EDITORS
Prof. Dr. Hasan AKGÜL
Prof. Dr. Zeliha SELAMOĞLU

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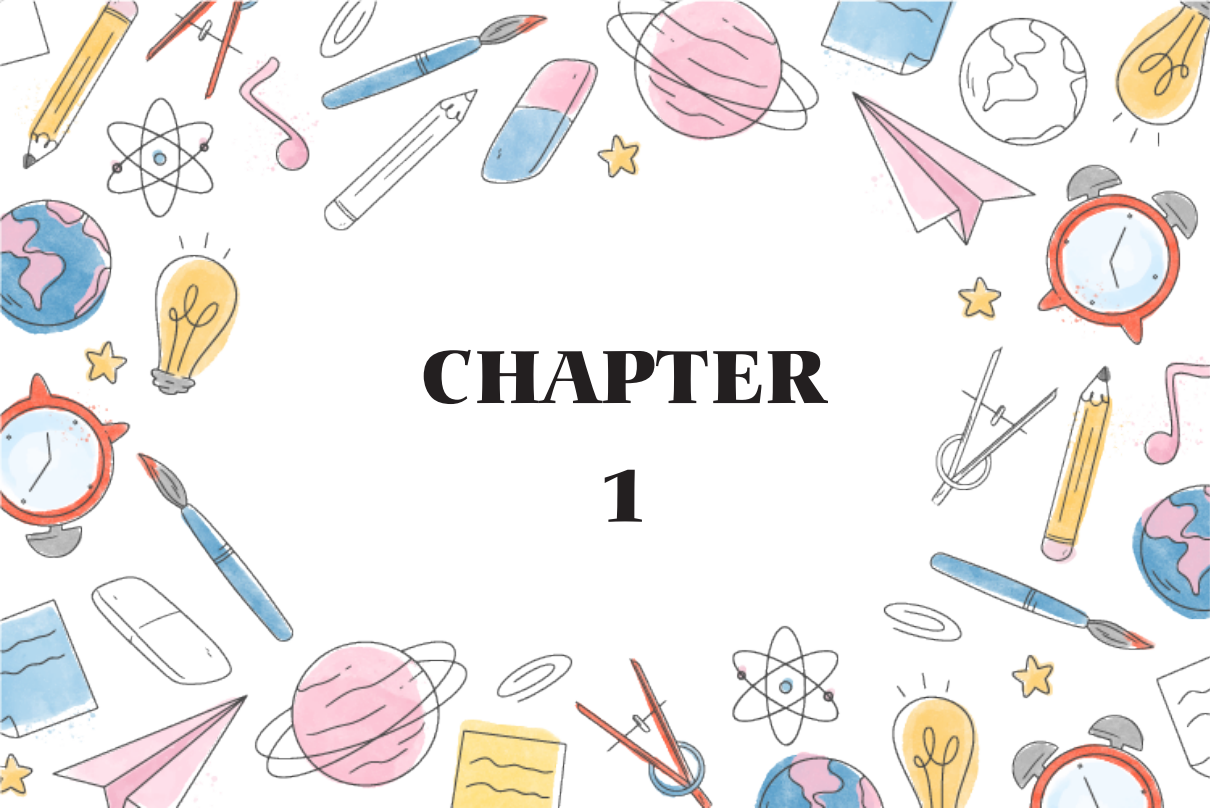

CHAPTER 1

BIOFILM AND RESISTANCE: NEW-GENERATION DETECTION AND INTERVENTION STRATEGIES

Erman ORYAŞIN[1]

1 Assoc. Prof. Dr., Aydin Adnan Menderes University, Aydin Vocational School of Health Services, 0000-0003-1242-7434.

1. Introduction

Complex microbial communities, known as biofilms, stick to surfaces and form a self-forming matrix composed of extracellular polymers (EPs), which are considered a major source of antimicrobial resistance (AMR) (Fleming et al., 2016). These structures not only provide a physical barrier but also provide genetic and metabolic changes that render conventional antibiotics ineffective, which is a key factor in the emergence of chronic infections (Hall-Stoodley et al., 2004). Biofilm-associated infections, including long-term wounds and medical device-related problems, are a major burden on health systems worldwide (Høiby et al. 2015). Systematic reviews indicate that approximately 65 percent of all bacterial infections are associated with biofilms, and this percentage rises to 80 percent in chronic wounds (Jamal et al., 2018; Malone et al., 2017). This challenge is exacerbated by pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella pneumoniae*, which have the ability to form thick biofilms (Oryasin et al., 2018). The persistence of biofilms contributes significantly to the growing AMR crisis, a global health threat highlighted by extensive research over the last decade (Høiby et al. 2015).

The long-term quality of biofilms requires new solutions in both the clinical and environmental settings. Research on biofilms has intensified in recent years, focusing on understanding the underlying mechanisms of biofilm formation and developing novel methods that go beyond conventional methods. Methods such as bioluminescence and fluorescence testing have become fast and accurate for detecting biofilms and checking how well antibiotics affect bacteria (Oryasin, 2020). New therapies - such as nanoparticles, bacteriophage therapy and quorum-sensing inhibitors - show great promise in the fight against biofilms. (Şendal et al., 2024; Costerton et al., 2005). The complete eradication of infections associated with biofilms remains an unattainable goal. Therefore, the critical question is how can these complex systems be effectively managed? A deeper understanding of their molecular processes may be a key factor in biofilm degradation, and the integration of advanced technologies into medical care may be a key factor. The continuing scale of biofilm threats reinforces the need for urgent inquiry.

This chapter provides a detailed overview of the molecular processes involved in the biofilm formation and reviews the latest detection and treatment approaches developed in recent years. This survey seeks to guide readers through the current knowledge gap and the potential opportunities it represents, covering a wide range of topics, from nanoparticle capabilities in biofilms to phage therapy, AI-based modelling, and combinatorial approaches. The challenge posed by biofilms transcends purely academic research and has become a key element in the effort to protect human health. The following sections aim to clarify whether these developments could lead to major breakthroughs in biofilm eradication.

2. Biofilm Formation: Molecular Mechanisms

Biofilm formation is a complex, multi-step process that allows microorganisms to withstand exposure to environmental stress and antimicrobials. The process is usually divided into five different phases: *(a)* formation of the initial bond with the surface, *(b)* irreversible binding, *(c)* formation of the matrix and development of microcolonies, *(d)* maturation of the biofilm, and *(e)* dispersal. (Flemming et al., 2019; Sauer et al., 2022) (Figure 1). These phases, traditionally thought to be linear, are now understood to be part of a more complex life cycle shaped by environmental signals and interactions with microorganisms, with changes in each phase influenced by both random and predictable factors (Sauer et al., 2022). In addition to surface-attached biofilms, free-floating microbial aggregates without attached biofilms also exhibit similar developmental and resistance stages, further improving the conceptual model for biofilm formation (Kragh et al., 2023). Genetic processes, Quorum Sensing (QS), and environmental cues tightly regulate these stages. Communication between cells through quorum sensing is essential for the regulation of biofilm formation. Bacteria can sense their population density and coordinate the production of extracellular matrix polymers (EPS) by producing and detecting autoinducer molecules, such as acyl homoserine lactones in Gram-negative bacteria and oligopeptides in Gram-positive bacteria (Li and Tian, 2012). LasI and RhlI-rhLR quorum sensing systems in *Pseudomonas aeruginosa* control the synthesis of polysaccharides, such as PSE and PSE. (Oryasin et al., 2018; Davies et al., 1998). Similarly, the *Staphylococcus aureus* agr system influences the formation and breakdown of biofilms via signalling pathways dependent on quorum sensing (Boles and Horswill, 2008). Recent studies provide further insight and show that QS not only induces biofilm development but also fine-tunes its structural stability and dispersal pattern (Mukherjee and Bassler, 2019).

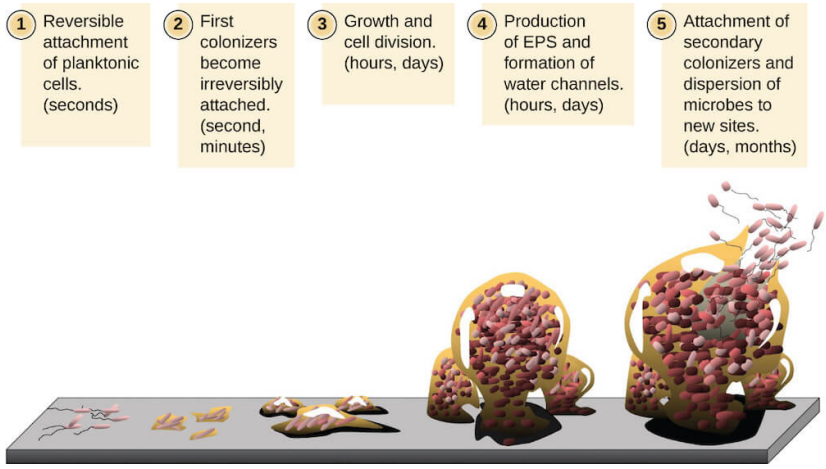

Figure 1: Biofilm is formed when planktonic (floating) bacteria of one or more species attach to the surface, produce a slime, and form a colony. (ASM Microbiology textbook, freely available at openstax.org).

The development of resistance to antimicrobials in biofilms is mainly due to the protective properties of the EPS matrix, which acts as a physical barrier to antibiotic penetration (Mah and O'Toole, 2001) (Figure 1). In addition, biofilms have various resistance mechanisms such as persister cells, dormant subpopulations with reduced metabolic activity, and efflux pumps, which actively expel antimicrobials (Lewis, 2007). Recent research has shown that the EPS matrix not only inhibits drug diffusion but also traps antibiotics, reducing their effective concentration in the biofilm (Liu et al., 2024). In *Pseudomonas aeruginosa*, efflux pumps such as MexAB-OprM, MexCD-OprM and MexEF-OprN are major contributors to multidrug resistance, and their overexpression in biofilms significantly reduces the efficacy of antibiotics (Lorusso et al., 2022). Recent studies have highlighted the role of cyclic di-GMP (CDG), a ubiquitous secondary messenger, as a key regulator of biofilm development. Increased c-di-GMP levels mediated by diguanylate cyclases promote EPS formation and biofilm stability, while phosphodiesterase degradation of EPS induces dispersal (Römling et al., 2013).

In *Escherichia coli* and *P. aeruginosa*, c-di-GMP signalling has been demonstrated to be involved in the pathway of transition from planktonic to biofilm-like life (Römling et al., 2013). In addition, metabolic diversity within biofilms, driven by gradients of oxygen and nutrients, increases resistance by creating a microenvironment in which antibiotics are less effective (Stewart and Franklin, 2008). The diversity of this biofilm is also enhanced by cell differentiation within subpopulations, with specialized cells contributing to both structural integrity and resistance to different

stresses (Sauer et al., 2022). Researchers conducted that heterogeneity in mature bacteria may increase their antibiotic resistance to beta-lactams by up to 100-fold (Santos-Lopez et al., 2019). Excessive expression of efflux pump genes, such as those in *Pseudomonas aeruginosa* MexAB-OprM, contributes to the development of multidrug resistance in biofilms (Liu et al., 2024). Additionally, biofilms facilitate the horizontal transmission of antibiotic resistance genes via mechanisms such as conjugation and transformation, thereby increasing resistance in microbial communities (Michaelis and Grohmann, 2023). Research has shown that these mechanisms of resistance interact synergistically to allow biofilms to elude conventional treatments, underlining the need for advanced molecular analysis to understand their behavior (Almatroudi, 2024). Together, these molecular mechanisms underlie biofilm resilience and are potential targets for new intervention strategies.

