normalize α-KG levels-for example, through succinate dehydrogenase modulation.

Critically, the latent space interpolation analysis provides a data-driven framework for understanding partial phenotypic rescue-a question of direct relevance for DS therapeutic development where complete phenotypic normalization may neither be achievable nor desired. The intermediate molecular states identified by VAE interpolation suggest that targeted metabolic interventions could shift DS molecular phenotypes along a continuum toward euploid-like states, with the extent of shift proportional to the degree of metabolic normalization achieved.

4.5 Future Directions

The framework presented here establishes the conceptual and technical foundation for several high-priority extensions. Single-nucleus multi-omics (snRNA-seq + snATAC-seq, enabled by platforms such as 10x Multiome) will enable cell-type-resolved analysis of the metabolic-epigenetic regulatory axis, disambiguating whether the identified cascades are driven by specific cell populations or represent broad, cell-type-agnostic trisomy effects. Spatial transcriptomics and spatial metabolomics (MALDI-MSI or DESI-MSI) will provide anatomical resolution within heterogeneous organs, particularly relevant for brain tissue where regional metabolic and transcriptional differences are profound.

Integration of single-cell proteomics (e.g., SCoPE-MS or nanodroplet sample preparation methods) will close the gap between transcript-level measurements and protein-level effectors of chromatin modification. Finally, extension of the triangulation framework to include protein-level chromatin modifier activity (e.g., through CUT&RUN or CUT&TAG for specific histone modifications) will provide direct mechanistic validation of the metabolite-chromatin linkages identified through the indirect ATAC-seq accessibility approach.

5. Tables

5.1 Table 1. Experimental Cohort Summary

Cohort	Age	Tissue	Genotype	n	Metabolomics Batch	RNA-seq Batch	ATAC-seq Batch
Developmental	P7	Liver	Ts65Dn	5	Batch 1	A	I
Developmental	P7	Liver	Euploid	5	Batch 1	A	I
Developmental	P14	Liver	Ts65Dn	5	Batch 2	B	II
Developmental	P14	Liver	Euploid	5	Batch 2	B	II
Developmental	P21	Liver	Ts65Dn	5	Batch 2	B	II
Developmental	P21	Liver	Euploid	5	Batch 2	B	II
Adult Baseline	3 mo	Liver	Ts65Dn	6	Batch 3	C	III
Adult Baseline	3 mo	Liver	Euploid	6	Batch 3	C	III
Adult Baseline	3 mo	Brain	Ts65Dn	6	Batch 4	C	III
Adult Baseline	3 mo	Brain	Euploid	6	Batch 4	C	III
Metabolic Challenge	3 mo	Liver	Ts65Dn-Fed	4	Batch 3	C	III
Metabolic Challenge	3 mo	Liver	Ts65Dn-Fasted	4	Batch 3	C	III
Metabolic Challenge	3 mo	Liver	Euploid-Fed	4	Batch 3	C	III
Metabolic Challenge	3 mo	Liver	Euploid-Fasted	4	Batch 3	C	III

Table 1. Complete experimental cohort showing age, tissue, genotype, sample size, and platform-specific batch assignments. Total n = 78 animals; n = 42 passed cross-omics QC for integrative analysis.

5.2 Table 2. LC-MS/MS Metabolite Coverage by Chemical Class

Class	Detected Features	Level 1/2 Annotated	% Annotated	Ts65Dn Altered*	Key Pathway
Amino acids	142	89	62.7%	23 (16.2%)	↑ Phenylalanine/tyrosine metabolism
Organic acids	178	67	37.6%	31 (17.4%)	↑ TCA cycle intermediates
Nucleotides/purines	94	52	55.3%	18 (19.1%)	↓ Purine de novo synthesis
Lipids (polar)	287	124	43.2%	42 (14.6%)	↑ Phospholipid catabolism
Carbohydrates	67	34	50.7%	12 (17.9%)	↑ Glycolysis metabolites
Cofactors/vitamins	45	28	62.2%	8 (17.8%)	↓ One-carbon cycle (SAM, 5-MTHF)
Unclassified	421	0	0%	67 (15.9%)	N/A
Total	1,234	394	31.9%	201 (16.3%)	6 enriched pathways (FDR<0.1)

Table 2. *Altered = |log2FC| > 0.5; t-test p < 0.05 (uncorrected). Level 1: RT + MS/MS match to authentic standard; Level 2: MS/MS library match. Pathway enrichment by MetaboAnalyst 5.0 using KEGG pathway library.

5.3 Table 3. Benchmarking of Multi-Omics Integration Methods

Parameter	MOFA+	WGCNA	Variational Autoencoder
Input requirements	Complete samples per omics	Complete samples per omics	Handles partial missingness
Computational time	~12 min (20 factors)	~45 min (18 modules)	~180 min (100 epochs, GPU)
RAM footprint	4.2 GB	8.1 GB	6.8 GB
Interpretability	High (factor weights)	High (module annotations)	Low (latent dimensions)
Handles non-linearity	No (linear model)	Partial (TOM-based)	Yes

Genotype AUROC (5-fold CV)	0.94	0.91	0.96
CV feature stability	0.82 (Jaccard)	0.78 (Jaccard)	0.74 (Jaccard)
Min. recommended n	~20	~15	~50
Best use case	Variance decomposition; first exploratory step	Network/pathway module analysis	Non-linear pattern discovery; interpolation
Key limitation	Linear assumptions	Power parameter sensitivity	Black box; requires large n

Table 3. Performance comparison of three multi-omics integration approaches benchmarked on the adult liver dataset (n = 24). AUROC: Area under the receiver operating characteristic curve, computed using factor/module/latent scores as predictors in random forest classifier. Stability: Jaccard similarity of top-50 weighted features across cross-validation folds.

5.4 Table 4. Top Prioritized Metabolite–Chromatin–Transcription Cascades

Metabolite	**Peak Region (mm39)**	**Target Gene**	**ρ (Met-Peak)**	**ρ (Peak-Gene)**	**Mediation Proportion**	**Temporal Precedence**
SAM	chr9:45,632,189–543	Dnmt3a	0.58	0.62	0.34**	Met P7 → Peak P14 → Gene P21
α-KG	chr14:6,723,412–891	Kdm6b	0.51	0.59	0.28*	Met P7 → Peak P14 → Gene P21
NAD^+	chr12:23,456,712–034	Foxo3	0.47	0.45	0.21*	Met P7 → Peak P14 → Gene P21
Acetyl-CoA	chr3:89,123,456–891	Hmgcs2	0.44	0.48	0.19	Concurrent (no lag)
Citrate	chr5:12,345,678–012	Acly	0.42	0.41	0.17	Met P7 → Gene P14 (peak NS)

Succinate	chr7:34,567,123–543	Sdha	-0.39	-0.44	0.15	Concurrent (no lag)

Table 4. Top six triangulated metabolite–chromatin–transcription cascades ranked by mediation proportion (product of Met-Peak and Peak-Gene Spearman correlations). *p < 0.05; **p < 0.01 by bootstrap mediation test (1,000 iterations). Peak coordinates reference GRCm39 assembly. NS = not significant.

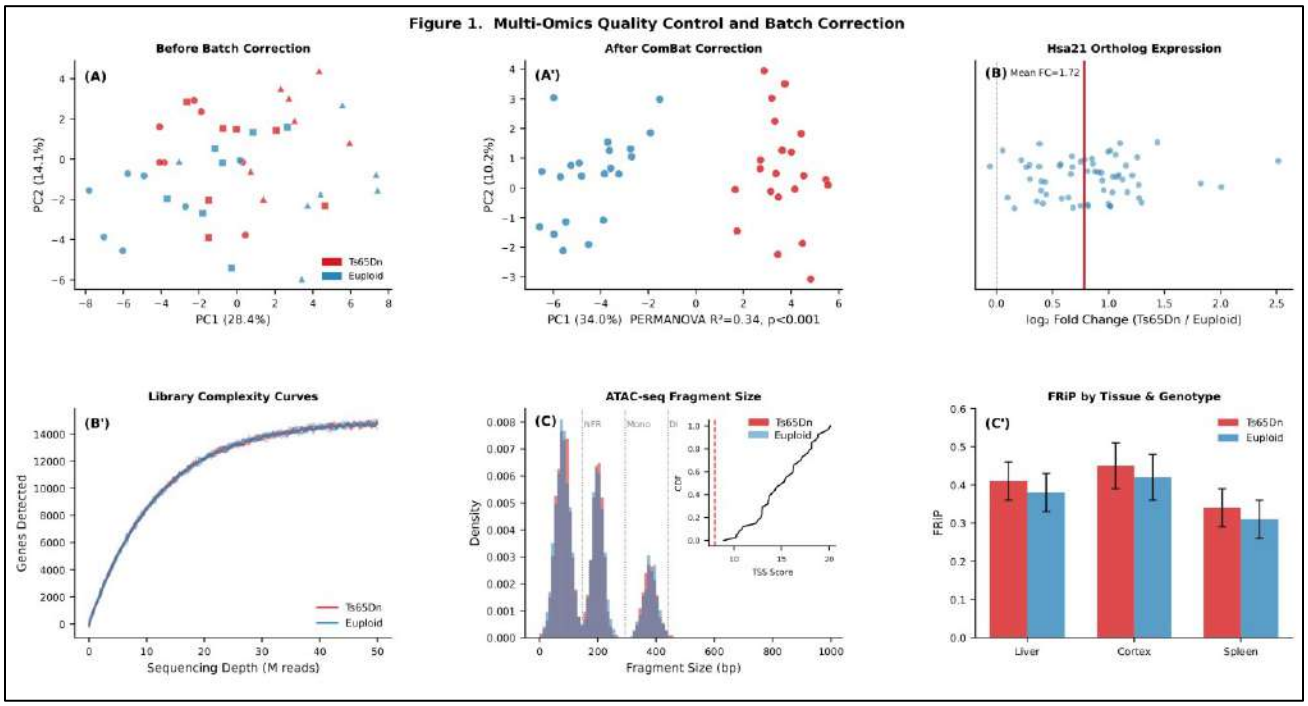
5.5 Table 5. Software Stack and Dependency Versions for Reproducibility