3. New Generation Detection Methods

The complexity and persistence of biofilms require the use of rapid, accurate, and sensitive detection methods to inform clinical treatment and therapy strategies. Traditional approaches, such as culture and microscopy, often struggle to capture the dynamic characteristics and diversity of biofilms (Flemming et al., 2016). In recent years, the trend has been towards more advanced detection techniques using innovative, high-throughput, and non-invasive techniques, which have led to improved resolution and specificity. These advanced tools, including bioluminescence and fluorescence tests, high-resolution imaging systems, and molecular profiling methods, have greatly improved our ability to detect biofilms and to assess their antimicrobial resistance.

Bacteria that form biofilms may be identified, and their minimum inhibitory concentration (MIC) is determined using bioluminescence methods. In biofilms, real-time monitoring of bacterial viability and metabolic activity is facilitated by tests using genetically engineered bacteria the express luciferase genes (Oryasin, 2020). The study showed that the identification of bioluminescence allows for the rapid detection of small changes in the biofilm response of Pseudomonas aeruginosa and offers a quicker alternative to standard plating (Oryasin, 2020). In addition, fluorescent methods using green fluorescent protein (GFP) or fluorescent dyes (such as SYTO9 and propidium iodide) allow a more complete understanding of the viability and structure of biofilms by distinguishing between living and dead cells (Azeredo, 2016). Researchers have developed a new method for dual-staining microbial biofilms with cost-effective coloring agents to increase the visibility and differentiation of microbial biofilms and thus improve diagnostic accuracy (Nirmala et al., 2024). Biofilm-specific sen-

sors, utilizing electrochemical and optical platforms, have also emerged as promising tools for real-time detection and characterization, particularly in clinical and industrial settings (Funari and Shen, 2022). Additionally, advanced microscopic techniques, such as optical tweezers, allow precise manipulation and analysis of biofilm formation at the single-cell level, offering new insights into microbial interactions (Camba et al., 2024). There are significant limitations in real-time online monitoring systems used in technical applications, such as water treatment and industrial pipelines, which prevent biofilm dynamics from being assessed in the operational environment (Pereira and Melo, 2023). The use of modern microbiological techniques, including sonication and next generation sequencing (NGS), has made it easier to detect biofilms on implant surfaces in orthopaedic settings, such as prosthetic joint infections (PJI), and thus to support antibiotic-targeted treatment (Mikziński et al., 2024). In addition, advanced molecular profiling techniques, including metabolomics and transcriptomics, have improved our understanding of the behavior of biofilms and their resistance mechanisms (Almatroudi, 2024).

Advanced imaging techniques, including confocal laser scanning microscopy (CLSM), have greatly improved biofilm analysis. When combined with fluorescence dyes, CLSM facilitates the generation of three-dimensional models of biofilm structure, providing insight into the spatial organization of extracellular polymers and microbial communities (Bridier et al., 2017). Polymerase chain reaction (PCR) and other molecular methods have become valuable tools for the investigation of biofilm communities. Real-time quantitative PCR (qPCR) allows for the sensitive detection of specific genes involved in biofilm formation, such as those involved in the detection of quorum sensing and virulence (Oryasin et al., 2018). These methods provide basic information for the tailoring of targeted interventions. The integration of advanced detection techniques provides a comprehensive framework for biofilm studies, allowing a link between laboratory research and clinical practice. It is expected that refining these approaches will improve diagnostic accuracy and inform the development of next-generation therapies.

4. New Generation Intervention Strategies

The resistance of biofilms to conventional antimicrobial treatments has led to the development of new methods to break their structure, prevent their formation, and make them more susceptible to existing treatments. These modern methods exploit advances in nanotechnology, phage therapy, quorum sensing inhibition, and mixed therapies to overcome complex biofilm challenges. These strategies, which target the molecular and physical basis of biofilm persistence, are promising alternatives to conventional

antibiotics that often cannot penetrate the extracellular matrix of polymers or remove persister cells (Flemming et al., 2016).

Biofilms are efficiently degraded by nanoparticles because of their specific physical and chemical properties, particularly their large surface area and ability to modify their reactivity. Metallic nanoparticles, including zinc oxide nanoparticles (ZnO) and silver nanoparticles (AgNPs), exhibit broad-spectrum antibacterial properties by producing reactive oxygen species (ROS), disrupting cell membranes, and disrupting biofilm matrix integrity (Siddiqi et al., 2018). Research has shown biosynthesized ZnO nanoparticles, whether doped or not, have the capacity to effectively inhibit the growth of *Pseudomonas aeruginosa* and *Staphylococcus aureus* and also have anti-tumor properties, which underline their dual therapeutic use (Şendal et al., 2024). Progress in the design of nanomaterials, including surface-modified nanoparticles and hybrid nanostructures, has improved biofilm penetration and inhibition, providing long-term solutions for both water treatment and medical applications (Cao et al., 2024). Machine learning tools, including Molib, have been developed to predict biofilm inhibitors, allowing the development of new therapies using nanoparticles (Srivastava et al., 2020). The ability of NPs to penetrate the EPS matrix increases their efficacy over traditional antibiotics, making them the cornerstone of the next generation of therapeutic approaches.

Bacteriophage therapy is another innovative approach that uses viruses that can specifically target and kill bacteria that form biofilms. Phages can penetrate biofilms by breaking down the EPS matrix using depolymerase enzymes and infecting sporulation cells, making them a highly targeted alternative to broad-spectrum antimicrobial therapy. (36) Recent research has shown that phage cocktails that combine several types of phages can significantly reduce the biomass of *P. aeruginosa* in vitro, with potential applications in chronic wound infections (Chan et al., 2016). Synthetic biology approaches have further increased the efficacy of phages by modifying them to express enzymes that disrupt the biofilm and thus increase their therapeutic potential (Aboobacker et al., 2023). The specificity and adaptability of phages make them attractive alternatives to personalized therapeutic approaches.

Quorum-sensing inhibitors (QSIs) target signaling systems that regulate the emergence and virulence of biofilms and provide a non-lethal control mechanism. Compounds such as furanones interfere with QS pathways and inhibit the production of EPS and the maturation of biofilms. Studies have shown that QSIs in combination with antibiotics increase the dispersal and susceptibility of biofilms and offer a synergistic approach for the treatment of infections (Brackman and Coenye, 2015). Synthetic biology has also introduced QS-degrading enzymes and small molecules

that add to the arsenal of resistance to biofilm formation. (Aboobacker et al., 2023).

Combination therapy incorporates multiple strategies to exploit the synergistic effects and address the limitations of single-agent therapy. For example, in preclinical models, the combination of nanoparticles with antibiotics or phages with QSIs has been shown to increase the eradication of biofilms (Şendal et al., 2024; Chan et al., 2016). Strategies targeting efflux pumps and persister cells, such as efflux pump inhibitors and metabolic stimulators, further improve the efficacy of biofilm-based antibiotics (Grooters et al., 2024). Current approaches for medical surfaces, including antimicrobial coatings and biofilm-disrupting agents, aim to prevent and control the emergence of biofilms, and thereby reducing the risk of infections (Wang et al., 2024). These approaches rely on different mechanisms of action to break down biofilms, kill embedded bacteria, and prevent their re-growth. As these new intervention strategies evolve, their implementation in clinical practice has the potential to revolutionize the treatment of infections associated with biofilms (Zafer et al., 2024).

5. Current Findings and Future Perspectives

Biofilm-associated infections continue to be a major challenge in the healthcare, agriculture, and industrial sectors, requiring continuous progress in detection and intervention strategies. Recent findings highlight the effectiveness of a new generation of tools, but also highlight the gaps in their clinical translation. Nanoparticles such as ZnO have shown strong anti-biofilm activity, and studies have shown that they can reduce the viability of *Pseudomonas aeruginosa* biofilms by up to 90 percent in vitro Phage therapy is gaining momentum and clinical studies have reported successful results in the treatment of chronic wound infections caused by multidrug-resistant pathogens (Jault et al., 2019). Quorum-sensing inhibitors, in combination with antibiotics, increased the clearance rate of biofilms in preclinical models, suggesting that this is a viable add-on (Brackman and Coenye, 2015). In addition to their pathogenic role, biofilms offer useful applications, such as bioremediation and wastewater treatment, where their resilience can be harnessed for environmental sustainability (Philipp et al., 2024).

The integration of artificial intelligence (AI) and biofilm research has transformative potential. Image analysis has become a powerful tool for characterizing biofilm structures, allowing for rapid and accurate identification of microbial populations and their spatial organization on biotic and abiotic surfaces (Ragi et al., 2023; Mishra et al., 2024). For example, machine learning algorithms can process complex imaging data to quantify

biofilm biomass and predict antimicrobial responses, thereby accelerating the development of targeted therapies (Ragi et al., 2023; Ding and Chen, 2025; Srivastava et al., 2020). The scalability of phage and nanoparticle-based therapies remains an obstacle, requiring advances in production and delivery systems (Highmore et al., 2022). Similarly, the economic burden of biofilms, estimated to cost billions of dollars annually in the health and industry sectors, highlights the need for cost-effective solutions (Cámara et al., 2022). Future research should focus on in vivo validation, regulatory frameworks, and the development of real-time monitoring technologies to address translational challenges (Pereira and Melo, 2023). In addition, understanding non-enveloped biofilm aggregates and their role in the spread of resistance could inform new therapeutic strategies (Kragh et al., 2023). Multi-drug-resistant biofilms, especially in clinical settings such as prosthetic joint infections (PJI), highlight the need for integrated approaches combining advanced diagnostic and therapeutic approaches (Zafer et al., 2024; Mikziński et al., 2024). Synthetic biology offers novel tools such as engineered microbial systems to combat resistance to biofilms, paving the way for future discoveries (Aboobacker et al., 2023). Ultimately, a multidisciplinary approach is necessary to improve the results of the biofilm challenges.

6. Conclusion

Biofilms are a persistent and multifaceted challenge in the fight against AMR, and require a comprehensive approach that includes advanced detection and intervention strategies. This chapter examines the molecular mechanisms that drive biofilm formation and highlights the critical role of quorum sensing, cyclic-di-GMP signaling, and the extracellular matrix of polymers in providing resistance throughout the complex life cycle (Flemming et al., 2016; Sauer et al., 2022). Notably, EPS not only acts as a physical barrier but also actively binds to antibiotics, while efflux pumps, persister cells, and horizontal gene transfer further increase resistance, highlighting the complexity of AMR mediated by biofilms (Liu et al., 2024; Lorusso et al., 2022; Michaelis and Grohmann, 2023). New detection techniques such as bioluminescence tests, double-staining techniques, optical sensors, and biofilm-specific sensors have revolutionized our ability to characterize biofilms with unprecedented accuracy and rapidly assess their structure and sensitivity (Oryasin, 2020; Nirmala et al., 2024; Funari and Shen, 2022). At the same time, innovative intervention strategies, including nanoparticles, phage therapy, quorum-sensing inhibitors, synthetic biology approaches, and combination therapies offer promising avenues for disrupting biofilms and increasing the efficacy of treatment (Cao et al., 2024; Chan et al., 2016; Brackman and Coenye, 2015; Grooters et al.

2024). Recent findings further highlight the potential of these approaches, with nanoparticles achieving significant reductions in biofilm and phage therapy demonstrating clinical success, while artificial intelligence (AI) is emerging as a transformative tool for biofilm research (Şendal et al., 2024; Ragi et al., 2023; Mishra et al., 2024).

Despite these advances, challenges remain in the translation of these technologies to widespread clinical use. The scalability of phage therapy, cost of nanoparticles, and need to validate artificial intelligence models in vivo highlight critical areas for further investigation (Highmore et al., 2022). Regulatory frameworks and standardized protocols are necessary to bridge the gap between laboratory innovation and patient care, and the economic importance of biofilms in different sectors requires cost-effective solutions (Cámara et al. 2022). Moreover, the integration of multidisciplinary approaches combining microbiology, nanotechnology, and computational biology is key to tackling the persistent biofilm threat, especially in multidrug-resistant infections (Zafer et al., 2024). As research progresses, the focus on cost-effective, scalable, and clinically viable solutions, together with the exploration of beneficial applications of biofilms and advanced surveillance strategies, will be of paramount importance for improving the results of biofilm-associated infections and ultimately advancing global health (Wang et al., 2024; Philipp et al., 2024).