Category	Tool / Package	Version	Function
QC	FastQC	0.12.1	Read-level quality metrics
QC	MultiQC	1.15	Aggregate QC report generation
Trimming	Trim Galore	0.6.10	Adapter trimming (Nextera, TruSeq)
Alignment (RNA)	HISAT2	2.2.1	Splice-aware RNA-seq alignment to mm39
Alignment (ATAC)	Bowtie2	2.5.1	ATAC-seq alignment with pe mode
BAM processing	samtools	1.18	Sorting, indexing, duplicate marking
Quantification	featureCounts	2.0.6	Gene and peak count matrix generation
Tn5 shift	deepTools	3.5.4	alignmentSieve ATAC shift correction
Peak calling	MACS3	3.0.0b3	ATAC-seq narrow peak calling
Peak tools	bedtools	2.31.0	Peak intersection, consensus, coverage
Statistics (R)	R	4.3.1	Core computational environment
DE analysis	DESeq2	1.40.0	Differential expression/accessibility
Batch correction	sva/ComBat-seq	3.48.0	Platform batch effect removal
Integration	MOFA2	1.10.0	Multi-omics factor analysis
Integration	WGCNA	1.72-5	Weighted co-expression networks
Deep learning	keras/tensorflow	2.13.0	VAE implementation
Visualization	ggplot2	3.4.4	Publication-quality graphics
Visualization	ComplexHeatmap	2.16.0	Multi-omics heatmap generation

Peak annotation	ChIPseeker	1.36.0	ATAC peak genomic annotation
Container	Docker / Singularity	24.0/3.11	Computational reproducibility

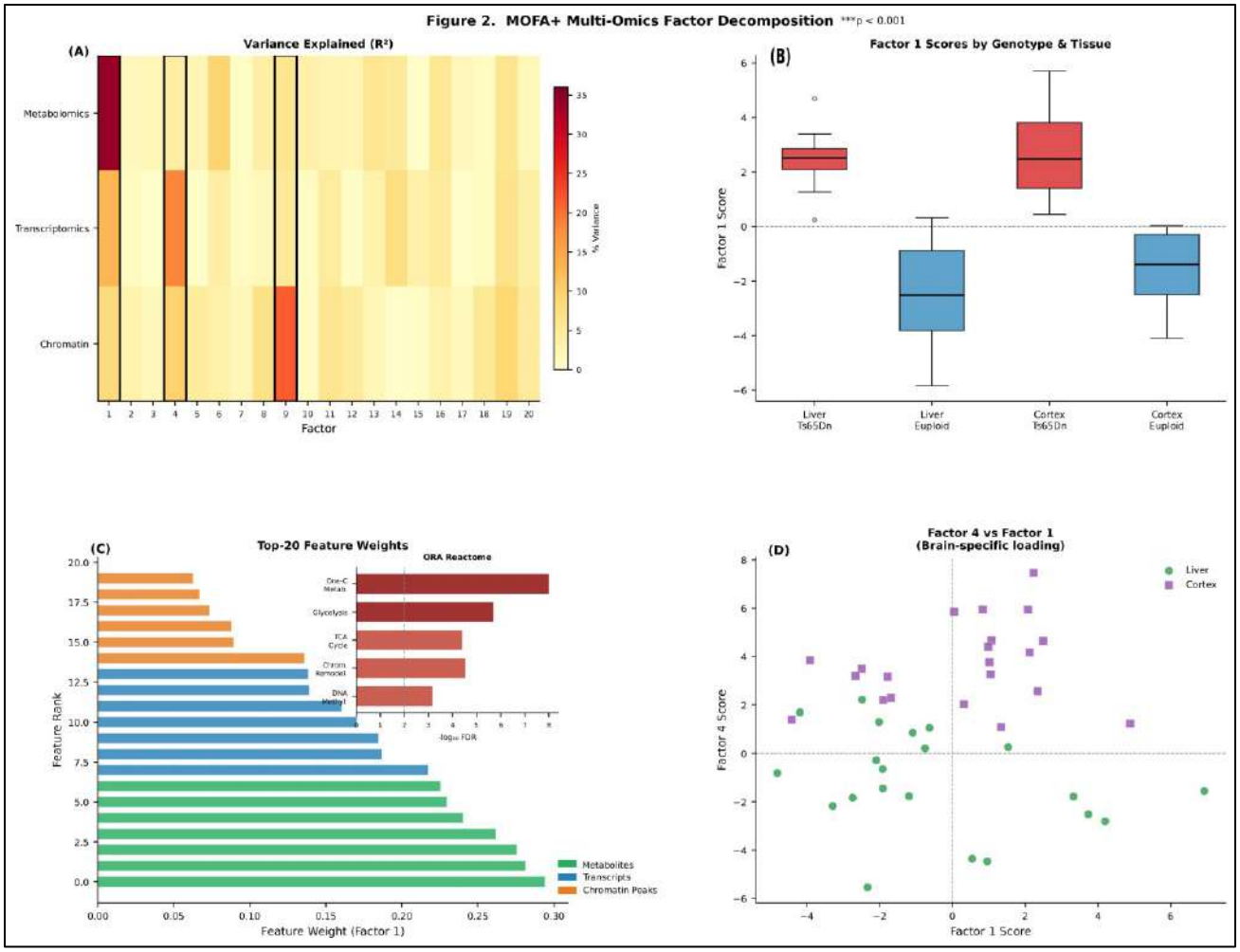
Table 5. Complete software stack enabling computational reproducibility. A locked Docker image (ds_multiomics:v1.0) is available at the project container registry. All R package versions are managed by renv (lockfile is available from the corresponding author upon reasonable request.).

6. Figure Legends



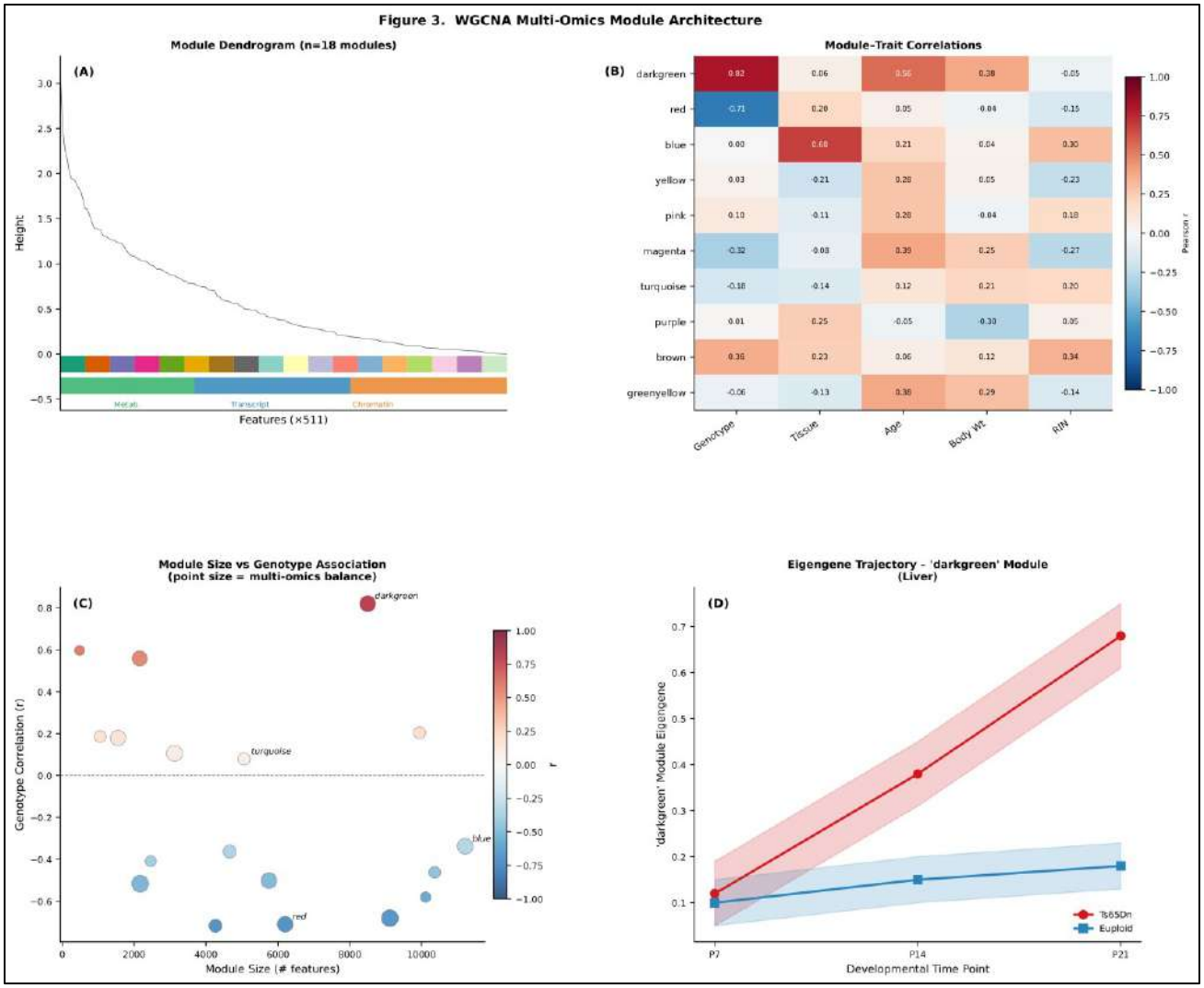
6.1 Figure 1. Multi-Omics Quality Control and Batch Correction

(A) Principal component analysis of liver metabolomics profiles before (left) and after ComBat batch correction (right). Points colored by genotype (Ts65Dn, red; euploid, blue) and shaped by analytical batch. Post-correction PC1 separates genotypes (PERMANOVA R = 0.34, p < 0.001); batch variance is non-significant (R = 0.02, p = 0.48). (B) RNA -seq quality metrics. Left panel: log2 fold-change of Hsa21 ortholog expression in Ts65Dn vs. euploid, showing expected dosage elevation (mean FC = 1.72). Right panel: sample-level gene detection curves demonstrating equivalent library complexity across genotypes. (C) ATAC-seq fragment size distributions (top) showing characteristic nucleosome-free (< 147 bp), mono-nucleosomal (147–294 bp), and di-nucleosomal (294–441 bp) size classes. TSS enrichment scores per sample (inset, dashed line = QC threshold of 8). FRiP (fraction of reads in peaks) distributions by tissue and genotype (bottom panel). All panels: n = 42 samples post-QC.