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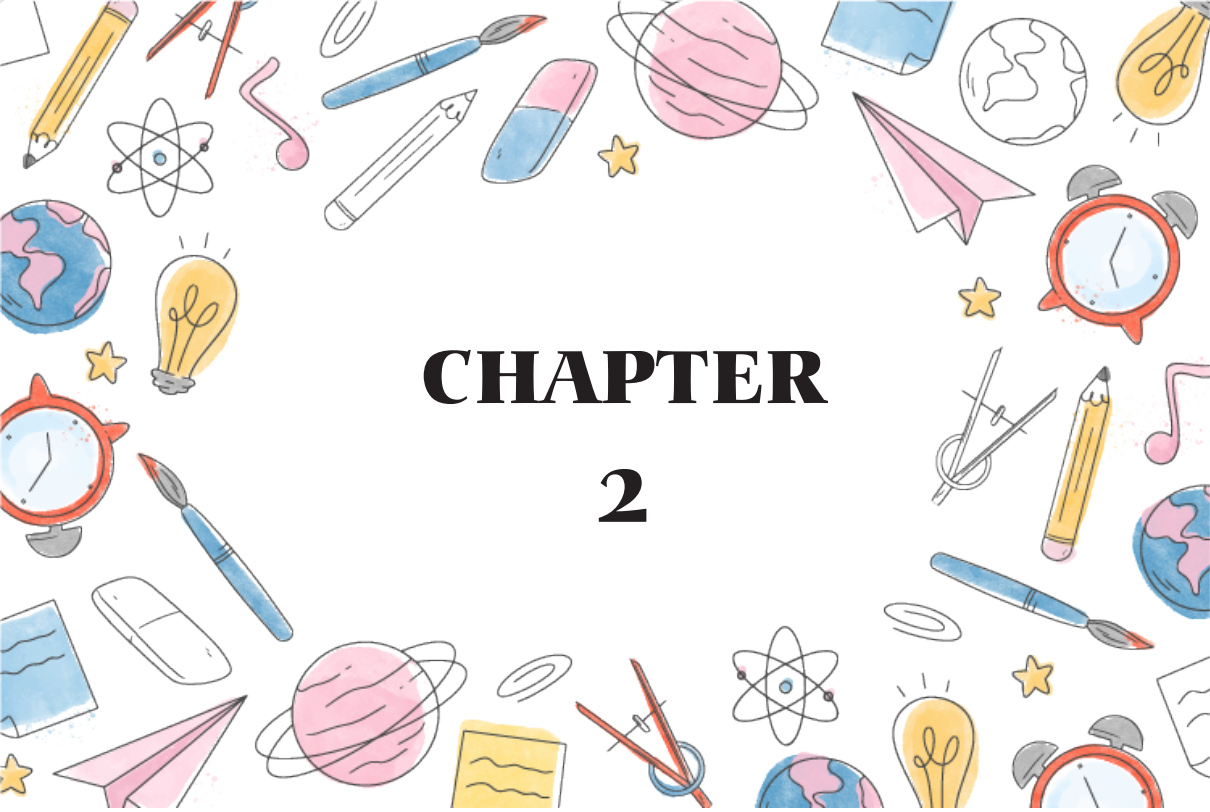

CHAPTER 2

LONG NON-CODING RNAS IN IDIOPATHIC GENERALIZED EPILEPSY

Fatma SÖYLEMEZ[1]

1 Asst. Prof., Mersin University, Medical Biology and Genetics (PhD)
soylemez_fatma@yahoo.com
Orchid ID: https://orcid.org/0000-0002-1939-8691

INTRODUCTION

Epilepsy is a serious multifactorial and common neurological disorder with a strong genetic predisposition. Characterized by symptomatic seizures, epilepsy represents a group of neurological disorders that can be caused by many conditions such as traumatic brain injury, brain tumors, brain infections, stroke, birth defects. Unpredictably severe recurrent seizures involve part or all of the body and lead to various developmental and functional effects (World Health Organization, 2022). Such an anomaly, occurring simultaneously in neurons, is partly due to imbalances in excitation and inhibition in the brain. Epileptogenesis, the process by which the normal brain becomes capable of spontaneous seizures after damage, is associated with large-scale changes in gene expression. These permanently contribute to the remodeling of neuronal networks and alter excitability. The progression of epilepsy also involves a series of changes involving ion channels, signal transduction, synaptic transmission, gap junctions, the autoimmune system, inflammatory reactions, apoptosis and more. These changes also affect treatment processes, including the type, onset and progression of epilepsy. Patients with epilepsy are treated with antiepileptic drugs and anticonvulsants to control seizures by stabilizing neuronal excitability, particularly the concentration of transmitters such as g-aminobutyric acid (GABA) and glutamate (Glu). But the drugs have many side effects and a third of patients have drug-resistant epilepsy. Approximately 130,000 people die from epilepsy each year and the costs of treating people with epilepsy are rising (Allers et al., 2015; Singh and Sander, 2020). Therefore, epilepsy is a major public health problem worldwide and a better understanding of its pathogenesis and therapeutic targets is needed. The search for epigenetic markers seems to be a great hope to diagnose, modify, and even cure epilepsy with a growing understanding of the biological mechanisms of epileptogenesis, as well as the development of better and precise pharmacological methods and non-pharmacological strategies (Duncan, 2022; Proix et al., 2021).

Idiopathic generalized epilepsies (IGE) account for one-fifth of all epilepsies but <1% of epilepsy research. This skew reflects misperceptions: the diagnosis is simple, the pathophysiology is understood, seizures are easily controlled, epilepsy is survivable, morbidity and mortality are low, and surgical interventions are impossible. Emerging evidence suggests that patients with IGE may go undiagnosed or misdiagnosed with focal epilepsy if the EEG or semiology has asymmetric or focal features. Genetic, electrophysiologic and neuroimaging studies provide insights into the pathophysiology, including overlaps and differences with focal epilepsies. IGE can begin in adulthood and patients have chronic and drug-resistant seizures. Rates of psychiatric and other comorbidities, including sudden

unexpected death in epilepsy, parallel those in focal epilepsy. IGE is an understudied spectrum in which our diagnostic sensitivity and specificity, scientific understanding and treatments fall short. Physical examination and cranial imaging are normal. Due to reasons such as EEG findings, deficiencies in the description of seizure indicators, lack of detailed questioning of the presence of myoclonia, misdiagnosis and continuation of treatment, the diagnosis of IGE may be delayed and/or inappropriate treatments may be applied.

Long non-coding RNAs (lncRNAs) are RNAs with a length of more than 200 nucleotides whose transcripts do not encode proteins (Liu et al., 2022). Changes in lncRNAs can be measured by RNA sequencing technology. So far only a small amount of lncRNAs have been fully characterized, however, their alterations, including overexpression, deficiency or mutation, have been associated with numerous diseases (Esteller, 2011; Ang et al., 2020). Recently, the role of lncRNAs in epileptogenesis has been gradually demonstrated, mainly related to the pathophysiological process of neuroinflammation and neuronal apoptosis and the balance of transmitters (Liu et al., 2022). Especially in the IGE patient group, there is a need for early diagnosis opportunities and new treatment options. Possible epigenetic mechanisms that will be determined to be associated with the disease may be important for the development of treatment options. Therefore, lncRNAs offer hope for epilepsy. Studies on lncRNAs suggest that they may be associated with certain processes involved in the pathogenesis of epilepsy and that lncRNAs may play a role in neurodevelopmental disorders, including epilepsy.

1. IDIOPATHIC GENERALIZED EPILEPSY (IGE)

1.1. Etiology and Classification

Idiopathic epilepsies have been recognized as genetic disorders triggered by emotional stress, pain, hormonal factors and alcohol. Myoclonic seizures and juvenile myoclonic epilepsy (JME) were recognized in the late 19th century in patients with upper body jerks upon awakening and progressing to convulsions (Reynolds, 1861). In the 1930s, a distinctive feature of idiopathic generalized epilepsy (IGE) was identified on EEG: bilateral, symmetrical 2.5-6 Hz generalized spike wave discharges (GSWD), which also distinguished absence from focal seizures (Devinsky et al., 2024). The 1989 International League Against Epilepsy (ILAE) classification revealed a genetic etiology for idiopathic epilepsies with focal onset and generalized onset (Scheffer et al., 2017). In 2010, ILAE used the term genetic instead of idiopathic. Clinically, patients with IGE have only absence, myoclonic and tonic- clonic seizures. Patients with epileptic

encephalopathy have generalized onset atypical absence seizures, absence with eyelid myoclonus, myoclonic absence, and tonic and atonic seizures. IGE accounts for approximately 20% of all epilepsies, but less than 1% of the scientific literature on epilepsy. This difference reflects widespread perceptions that anti-seizure medications (ASMs) control seizures in patients with IGE, that comorbidities are infrequent and insignificant, and that surgical procedures are ineffective for seizure control.

Epilepsies are classified as generalized, focal, generalized and focal and unknown (idiopathic) (Scheffer et al., 2017). The next level of classification is epilepsy syndrome, which cannot always be diagnosed but provides information on prognosis and genetic counseling. Genetic generalized epilepsies (GGEs) include idiopathic generalized epilepsy (IGE) and epileptic encephalopathies (Hirsch et al., 2022). IGE specifically refers to epilepsy syndromes such as juvenile myoclonic epilepsy (JME), juvenile absence epilepsy (JAE), childhood absence epilepsy (CAE) and generalized tonic-clonic seizures alone (Scheffer et al., 2017) (Figure 1). The new classification emphasizes etiology as a tool for diagnosis, prognostic counseling and epilepsy management. It is also vital in determining the risk of recurrence of single seizures and diagnosing epilepsy versus seizures caused by something other than underlying brain damage (Balestrini et al., 2021). These groups may overlap in patients with multiple contributing etiologies and etiology should always be considered in conjunction with classification. Etiology is divided into six subgroups: structural, genetic, infectious, metabolic, immune and unknown (Scheffer et al., 2017). In patients with idiopathic (genetic) generalized epilepsy, by definition, there is no evidence of structural brain lesions on magnetic resonance imaging (MRI), and there is a lack of interictal symptoms and signs that rule out most etiological groups (Guerrini et al., 2019; Koutroumanidis et al., 2005). A genetic role has been suggested due to twin studies showing higher concordance rates in monozygotic twins than dizygotic twins (Berkovic et al., 1998; Corey et al., 1991). Studies on juvenile myoclonic epilepsy (JME) have found that BRD2 gene polymorphisms on chromosome 6p21.3 and connexin-36 gene polymorphisms on chromosome 15q14 are associated with increased susceptibility to JME (Delgado-Escuta et al., 1999; Hempelmann et al., 2006). However, most epilepsies are not linked to known genetic mutations. Another factor linked to idiopathic generalized epilepsies is sleep quality. Studies have shown that sleep deprivation and underlying abnormal sleep causes are strong triggers for seizures (Lenher et al., 2022; Tavşanlı and Kınay., 2023). However, effective antiepileptic treatment can improve sleep quality as well as reduce the likelihood of seizures.