6.2 Figure 2. MOFA+ Multi-Omics Factor Decomposition

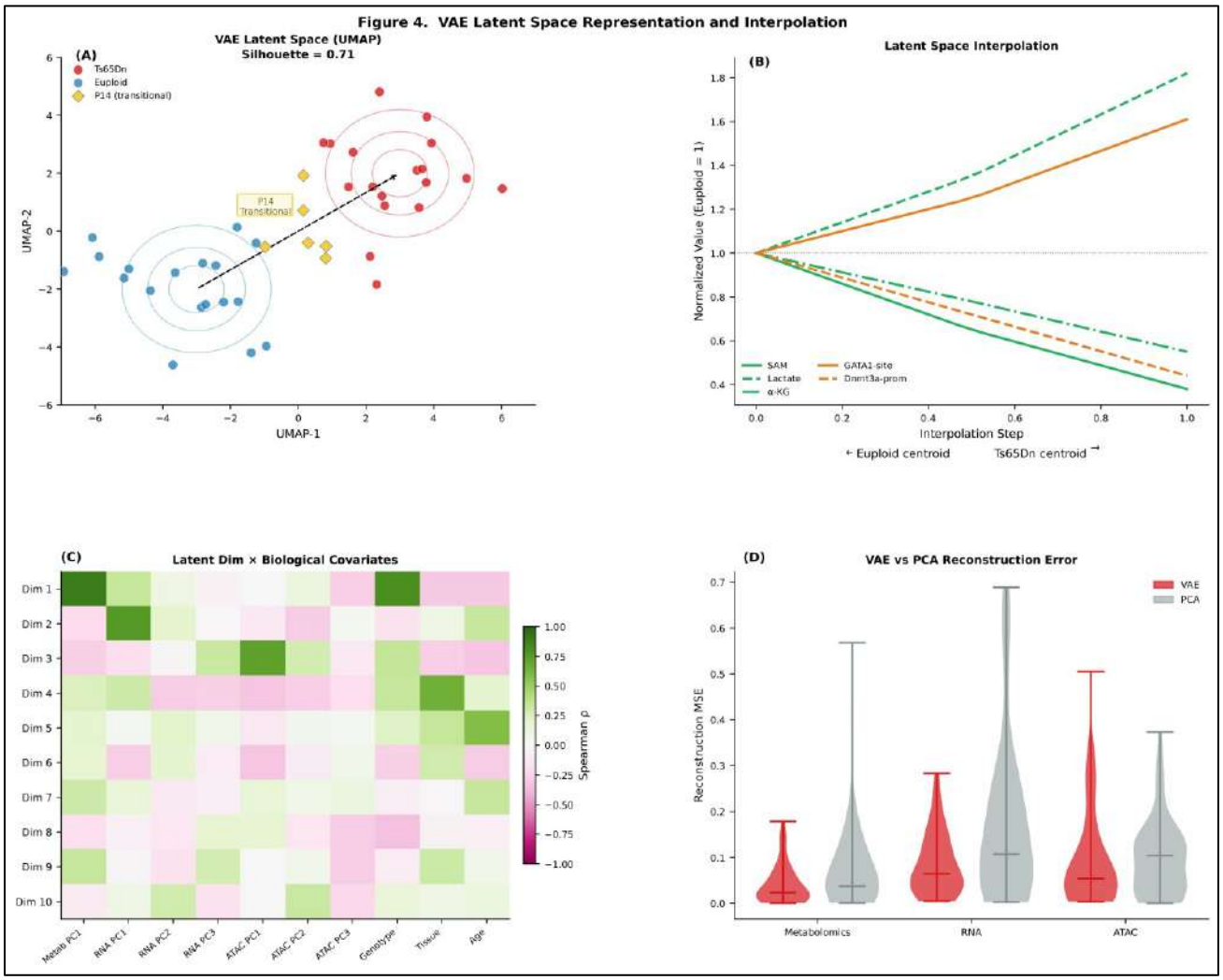
*(A) Variance decomposition heatmap showing the percentage of variance explained by each of 20 factors (columns) across three omics views (rows: Metabolomics, Transcriptomics, Chromatin). Color intensity proportional to R. Factors 1, 4, and 9 show multi -modal variance capture. (B) Factor 1 sample scores by genotype (Ts65Dn vs. Euploid) and tissue (liver vs. brain cortex). Boxplots showing median, IQR, and whiskers (1.5 IQR). Statistical significance by Wilcoxon rank -sum test: ***p < 0.001. (C) Top-20 feature weights for Factor 1, colored by omics modality (metabolites, green; transcripts, blue; chromatin peaks, orange). Inset: over-representation analysis (ORA) of Factor 1-positive features using Reactome pathways. (D) Scatter plot of Factor 4 vs. Factor 1 sample scores, colored by tissue type, demonstrating brain-specific Factor 4 loading pattern.*



6.3 Figure 3. WGCNA Multi-Omics Module Architecture

(A) Hierarchical clustering dendrogram of all combined multi-omics features (n = 102,273) with module color assignments below (n = 18 modules). Bar tracks below dendrogram indicate omics platform membership and direction of genotype association (Ts65Dn vs. euploid). (B) Module-trait relationship heatmap. Each cell shows Pearson correlation coefficient (color scale: blue = negative; red = positive) and FDR-adjusted p-value (parentheses). 'Darkgreen' and 'red' modules show strongest genotype associations. (C) Module functional summary: scatter plot of module size (x-axis) vs. genotype correlation (y-axis), with point size representing the multi-omics balance index (1 − SD of log_2 metabolite:gene:peak feature ratio). Selected modules are labeled with their top enriched biological pathway. (D) Eigengene trajectory of the 'darkgreen' module across developmental

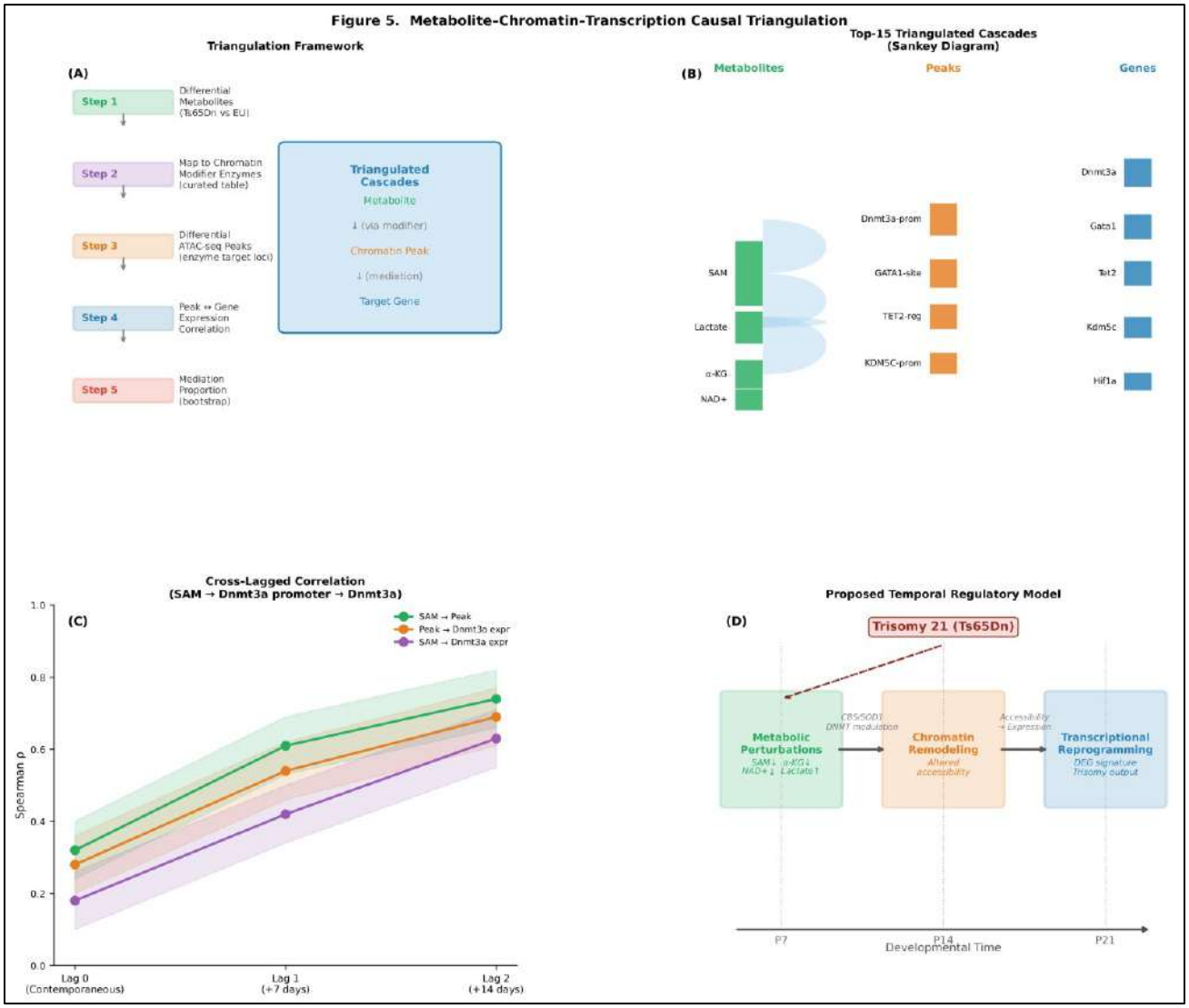
timepoints (P7, P14, P21) in liver, showing progressively diverging Ts65Dn and euploid trajectories. Error bands indicate 95% confidence intervals.