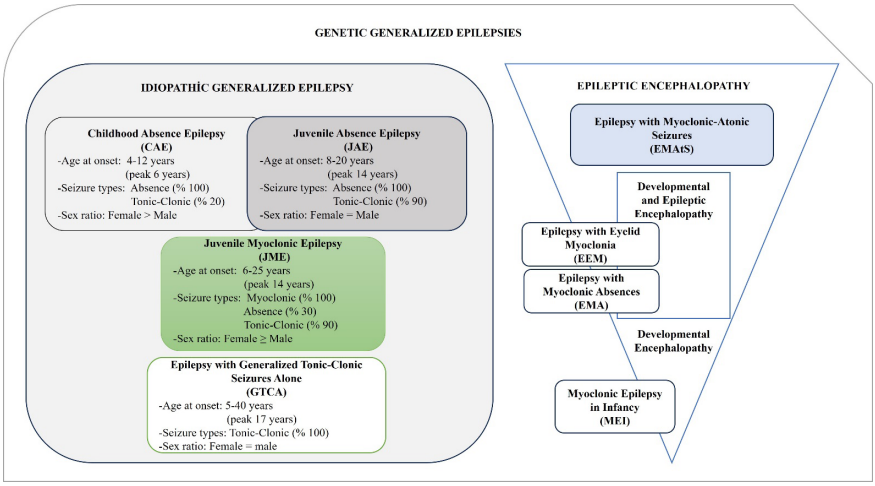

Figure 1. *Classification of genetic generalized epilepsies (Hirsch et al., 2022).*

1.1.1. Juvenile Myoclonic Epilepsy (JME)

JME is a common epilepsy syndrome that occurs between the ages of 8 and 26, with the highest incidence between the ages of 12 and 16. JME accounts for 25 to 30 percent of idiopathic generalized epilepsies and 10 percent of all epilepsy cases. It is characterized by three potential seizure types myoclonic seizures (usually immediately after waking up or when the individual is tired), generalized tonic-clonic seizures and typical absence seizures. Myoclonic seizures are sudden, short jerks affecting the arms, legs, face or the whole body. Generalized tonic-clonic seizures occur in about two-thirds of patients. Roughly one third have typical absence seizures, which are more likely to occur in the morning (Koutroumanidis et al., 2005). Approximately 30% of JME patients show photosensitivity (Bauer et al., 2017). Family history of epilepsy is associated with an earlier age of onset in JME patients (Najafi et al., 2016). IGE patients are thought to have normal cognitive functioning; however, JME patients showed lower advanced cognitive function tests than controls. Complementary functional neuroimaging studies and higher cortical function tests should be performed for a comprehensive diagnosis. Prognosis is good, with 85% to 90% of patients remaining seizure-free on a single medication (Chawla et al., 2021). It is not associated with conditions such as head trauma, brain tumors or encephalitis. Patients are inoperable and require lifelong treatment with anticonvulsants. Therefore, there is a need to search for new methods to treat the pathogenesis of this disease.

1.1.2. Childhood Absence Epilepsy (CAE)

Childhood absence epilepsy occurs in early childhood, with peak onset between 4 and 7 years of age, usually before the age of 10. Clinically, patients experience staring and impaired awareness. They usually have only absence seizures and have fewer than 20 seizures per day at the time of diagnosis. Hyperventilation test triggers seizures (Bashiri et al., 2022). Typical absence seizures are short (4 to 30 seconds), sudden-onset blank episodes with impaired consciousness (loss of awareness and unresponsiveness) and behavioral stopping or staring. These episodes may be associated with orofacial automatisms (Koutramanidis et al., 2017). EEG will show a typical 3 Hz spike and wave pattern. Ethosuximide is used as first-line treatment and is effective in more than 50% of seizures. However, ethosuximide is ineffective against non-absence seizures, so valproate is the drug of choice if another type is also present. Poor prognosis includes abnormal baseline slowing and generalized- tonic-clonic or myoclonic seizures as well as EEGs with absence seizures (Kessler and McGinnis, 2019).

1.1.3. Juvenile Absence Epilepsy (JAE)

The age of onset of seizures distinguishes childhood absence epilepsy from juvenile absence epilepsy (Operto et al., 2022). JAE occurs between 7 and 16 years of age, with a peak onset between 10 and 12 years of age. The predominant seizure type is absence seizures. These can occur many times a day but are typically not as frequent as in childhood absence epilepsy. Automatisms, more or less coordinated involuntary motor activities that occur during or after an epileptic seizure in a state of impaired consciousness, are more common (especially perioral or hand automatisms). Generalized tonic-clonic seizures occur infrequently and myoclonic seizures may occur in a small proportion (Operto et al., 2022).

1.1.4. Generalized Tonic-Clonic Seizure Epilepsy (GTCA)

Generalized tonic-clonic seizures, previously known as grand mal seizures, are defined as seizures in which the tonic phase is followed by clonic muscle contractions. They occur in bilateral cortical, subcortical and brainstem networks of the brain and rapidly affect them (Kodankandath et al., 2023). The most common age of onset is the mid-teens. Seizures tend to occur shortly after waking (within 1 to 2 hours), but can occur at any time. Similar to JME, triggering factors include sleep deprivation, fatigue and excessive alcohol consumption (Koutramanidis et al., 2017).

1.2. Pathophysiology, Diagnosis and Treatment

Animal models of IGE have shown that seizures can be caused by variants in calcium channel and γ-aminobutyric acid (GABA) receptor genes (Huguenard, 2019; Lindquist et al., 2023). Other mechanisms are also involved, including increased thalamic glutamate and decreased N-acetyl aspartate (Blumenfeld, 2005). The term generalized suggests that all brain neurons are affected simultaneously and homogeneously in IGE, but electrical, neuroimaging and molecular studies support the involvement of specific thalamocortical networks with preservation of others (Blumenfeld, 2005; Larivi`ere et al., 2020) In patients with IGE, structural imaging studies have revealed atrophy and functional imaging studies have revealed abnormal nodes in frontocentral cortical areas (Larivi`ere et al., 2020; Gong et al., 2021; Sone et al., 2019). Basal ganglia and cerebellar regions may also be functionally impaired in IGE (Gong et al., 2021).

IGE is characterized by generalized spike-wave discharges (GDDD) and absence, myoclonic or generalized tonic-clonic seizures (GTCS). The main EEG finding of IGE is these tonic-clonic seizures with normal background rhythms (Scheffer et al., 2017; Hirsch et al., 2022) The clinical features of the main IGE syndromes are summarized in Figure 1. Seizures are often triggered by fatigue, stress and environmental stimuli (e.g. driving while the sun reflects its light through the trees) (Wassenaar et al., 2014). However, EEG cannot reliably distinguish between IGE syndromes. For example, spike-wave discharges of 3.5 to 6 Hz occur with similar frequency in juvenile myoclonic epilepsy alone or with polypics, tonic-clonic epilepsy alone, juvenile absence and childhood absence epilepsy. Normal EEGs may be more common in JME than in other syndromes (Devinsky et al., 2024; Asadi-Pooya et al., 2013). These differences can often have the effect of complicating the diagnosis and excluding epilepsy. Once diagnosed, the first step for the treatment of epilepsy patients is the use of antiepileptic drugs and anticonvulsants to control seizures by stabilizing neuronal excitability, especially the concentration of transmitters such as γ-aminobutyric acid (GABA) and glutamate (Glu). But the drugs have many side effects and one third of patients have drug-resistant epilepsy. Furthermore, the lack of professional medical care is still the most important reason for the increased risk of epilepsy (Singh and Trevick, 2016).

Anti-seizure drugs reduce neuronal hyperexcitability and are the primary IGE treatment. Decisions regarding the initiation and selection of these medications, dosage and discontinuation depend on seizure type and epilepsy syndrome, patient demographics, lifestyle and comorbidities, drug interactions and side effects. Treatment outcomes for most childhood-onset IGE are good; many patients with childhood absence epilepsy discontinue anti-seizure medications. Patients with IGE whose seizures begin or persist

into adolescence and young adulthood (e.g. JME) usually require lifelong treatment. However, many can reduce or eliminate medication by improving lifestyle factors such as sleep, addiction and limited alcohol intake. Studies evaluating anti-seizure medications for IGE are limited (Glauser et al., 2013). One study compared valproate, lamotrigine and topiramate in 450 patients with IGE (Marson et al., 2007). Valproate was superior to lamotrigine and topiramate in time to treatment failure; valproate was superior to lamotrigine and similar to topiramate in time to 12-month remission (Marson et al., 2007). While ethosuximide and valproate had superior efficacy compared to lamotrigine for childhood absence epilepsy, ethosuximide was the preferred treatment with fewer side effects. The failure of lamotrigine was usually due to poor seizure control, whereas the failure of valproate was usually due to side effects. Seizure freedom rates in JME are better for valproate, but lamotrigine is better tolerated (Silvennoinen et al., 2019). Seizure recurrence in adolescent and young adult patients with IGE is often associated with irregular medication use, sleep deprivation, alcohol withdrawal or stress (Wassenaar et al., 2014). These factors often coexist. For example, a person who is sleep deprived due to overwork may consume excessive alcohol; intoxication promotes maladaptation and alcohol deprivation impairs sleep quality and lowers the seizure threshold. In women of reproductive age, anti-seizure medications pose therapeutic challenges because they can interact with hormonal contraceptives, are potentially teratogenic and pass into breast milk. These enzyme-inducing drugs (e.g. phenobarbital, phenytoin, carbamazepine, topiramate, lamotrigine) increase hepatic metabolism of oral contraceptives, increasing the risk of unplanned pregnancy. All anti-seizure drugs have a potential teratogenic risk. The lowest risk exists for levetiracetam, lamotrigine and oxcarbazepine (risk of major congenital malformations approximately 1%-3%; 0%-1% higher than the general population). Topiramate, carbamazepine and phenytoin have a higher risk (3%-5%), while phenobarbital (6%) and valproate (7%-10%) have the highest risk (Silvennoinen et al., 2019; Vossler et al., 2020). Valproate is highly effective in controlling seizures, but is highly teratogenic and is associated with intellectual decline, autism and intellectual disability in children exposed in utero (Bjørk et al., 2022). These children have reduced intelligence (7-10 points) and impaired verbal and memory function compared to children exposed prenatally to phenytoin, lamotrigine or carbamazepine (Meador et al., 2013). Men with epilepsy face little reproductive risk from anti-seizure medications or epilepsy itself. However, enzyme-inducing drugs can reduce testosterone levels. Valproate may also be associated with reversible male infertility (Tallon et al.)

Approximately 130,000 people die from epilepsy each year and the cost of treating epilepsy patients is rising (Allers et al., 2015; Singh and Sander, 2020). Therefore, epilepsy is a major public health problem worldwide and there is a critical need to better understand the pathogenesis and therapeutic targets, and more effective and noninvasive treatments should be explored and developed. Although several antiepileptic drugs have been developed in recent years, clinical assessments of physiologic status still predict poor outcomes in 30-40% of epilepsy patients treated with current antiepileptic drugs. Even with treatment, patients can develop treatment-resistant epilepsy, as well as numerous serious complications such as memory loss, suicidal feelings and sudden unexpected death (Laxer et al., 2014). However, with an increasingly better understanding of the biological mechanisms of epileptogenesis, including epigenetic determinants and pharmacogenomics, better and precise pharmacological methods and non-pharmacological strategies seem to be a great hope to predict, modify and even cure epilepsy (Duncan, 2022; Proix et al., 2021).

2. LONG NON-CODING RNAs (lncRNA)

Long non-coding RNAs (lncRNAs) are RNAs with a length of more than 200 nucleotides whose transcripts do not encode proteins (Liu et al., 2022). Changes in lncRNAs can be measured by RNA sequencing technology. So far only a small amount of lncRNAs have been fully characterized, however, their alterations, including overexpression, deficiency or mutation, have been associated with numerous diseases (Esteller, 2011; Ang et al., 2020). Recently, the role of lncRNAs in epileptogenesis has been gradually demonstrated, mainly related to the pathophysiological process of neuroinflammation and neuronal apoptosis and the balance of transmitters (Liu et al., 2022). Neuroinflammation, known to play a role in many neuropsychiatric diseases, including Alzheimer's disease and depression, has also been implicated in the pathogenesis of epilepsy (Vezzani et al., 2019; Kumar et al., 2022). Furthermore, abnormal neuronal function and survival are also critical for the development of epilepsy. Increased neuronal apoptosis has been strongly associated with epilepsy progression (Henshall and Simon, 2005).