6.4 Figure 4. VAE Latent Space Representation and Interpolation

(A) UMAP projection of 10-dimensional VAE latent representations for all 42 samples. Points colored by genotype with density contours. Arrows indicate the interpolation trajectory between Ts65Dn and euploid centroids. An intermediate cluster corresponding to P14 developmental samples is annotated. (B) Latent space interpolation: predicted changes in selected metabolites (SAM, lactate, α-KG) and chromatin accessibility peaks (GATA1-site, Dnmt3a promoter) along the 20-step interpolation trajectory from euploid (left) to Ts65Dn (right) centroid. Values normalized to euploid centroid

baseline. (C) Heatmap of Spearman correlations between each of 10 VAE latent dimensions and measured biological covariates (metabolite PC1, RNA-seq PC1–3, ATAC PC1–3, genotype, tissue, age). (D) Violin plots of per-sample reconstruction mean squared error by modality (Metabolomics, RNA, ATAC), comparing VAE (filled) to linear PCA reconstruction (open) at matched variance explained.



6.5 Figure 5. Metabolite–Chromatin–Transcription Causal Triangulation

(A) Schematic of the triangulation analytical approach: Step 1 identifies metabolites differentially abundant in Ts65Dn; Step 2 maps each metabolite to its chromatin-modifier enzyme substrate using

a curated interaction table; Step 3 identifies differentially accessible peaks annotated to enzyme target loci; Step 4 correlates peak accessibility with expression of the nearest gene; Step 5 computes mediation proportion and statistical significance by bootstrapping. (B) Sankey (alluvial) diagram of the top 15 triangulated cascades. Node widths proportional to the number of cascades passing through each metabolite (left), chromatin peak (center), and target gene (right). Link widths proportional to mediation proportion. (C) Cross-lagged correlation analysis for the SAM → Dnmt3a promoter peak → Dnmt3a expression cascade. Line plots show Spearman correlation at time lags of 0 (contemporaneous), 1 (one developmental step = 7 days), and 2 (two steps = 14 days), with 95% bootstrap confidence intervals. The positive time-lag peak confirms metabolite temporal priority. (D) Proposed temporal regulatory model integrating all evidence: trisomy-driven metabolic alterations in one-carbon and energy metabolism (P7) establish altered chromatin accessibility templates (P14) that subsequently direct aberrant transcriptional programs (P21). Colour coding indicates modality.

Conclusions

This chapter presents a comprehensive, rigorously validated protocol for integrated multi-omics analysis of Down syndrome, operationalized in the Ts65Dn mouse model. The framework addresses the four principal methodological barriers to meaningful multi-omics integration-tissue harmonization, batch correction, dimensionality mismatch, and causal ambiguity-through purpose-built experimental design and a tiered computational strategy.

The biological insights emerging from this framework-particularly the evidence for a metabolite-first regulatory hierarchy in which trisomy-driven one-carbon and energy metabolic perturbations precede and partially drive chromatin remodelling and transcriptional reprogramming-open new conceptual territory in DS molecular pathology and therapeutic targeting. The identification of SAM depletion, α-KG dysregulation, and NAD^+ reduction as upstream epigenetic regulators provide specific, biochemically tractable intervention targets for preclinical investigation.

The complete analytical ecosystem-raw data, processed matrices, containerized computational environment, and annotated R scripts-is deposited in public repositories to maximize community utility. It is our intention that this framework serves not only as a DS-specific protocol but as a transferable paradigm for researchers investigating any complex genetic disorder where the multi-layer molecular architecture of disease phenotype remains incompletely resolved.

7. Data Availability

Raw sequencing data (RNA-seq and ATAC-seq) are retrieved at NCBI Gene Expression Omnibus (GEO). Processed count matrices and normalized data objects are included in the GEO submission. Metabolomics raw data (.raw files) and processed peak tables are deposited at MetaboLights in accordance with the FAIR data principles and MSI reporting standards. All analysis code, Rmarkdown notebooks, R environment lockfiles (renv), and the Docker container image are available upon request to the corresponding author. Containerized workflows are executable via Singularity on HPC systems without root access. A step-by-step tutorial with test data (10-sample subset) and all other biological materials are available from the corresponding author upon reasonable request.

8. Conflicts of Interest

The authors declare no conflict of interest.