2.1. Biological Properties and Functions of lncRNAs

LncRNAs are transcribed by RNA polymerase II or III and lack open reading frame (ORF) and Kozak consensus sequence structures. Similar to mRNAs, a maturation process is required for lncRNAs, and mature lncRNA molecules can be found in the nucleus, cytosol and organelles including mitochondria (Villa et al., 2019). In general, lncRNAs in the

nucleus are mainly involved in epigenetic and transcriptional regulation, while lncRNAs in the cytoplasm are usually involved in the regulation of post-transcription, which regulates mRNA stability, protein translation and the competitive endogenous RNA (ceRNA) network (Gao et al., 2020). According to their location in the genome and their orientation to nearby protein-coding genes, lncRNAs can be categorized into sense, antisense, intronic, intergenic and bidirectional (Esteller, 2011).

LncRNAs can regulate the downstream transcription of genes or affect the expression of target genes by sponging miRNAs (Esteller, 2011). Moreover, lncRNAs can block a specific molecular pathway (Figure 2). Furthermore, lncRNAs can guide specific proteins to reach the target location or facilitate the interaction of numerous molecules and proteins, promoting the integration of information among different signaling pathways (Esteller, 2011; Quinn and Chang, 2016; Gao et al., 2020). For example, lncRNA H19 was reported to inhibit glial cell activation in the hippocampus of epileptic rats by targeting the STAT3 signaling pathway (Han et al., 2020), while lncRNA MEG3 can reduce neuronal apoptosis in the hippocampus of epileptic rats through the PI3K/AKT/mTOR pathway (Zhang et al., 2020). In addition to regulating target gene expression at the transcriptional and posttranscriptional level, some lncRNAs can also manipulate target gene expression in an epigenetic manner (Tsai et al., 2010; Peschansky and Wahlestedt, 2014). Some lncRNAs can recruit chromatin remodeling complexes to a specialized splicing site or recruit specific histone modification enzymes to determine DNA methylation pattern or histone status (Tsai et al., 2010; Chen and Xue, 2016).

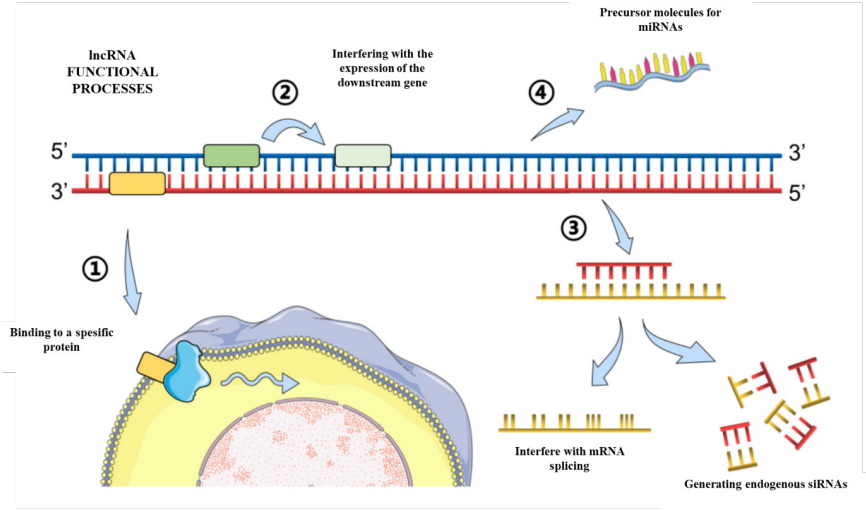

Figure 2. *Common models of the biological functions of lncRNAs (Liu et al., 2022). Long non- coding RNAs can act by affecting different biological mechanisms, mainly in these ways: 1) bind to a specific protein and regulate its activity directly or indirectly by altering cellular localization by forming nucleic acid-protein complexes; 2) It encodes the transcriptional process of the upstream promoter region of a protein-coding gene, inhibits RNA polymerase II or mediates chromatin remodeling and histone modification, or interferes with downstream expression of the gene; 3) Form complementary double helices with the transcription of the protein-coding gene and generate endogenous siRNAs under the action of the 'Dicer' enzyme or interfere with 'mRNA splicing' and generate different transcripts; 4) serve as precursor molecules for miRNAs.*

2.2. The Role of lncRNAs in the Idiopathic Generalized Epilepsy Process and Possible Mechanisms

Studies have shown that lncRNAs significantly participate in the molecular mechanisms of epilepsy development by regulating epigenetic, transcription and post-transcriptional gene expression. It is even thought that lncRNAs may serve as novel diagnostic markers and therapeutic targets for epilepsy in the future (Kuang, 2023).

Neuroinflammation is a contributing factor for many neuropsychiatric diseases, including Alzheimer's disease and depression, and increasing evidence has demonstrated its role in the pathogenesis of epilepsy (Vezzani et al., 2019; Kumar et al., 2022). Sun et al. (2017) identified through microarray screening that the lncRNA GAS5 can regulate microglia polarization by acting as an epigenetic regulator. In a rat model of bilateral chronic constriction injury, the expression levels of lncRNA 00311 and

lnc-AK141205 were upregulated in dorsal spinal microglia (Pang et al., 2020). By interacting with proteins, RNA and DNA, lncRNAs may serve as regulators of gene expression pathways involved in the regulation of both pro-inflammatory and anti-inflammatory process in the central nervous system (Tripathi et al., 2021). Acting as regulators of inflammation and neuronal differentiation pathways in the epileptic brain, lncRNAs are reported to play a role in the development of epilepsy (Zhao et al., 2019). Abnormal neuronal function and survival are the most important features of epilepsy. Increased neuronal apoptosis is strongly associated with epilepsy progression (Henshall and Simon, 2005). Studies have shown that lncRNAs may be involved in the pathogenesis of epilepsy through neuronal apoptosis. In the epileptic neuronal model not treated with Mg2C and in rat models induced with LiCl or pilocarpine, lncRNA SNHG1 and lncRNA MALAT1 expressions were significantly increased, while lncRNA FTX and lncRNA MEG3 expressions were significantly decreased (Wu and Yi, 2018; Li et al, 2019; Cai et al, 2020; Zhang et al, 2020). In another study, it was determined that lncRNA ZFAS1, in addition to promoting neuroinflammation, may also exacerbate the development of epilepsy by accelerating neuronal apoptosis (He et al., 2021). Another lncRNA, TUG1, regulated cell activity and apoptosis of hippocampal neuron by sponging miR-199a- 3p (Li et al., 2021). Besides the regulatory effect of lncRNA 17A in autophagy and apoptosis, about 100 dysregulated lncRNA transcripts were found in amyloid β peptide-treated SH-SY5Y cells (Wang et al., 2019). Furthermore, lncRNA H19 was highly expressed during the latent period of epilepsy and contributed to the apoptosis process of hippocampal neurons by targeting miRNA let-7b (Han et al., 2018). It was reported that lncRNA HOXA-AS2 and SPRY4-IT1 expressions were higher in male patients with JME compared to male controls (Hashemian et al., 2019).

Evidence from brain imaging and postmortem studies in animal models suggests that inhibitory GABAergic interneurons and excitatory Glu neurons are dysfunctional and contribute to epileptogenesis (Treiman, 2001; Pfisterer et al., 2020). Major clinical anticonvulsants, such as lacosamide, levetiracetam, phenobarbital, phenytoin and valproate, mainly aim to improve GABAergic activity or reduce Glu-ergic activity (De Deyn et al., 1990). Highly expressed lncRNAs in the brain are involved in important neurobiological processes, including neurotransmitter synthesis and transmission, neurogenesis and neural plasticity (Bond et al., 2009). It has been reported that lncRNAs associated with PICK1, GADL1 and PMD6 genes are abundant in pathways related to ionotropic Glu receptor and GABA synthesis (Muniz et al., 2022). In addition to abrogating astrocyte activation, overexpression of lncRNA UCA1 suppressed the expression of astrocyte glutamate aspartate transporter (GLAST) via the JAK/STAT sig-

naling pathway, resulting in a reduction in the frequency of epileptic seizures and the promotion of learning and memory in temporal lobe epilepsy rats (Wang et al., 2020). In addition, it has been shown that the regulatory effect of lncRNA GAS5 in epilepsy is also involved in the regulation of the Calmodulin-dependent protein kinase II (CaMKII)g/N-methyl-D-aspartate receptor (NMDAR) pathway (Zhao et al., 2022). All studies have shown that there is a close link between changes in lncRNAs, GABA/Glu balance in epilepsy and the effect of anticonvulsants. Genome-wide methylation assessment in nine resected hippocampal tissue samples from patients with refractory temporal lobe epilepsy (TLE) demonstrated methylation differences of UCA1, ADARB2-AS1, LINC324 and MAP3K14-AS1 lncRNAs in hippocampal tissue in human temporal lobe epilepsy (Miller-Delaney et al., 2015). DNA from 30 TLE patients (including drug-resistant or drug-responsive, MRI-negative or in the presence of hippocampal sclerosis) and peripheral blood of 30 controls was isolated for genome-wide methylation analysis and abnormal methylation patterns were observed in miRNA and lncRNA genes in this study (Xiao et al., 2018).

3. CONCLUSION

Although much energy and funds are spent each year on research and development of antiepileptic drugs, an effective and durable treatment for epilepsy has still not been found. Although seizures can be controlled with anti-epileptic drugs, especially in many of the idiopathic generalized subtypes, there are many patients who require continuous treatment. Drug resistance is present in a large proportion of these patients. Given the preponderance of evidence, the pathogenesis of epilepsy in most patients is due to epigenetic and/or environmental factors rather than genetic. In this context, perhaps some lncRNAs involved in epileptogenesis could be evaluated for their potential role as environmental sensors. Given their close links with neuroinflammation, apoptosis and the balance of transmitters, particularly GABA and Glu, lncRNAs are considered as potential therapeutic targets for epilepsy treatment. Although a large number of lncRNAs have been identified, the mechanistic and functional roles for most lncRNAs are still unknown. Furthermore, there is a need for increased knowledge about the dynamic changes of lncRNAs and their functions in the progression of epilepsy, especially in different types of attacks. Studies on the expression levels of lncRNAs in epilepsy and the pathways through which they participate in the epilepsy process have been largely excluded from IGE. LncRNA studies in idiopathic generalized epilepsy subtypes are limited. Considering the delays in the diagnostic process of these patients, the disadvantages of lifelong drug use since these patients can be controlled with drugs although there is no definitive treatment, mental activity disorders

seen in patients in some IGE subgroups and drug-resistant patients, it is evaluated that there is a need to study lncRNA and other epigenetic regulators in this disease group and contribute to the literature.

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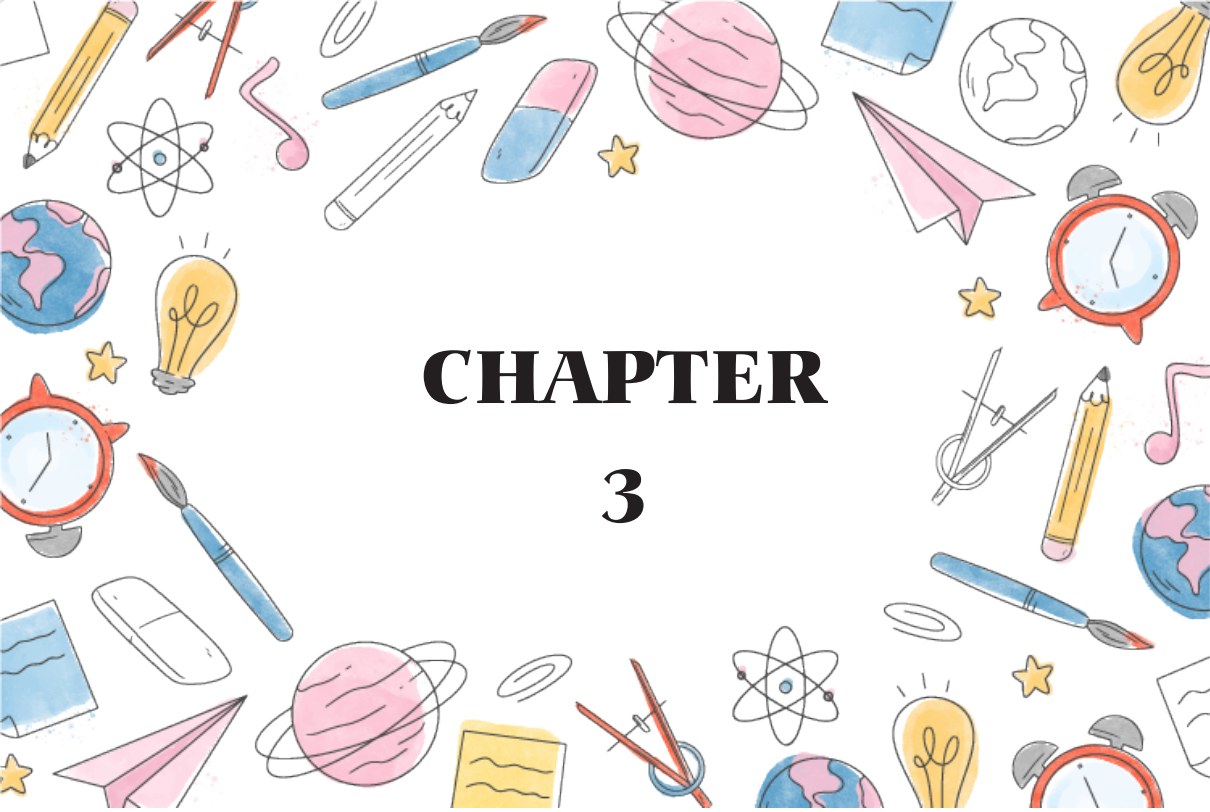

CHAPTER 3

INTESTINAL EPITHELIAL DAMAGE IN CELIAC DISEASE: APOPTOSIS, PYROPTOSIS, AND NECROPTOSIS

Dinara BOKENBAY[1]

Sonay TEMURHAN[2]

Çiğdem KEKİK ÇINAR[3]

1 Doktora öğrenci, İstanbul Ünivesitesi, Sağlık Bilimler Enstitüsü, Tıbbi Biyoloji Anabilim Dalı, E-mail: dinara.bokenbay@gmail.com

2 Dr. Öğr.Üyesi, İstanbul Ünivesitesi,Tıp Fakültesi, Doku Tipleme Laboratuvarı, E-mail: sonaytemurhan@gmail.com

3 Prof.Dr., İstanbul Ünivesitesi,Tıp Fakültesi, Doku Tipleme Laboratuvarı, E-mail: cigdem.kekik@istanbul.edu.tr

I. Celiac Disease

Celiac disease (CD) is a chronic autoimmune inflammatory enteropathy of the small intestine triggered by gluten ingestion in genetically predisposed individuals. Gluten is a protein complex found in prolamins, including gliadins in wheat, secalins in rye, hordeins in barley, and avenins in oats.

Celiac disease has a global prevalence of approximately 1% and can develop at any age (1,2). It manifests through diverse clinical symptoms such as diarrhea, abdominal pain, weight loss, growth retardation, bloating, nausea, or vomiting. If left undiagnosed, it can lead to severe health complications (3).

I-1. Role of HLA Alleles

Approximately 90–95% of the genetic predisposition to CD is attributed to the Human Leukocyte Antigen (HLA)-DQ2 allele, with 5–10% linked to HLA-DQ8. These alleles play a critical role in the disease's pathogenesis (1). They facilitate the efficient presentation of gluten peptides by HLA class II molecules to T cells, triggering an inflammatory response in the small intestinal mucosa. This response results in villous atrophy and impaired nutrient absorption (2).

I-2. Pathogenesis and Immune Response

Upon ingestion, gluten undergoes deamidation by tissue transglutaminase in the gastrointestinal tract. Deamidated gluten peptides, when presented by HLA-DQ2 or HLA-DQ8 molecules, activate $CD4^+$ T cells. This activation leads to the secretion of proinflammatory cytokines and the production of anti-gluten and anti-transglutaminase antibodies by B cells. The subsequent autoimmune response damages the intestinal mucosa and underlies the clinical manifestations of CD (3).

I-3. Clinical Diagnosis and Genetic Testing

The diagnosis of CD relies on clinical presentation, serological tests, and small intestinal biopsy. In cases with diagnostic challenges or seronegative presentations, the presence of HLA-DQ2 and HLA-DQ8 alleles provides valuable genetic evidence (4). These tests are typically conducted using PCR-based analysis of DNA isolated from blood samples (3). A negative HLA test has a high predictive value, as individuals lacking HLA-DQ2 or HLA-DQ8 have an extremely low likelihood of developing CD (5). While HLA testing identifies genetic susceptibility, it does not

confirm the diagnosis. It can, however, prevent unnecessary biopsies and follow-ups. Importantly, not all individuals with a positive HLA test develop CD, as environmental and other genetic factors also contribute to disease onset (6).

II. Mechanisms of Cell Death

The maintenance of tissue homeostasis in adult organs depends on a delicate balance between cell production and cell loss. Although extensive research has been conducted on cell production in the intestinal epithelium, the mechanisms of cell loss remain insufficiently understood. Continuous cell death occurs in the intestinal mucosa under both physiological and pathological conditions, and this process is tightly regulated to preserve epithelial barrier integrity (7,8).

II-1. Apoptosis

Apoptosis is a programmed form of cell death that eliminates dying cells without eliciting inflammation. In the intestinal epithelium, apoptotic cells are rapidly removed by neighboring enterocytes, preventing gaps in the epithelial layer and maintaining barrier integrity (7,8). Anoikis, a specialized form of apoptosis, occurs when cells lose survival signals from their interactions with the extracellular matrix.

In CD, apoptosis is triggered by interferon-gamma (IFN-γ) produced by gluten-specific $CD4^+$ Th1 cells, accompanied by elevated levels of type I IFNs, IL-15, and IL-18 (9,10). These mediators enhance the cytotoxic activities of $CD8^+$ T lymphocytes, γ/δ T cells, and natural killer (NK) cells, causing mucosal damage. They induce enterocyte apoptosis via the FAS/FASL axis and through perforin/granzyme B secretion (11–16) (Figure 1).

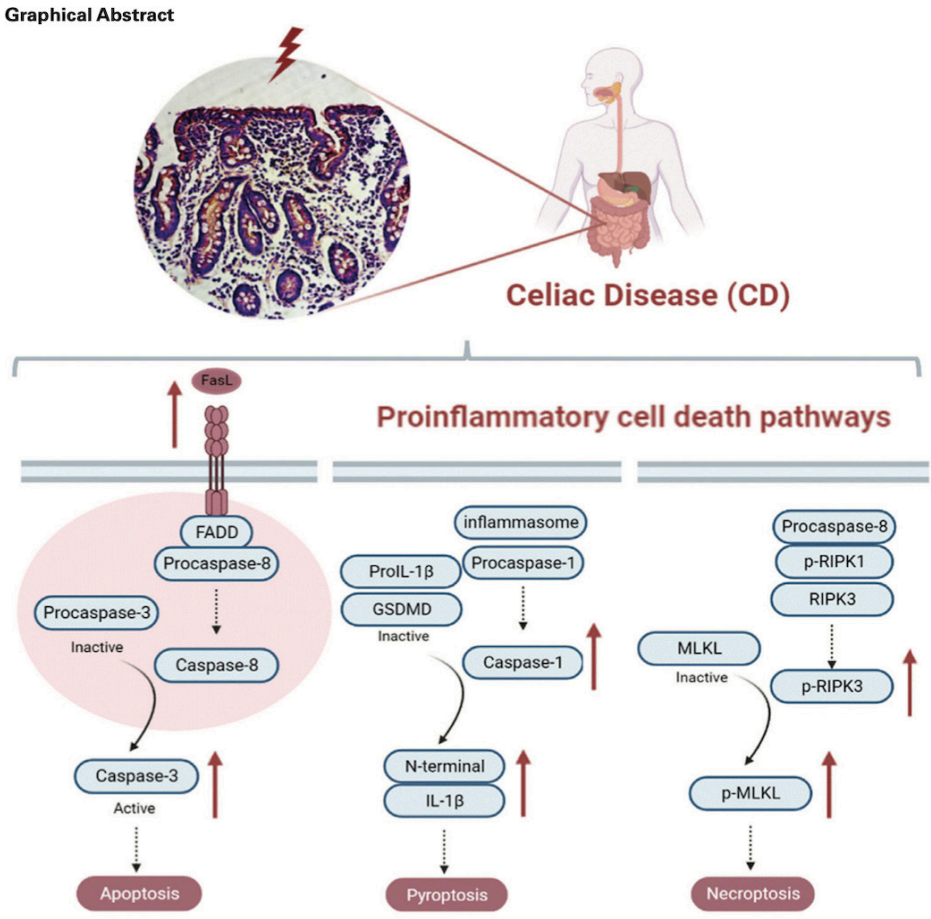

Figure 1. Mechanisms of programmed cell death pathways (apoptosis, pyroptosis, and necroptosis) [16].

Pyroptosis

In recent years, other programmed cell death (PCD) mechanisms with necrosis-like phenotypes have been identified. These pathways can trigger the release of proinflammatory mediators such as IL-1β, IL-18, and alarmins even before cell death occurs (17,18,19). Some of these non-apoptotic PCD pathways are thought to play a role in the pathogenesis of celiac disease (CD). Pyroptosis, a programmed lytic cell death pathway, facilitates the rapid clearance of damaged cells during infections. Unlike apoptosis, pyroptosis leads to inflammation through the release of cellular components and damage-associated molecular patterns (DAMPs). In the intestine, this can result in barrier dysfunction and excessive translocation

of macromolecules and microbiota components from the lumen (20). Such uncontrolled translocation can initiate and sustain chronic inflammation associated with intestinal diseases.

In untreated CD, upregulated interferons (IFNs) stimulate the expression of inflammasome sensors such as AIM2, IFI16, NRLP2, and NLRC5, alongside the transcription factor IRF1 and cytokine IL-18. IRF1, a key player in CD pathology, also promotes the expression of caspase-4, caspase-5, NLRP3, and cytokine IL-1β, which are inflammatory mediators induced by IL-17A and Toll-like receptors (TLRs). These processes render target cells more sensitive to a range of inflammatory stimuli, including gliadin peptides such as p31-43, which can activate the NLRP3 inflammasome. Moreover, the activation of caspase-4 and caspase-5 may be driven by intracellular lipopolysaccharide (LPS) release, a mechanism yet to be explored in CD.

Ultimately, these pathways converge to activate caspases-1, -4, and -5, which process pro-IL-1β and pro-IL-18 into their biologically active forms. During this process, Gasdermin-D (GSDMD) is cleaved into N-terminal fragments that oligomerize within the cell membrane, forming pores that facilitate the release of IL-1β and IL-18. These cytokines subsequently promote pyroptotic cell death. Upon release, IL-1β and IL-18 interact with their respective receptors, IL-1R and IL-18R, activating immune cells that amplify the local inflammatory response (Figure 2). This mechanism not only explains a subset of epithelial cell death in CD but also contributes to the increased production and secretion of IL-1β and IL-18. These cytokines, in turn, activate Th1, Th17, and cytotoxic T lymphocytes, thereby fueling additional inflammatory pathways integral to CD pathogenesis (21-24).