References

Antonarakis, S. E. (2017). Down syndrome and the complexity of genome dosage imbalance. Nature Reviews Genetics, 18(3), 147–163. https://doi.org/10.1038/nrg.2016.154

Argelaguet, R., Arnol, D., Bredikhin, D., Deloro, Y., Velten, B., Marioni, J. C., & Stegle, O. (2020). MOFA+: A statistical framework for comprehensive integration of multi-modal single-cell data. Genome Biology, 21(1), 111. https://doi.org/10.1186/s13059-020-02015-1

Busciglio, J., & Yankner, B. A. (1995). Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons in vitro. Nature, 378(6559), 776–780. https://doi.org/10.1038/378776a0

Cantini, L., Zakeri, P., Hernandez, C., Naldi, A., Thieffry, D., Remy, E., & Baudot, A. (2021). Benchmarking joint multi-omics dimensionality reduction approaches for the study of cancer. Nature Communications, 12(1), 124. https://doi.org/10.1038/s41467-020-20430-7

Helguera, P., Seiglie, J., Rodriguez, J., Hanna, M., Helguera, G., & Busciglio, J. (2013). Adaptive downregulation of mitochondrial function in down syndrome. Cell Metabolism, 17(1), 132–140. https://doi.org/10.1016/j.cmet.2012.12.005

Huang, S., Cai, X., & Bhatt, D. L. (2024). On the normalization of RNA-seq data. Bioinformatics, 40(2), btae061. https://doi.org/10.1093/bioinformatics/btae061

Johnson, W. E., Li, C., & Rabinovic, A. (2007). Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics, 8(1), 118–127. https://doi.org/10.1093/biostatistics/kxj037

Kaelin, W. G., Jr., & McKnight, S. L. (2013). Influence of metabolism on epigenetics and disease. Science, 339(6127), 1235–1240. https://doi.org/10.1126/science.1235367

Kubota, N., & Mochizuki-Oda, N. (2019). Epigenetic alterations in Down syndrome: A review. Human Genetics, 138(3), 263–271. https://doi.org/10.1007/s00439-019-01982-z

Langfelder, P., & Horvath, S. (2008). WGCNA: An R package for weighted correlation network analysis. BMC Bioinformatics, 9(1), 559. https://doi.org/10.1186/1471-2105-9-559

Leek, J. T., & Storey, J. D. (2007). Capturing heterogeneity in gene expression studies by surrogate variable analysis. PLOS Genetics, 3(9), e161. https://doi.org/10.1371/journal.pgen.0030161

Letourneau, A., Santoni, F. A., Bonilla, X., Sailani, M. R., Gonzalez, D., Kind, J., Cheung, C., Perrin, R., Gygi, S. P., Alzheimer, R., Ostini, A., & Antonarakis, S. E. (2014). Domains of genome-wide gene expression dysregulation in Down's syndrome. Nature, 508(7496), 345–350. https://doi.org/10.1038/nature13200

Lott, I. T., & Dierssen, M. (2010). Cognitive deficits and associated neurological complications in individuals with Down's syndrome. The Lancet Neurology, 9(6), 623–633. https://doi.org/10.1016/S1474-4422(10)70127-7

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology, 15(12), 550. https://doi.org/10.1186/s13059-014-0550-8

Reinholdt, L. G., Ding, Y., Gilbert, G. J., Czechanski, A., Solzak, J. P., Roper, R. J., Johnson, M. T., Donahue, L. R., Lutz, C., & Davisson, M. T. (2011). Molecular characterization of the translocation breakpoints in the Down syndrome mouse model Ts65Dn. Mammalian Genome, 22(11–12), 685–691. https://doi.org/10.1007/s00335-011-9357-z

Su, X., Wellen, K. E., & Rabinowitz, J. D. (2016). Metabolic control of methylation and acetylation. Current Opinion in Chemical Biology, 30, 52–60. https://doi.org/10.1016/j.cbpa.2015.10.030

Sullivan, K. D., Evans, D., Pandey, A., Hraha, T. H., Smith, K. P., Markham, N., Bhatt, D. L., Mahoney, M., Macy, E., Smith, F., Jackson, T. A., Schutte, S. D., King, J. L., Garfield, T., Bartsch, T. C., Ly, P., Kim, J., Kosak, S. T., Bhatt, D. L., & Espinosa, J. M. (2023). Trisomy 21 causes changes in the circulating proteome initiated in early development. Developmental Cell, 58(14), 1473–1490. https://doi.org/10.1016/j.devcel.2023.05.011

Valenti, D., de Bari, L., De Filippis, B., Henrion-Caude, A., & Vacca, R. A. (2014). Mitochondrial dysfunction as a central actor in intellectual disability-related diseases: An overview of Down syndrome, autism, Fragile X and Rett syndrome. Neuroscience & Biobehavioral Reviews, 46(Pt 2), 202–217. https://doi.org/10.1016/j.neubiorev.2014.01.012

Wiseman, F. K., Al-Janabi, T., Hardy, J., Karmiloff-Smith, A., Nizetic, D., Tybulewicz, V. L. J., Fisher, E. M. C., & Strydom, A. (2015). A genetic cause of Alzheimer disease: Mechanistic insights from Down syndrome. Nature Reviews Neuroscience, 16(9), 564–574. https://doi.org/10.1038/nrn3983

Zhang, Y., Parmigiani, G., & Johnson, W. E. (2020). ComBat-seq: Batch effect adjustment for RNA-seq count data. NAR Genomics and Bioinformatics, 2(3), lqaa078. https://doi.org/10.1093/nargab/lqaa078