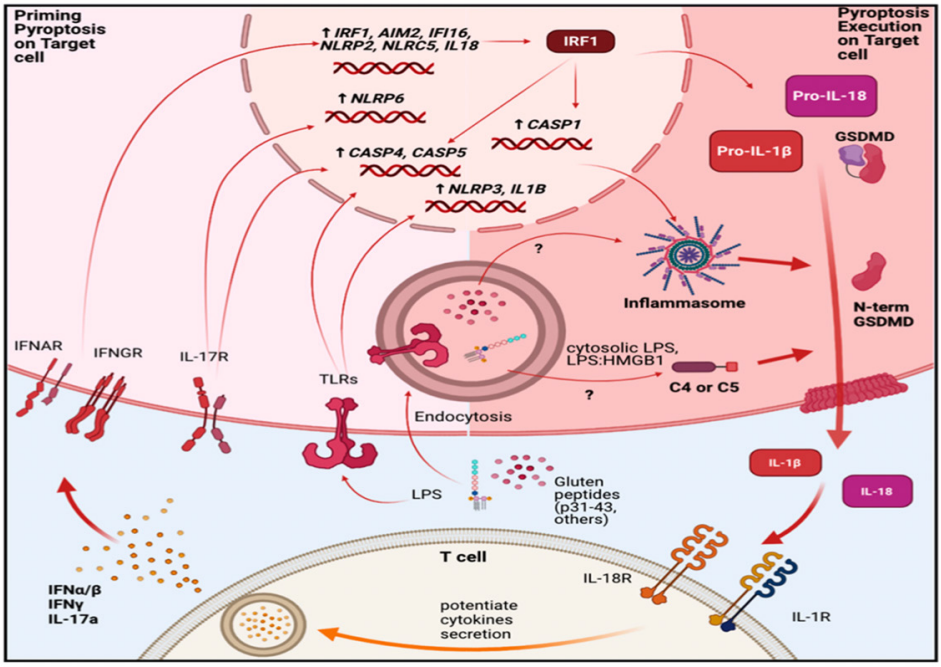

Figure 2. Pyroptosis: The mechanism of pyroptosis driving the inflammatory response in celiac disease (24).

II-3.Necroptosis

Necroptosis is a necrotic form of programmed cell death (PCD) mediated by the phosphorylation and activation of the membrane pore protein mixed lineage kinase domain-like pseudokinase (MLKL) by receptor-interacting serine/threonine kinase 3 (RIPK3) (17). Interferons (IFNs), characteristic of celiac disease (CD), can induce the expression of necroptotic mediators such as MLKL and Z-DNA-binding protein 1 (ZBP1) and promote the extracellular release of high mobility group box 1 (HMGB1), a nuclear protein. Moreover, ZBP1 plays a central role in directly activating necroptosis via the STAT1/ZBP1/RIPK3 pathway in the presence of caspase-8 inhibition (25–27).

Signal transduction through TLR/TRIF and death receptors (TNFR1, CD95)/TRADD/FADD also triggers necroptosis via RIPK3 activation when caspase-8 is inhibited. RIPK3 activation leads to MLKL phosphorylation, and the phosphorylated MLKL (pMLKL) is transported to the plasma membrane within vesicles in association with the regulatory protein ZO-1. Upon reaching the plasma membrane, pMLKL monomers polymerize, forming pores that induce necroptotic cell death and result in the ext-

racellular release of alarmins, including IL-1α, IL-33, and HMGB1 (28) (Figure 3).

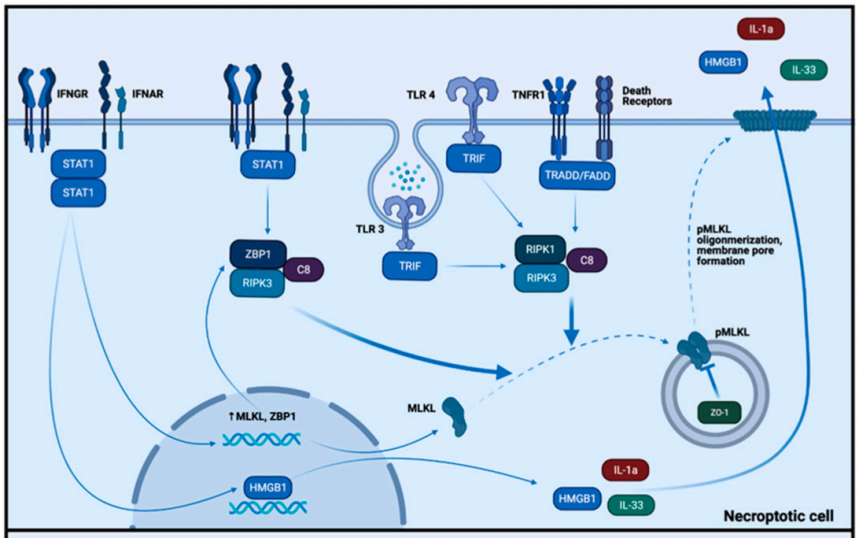

Figure 3. Necroptosis: A Programmed Cell Death Mechanism (24).

In CD, programmed cell death pathways such as apoptosis, pyroptosis, and necroptosis contribute to inflammation and tissue damage in the intestinal mucosa. Understanding these mechanisms could pave the way for novel therapeutic strategies aimed at modulating inflammation and promoting mucosal healing.

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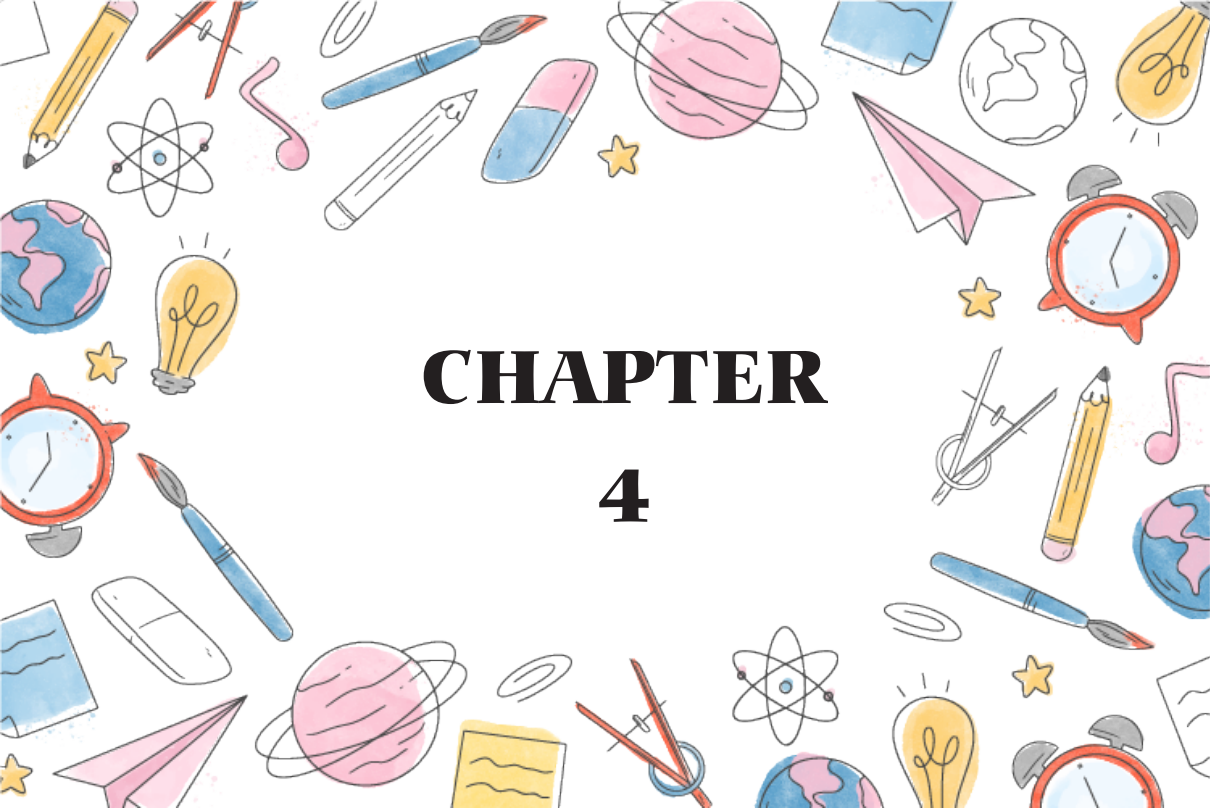

CHAPTER 4

THE ermTR GENE: AN UNIQUE PLAYER IN ANTIBIOTIC RESISTANCE

Erman ORYAŞIN[1]

Bülent BOZDOĞAN[2]

1 Assoc. Prof. Dr., Aydin Adnan Menderes University, Recombinant DNA ve Recombinant Protein Research Center (REDPROM), ORCID: 0000-0003-1242-7434.

2 Prof. Dr., Aydin Adnan Menderes University, Recombinant DNA ve Recombinant Protein Research Center (REDPROM), ORCID: 0000-0003-2469-9728.

1.Introduction

The *ermTR* gene, initially identified in *Streptococcus pyogenes* by Seppälä et al. (1998), is a member of the erm gene family associated with antibiotic resistance. This gene is key for bacteria, especially *S. pyogenes*, to adapt to antibiotics such as erythromycin and clindamycin. The importance of *ermTR* lies in its capacity to provide low-level resistance to erythromycin while allowing for high-level resistance to clindamycin (Oryaşın et al., 2020).

Lincosamides such as clindamycin are especially valuable for treating infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and other Gram-positive bacteria. The increasing prevalence of bacteria exhibiting resistance to both macrolides and lincosamides, often driven by *erm* genes such as *ermTR*, poses a significant clinical challenge particularly when treating streptococcal infections.

Macrolides, lincosamides, and streptogramins (MLS) are a group of antibiotics that inhibit bacterial growth by binding to the ribosome, demonstrating strong efficacy against Gram-positive bacteria (Oryaşın et al., 2020). The growing problem of bacteria developing resistance to antibiotics, especially those affecting streptococci, has become a major clinical issue (Cattoir & Leclercq, 2017).

2. The molecular mechanism behind *ermTR*-mediated resistance

The structure of a gene and its resulting protein product

The *ermTR* gene consists of 732 nucleotides, displaying 82.5% similarity with *ermA*, and is expected to encode a polypeptide of 243 amino acids (Seppälä et al., 1998). Its expression and induction can be influenced by the presence or absence of the gene's regulatory region, as noted by Oryaşın et al. in 2020.

Methylation of 23S rRNA

The resistance profile conferred by *ermTR* is primarily due to the methylation of adenine residues in the 23S rRNA, which alters antibiotic binding sites (Cattoir & Leclercq, 2017). Specifically, methylases encoded by *erm* genes methylate the adenine residue at position 2058 of the ribosomal RNA 23S subunit. The modification enables bacteria to endure antibiotic treatment.

The Erm family of methyltransferases (erythromycin resistance methyltransferase) facilitates the methylation of the N6 position of nucleotide A2058 in the 23S rRNA. This methylation process prevents macrolides

from interacting with the ribosome, effectively conferring high resistance to macrolide antibiotics (Golkar et al., 2018). Methylation also impacts the binding of lincosamides, which is why cross-resistance is often seen between macrolides and lincosamides. ErmTR's distinct resistance profile, characterised by both low- and high-level resistance to clindamycin, offers a potentially unique mechanism of action in comparison to other *erm* genes. The differing impacts of macrolides and lincosamides on antibiotic resistance underscore the multifaceted nature of resistance mechanisms and their practical effects on treatment.

So far, six mobile linezolid resistance classes, encompassing *lnu, cfr, erm, vga, lsa,* and *sal,* have been recognised. Lincosamide resistance genes are commonly encountered on mobile genetic elements, including plasmids, transposons, integrative and conjugative elements, genomic islands, and prophages (Yang et al., 2024). The Erms enzymes promote the addition of either one or two methyl groups to the A2058 residue, this process being influenced by the *erm* gene (Svetlov et al., 2021).

Svetlov et al. (2021) offered significant understanding of how the *ermTR* gene is responsible for macrolide resistance, primarily through the dimethylation of the A2058 residue in 23S rRNA. The high-resolution crystal structures show that this modification happens during ribosome assembly (30S and 70S), with Erm methyltransferases targeting the A2058 site, remaining accessible in the process. These results highlight the crucial phase of ribosomal maturation. This study questioned established beliefs about significant changes to ribosomal structure after post-dimethylation, finding that the modification does not cause major changes to ribosomal architecture or operation.

Svetlov et al. (2021) found that the dynamics of ribosomal interactions are precise and the desosamine moiety plays a vital role in macrolide binding. These insights lay the groundwork for developing rational drug design strategies to tackle the pressing issue of antibiotic resistance. These discoveries hold significant importance in medical settings, where the widespread presence of erm genes in disease-causing bacteria hinders treatment choices.

Comparison with Other *erm* Genes

To date, at least 55 *erm* genes have been identified, with *erm*(A), *erm*(B) and *erm*(C) being the most frequently encountered (Yang et al., 2024). Each class shows distinct resistance profiles, induction methods, and patterns of occurrence among bacterial species. Widely distributed among various Gram-positive and Gram-negative bacteria, *ermB* is found in contrast to *ermTR*, which is predominantly located in *Streptococci*.

In contrast to *ermA* and *ermB*, which encode methyltransferases that methylate adenine at position 2058 of 23S rRNA and impart high-level resistance to both macrolides and clindamycin, ermTR displays a distinct resistance profile (Oryaşın et al., 2020). Constitutive expression of *ermTR* results in low-level erythromycin resistance at 8 mg/L and high-level resistance to clindamycin at 128 mg/L (Oryaşın et al., 2020).

3. Regulatory Mechanisms and Induction

The expression of *erm* genes can be either constitutive or inducible, depending on specific stimuli. When the leader peptides are being translated before the genes that encode methyltransferases, such as *erm*(B), in the presence of erythromycin, ribosome stalling occurs, which in turn leads to the induction of downstream methyltransferase expression (Arenz et al., 2014). The *erm* genes can be expressed constitutively either with a mutated leader peptide or in the absence of the leader peptide (Yang X et al., 2024).

The Regulatory Region of ermTR

The regulatory region of *ermTR* is vital for its adaptive response to antibiotics. This area encompasses three key hairpin formations, with the third featuring a higher delta energy that conceals the ribosome's binding site and the *ermTR* gene's start codon on the mRNA (Oryaşın et al., 2020).

Induction Mechanism

Compounds such as erythromycin are hypothesized to induce conformational changes in the mRNA structure., enabling the ribosome to bind and start the synthesis of the ErmTR enzyme (Oryaşın et al., 2020). The induction mechanism enables a quick response to the presence of antibiotics in the environment.

The *ermTR* induction mechanism is particularly relevant to lincosamide resistance. Constitutive expression provides a high degree of resistance to clindamycin, and erythromycin induction can substantially boost this resistance. Inducible clindamycin resistance has significant clinical implications because it may result in treatment failure if clindamycin is administered to treat infections caused by bacteria that appear to be resistant to erythromycin but susceptible to clindamycin.

The induction mechanism of erm genes, encompassing *ermTR*, involves an intricate process including ribosome stalling and translational attenuation. Binding of an inducer antibiotic to the ribosome causes the ribosomal process to halt at a particular location within the leader peptide

region. This stalling event initiates a conformational shift in the mRNA structure, revealing the Shine-Dalgarno sequence and the erm gene start codon, which then enables its translation to proceed.

Differential Induction Effects

A noticeable impact of the regulatory region was evident in *S. pyogenes* NZ131, where the clindamycin MIC rose by more than 16-fold following induction with erythromycin (Oryaşın et al., 2020). The presence of the regulatory region (*ermTR*+rr) results in erythromycin and clindamycin MICs that are nearly identical, however, clindamycin MICs rise by more than 32-fold (from 4 to over 128 mg/L) following erythromycin induction as reported by Oryaşın et al. (2020).

This differential induction effect is not exclusive to *ermTR* and can also be seen in other *erm* genes. The induction profile for macrolide resistance can differ based on the *erm* gene and the specific bacterial species involved, which adds to the complexity of macrolide resistance in clinical environments.

Translational Attenuation and Inducible Expression of *ermTR*

The inducible expression of erm genes, such as *ermTR*, is primarily regulated by translational attenuation. This mechanism allows bacteria to detect the presence of macrolide antibiotics and activate resistance genes on demand, thereby reducing the metabolic strain of continuous expression.

The process of translational attenuation incorporates a leader peptide sequence positioned upstream of the erm gene coding region. A specific sequence of nucleotides is essential for controlling the initiation of translation by causing ribosomes to pause and altering the structure of messenger RNA (Bozdogan and Appelbaum, 2004).

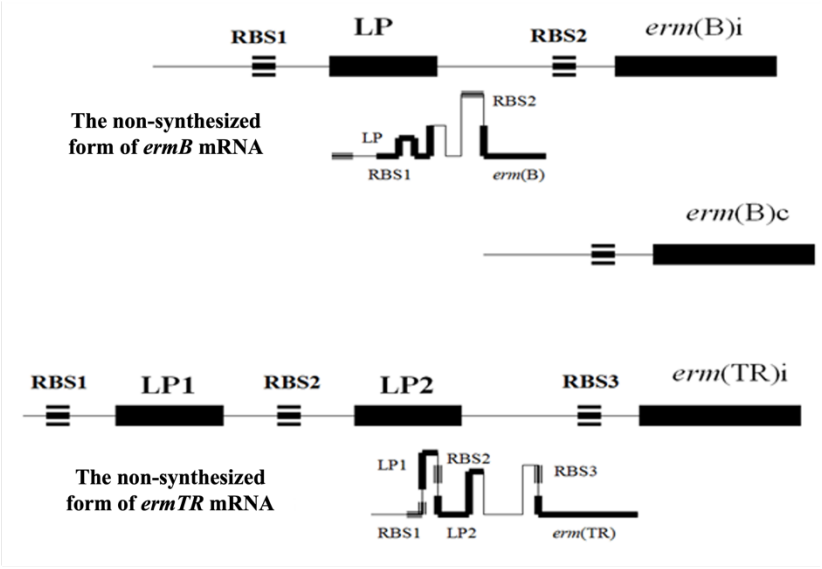

Figure 1: *The inducible and constitutive regulatory regions of the ermB gene and the inducible regulatory region of ermTR have been shown (Bozdogan and Appelbaum, 2004).*

The Mechanism of Ribosomal Stalling Induced by Macrolide Antibiotics

1. In the absence of a macrolide antibiotic, ribosomes are able to efficiently translate the leader peptide, which in turn prevents the downstream expression of *ermTR*. However, when a macrolide antibiotic, such as erythromycin, binds to the ribosome, it results in ribosome stalling at a specific codon within the leader peptide region.
2. The stalling of the ribosome causes a change in the mRNA structure, resulting in the unfolding of an inhibitory hairpin structure that normally blocks the Shine-Dalgarno sequence and the *ermTR* start codon. This structural change makes the Shine-Dalgarno sequence accessible, enabling ribosome binding and the initiation of *ermTR* translation.
3. Once the SD sequence is accessible, the full *ermTR* gene is transcribed and translated into an Erm methyltransferase enzyme, which then methylates the 23S rRNA at the A2058 position, preventing macrolide binding to the ribosome and thereby conferring resistance.

The distinctions between ermTR and other erm genes.

Unlike *ermA* or *ermB*, which display robust inducibility, *ermTR* has been documented to have a weaker ribosome stalling effect, resulting in partial constitutive expression even in the absence of macrolides. This distinctive regulatory feature may underlie its distinct resistance profile, which involves low-level erythromycin resistance and high-level clindamycin resistance.

The *ermA* and *ermC* genes are most commonly found in staphylococci, the *ermB* gene in *enterococci* and *streptococci*, while the *ermTR* gene was first identified in *Streptococcus pyogenes* strains and constitutes the most prevalent methylase group in this species. The synthesis of Erm methylase can be induced by the presence of macrolides in the environment. Typically, 14- and 15-membered macrolides induce resistance to a greater extent than 16-membered macrolides. In *staphylococci*, lincosamides do not induce resistance. The induction of resistance occurs during the protein synthesis phase, specifically at the translational stage. Upon erythromycin binding, conformational changes in the mRNA occur, and an RBS (ribosome binding site) previously inaccessible to the ribosome in the secondary RNA structure becomes accessible for ribosome binding (Bozdogan and Appelbaum, 2004) (Fig 1).

Translational attenuation has significant clinical implications.

- The presence of a working mechanism for slowing down translation means bacteria initially seem to be susceptible to clindamycin in standard tests but can develop resistance when exposed to macrolides. The correct interpretation of this phenomenon is essential for making informed decisions about antibiotic treatment, since misinterpretation could result in unsuccessful treatments.
- Developing novel antibiotics could be achieved by targeting the mechanism that causes attenuation, which would prevent ribosome stalling and block the activation of ermTR.

4. Experimental Findings and Clinical Implications

Isogenic Conditions Study

Oryaşın et al. (2020) investigated the impact of the *ermTR* gene on macrolides and lincosamides under isogenic conditions to clarify why ErmTR varies from other methylases. The researchers isolated *ermTR* from *Streptococcus pyogenes* C1 and then moved it to *S. pyogenes* NZ131, both instances occurring with and without its regulatory region intact.

MIC Determination

The results showed that ermTR, which is always expressed, provided low-level resistance to erythromycin at a concentration of 8 mg/L, but high-level resistance to clindamycin at a concentration of 128 mg/L (Oryaşın et al., 2020). The regulatory region's presence led to a substantial rise in clindamycin resistance under erythromycin induction, with resistance increasing from 4 mg/L to greater than 128 mg/L.

When interpreting the results of antibiotic susceptibility tests, it is essential to take into account both the inherent and acquired resistance that may be present. In healthcare environments, untreatable bacterial resistance can arise if it is not identified and managed correctly.

The distinct resistance characteristics of *ermTR*, most notably its influence on lincosamide resistance, have substantial clinical repercussions. The coexistence of low-level erythromycin resistance and high-level clindamycin resistance complicates traditional diagnostic approaches. There is a requirement for more advanced diagnostic methods, like molecular detection of *erm* genes or inducible clindamycin resistance testing, to inform the selection of suitable antibiotic treatments.

Double Disk Testing

The double disk test, also referred to as the D-test, is a significant diagnostic tool for identifying clindamycin resistance that can be induced in clinical isolates. The procedure entails positioning erythromycin and clindamycin disks in close proximity on an agar plate that has been inoculated with the test organism. A reduction or diminishment in the size of the clindamycin inhibition zone adjacent to the erythromycin disk (D-shaped) is indicative of inducible resistance.

Induction with erythromycin and azithromycin led to a decrease in the clindamycin inhibition zone for *S. pyogenes* transformed with *ermTR* and regulatory regions, but had no effect on telithromycin inhibition, according to Oryaşın et al. (2020). This provides additional evidence for the distinct induction profile of *ermTR*.

5. Environmental Factors and Resistance Spread

Antibiotic Pressure

Environmental factors have a substantial impact on *ermTR* gene expression in *Streptococcus pyogenes.* Antibiotics at levels below the minimum needed to inhibit bacterial growth can stimulate stress responses,

causing an increase in ermTR and allowing bacteria to adapt rapidly (Motallebirad et al., 2021).

Recent studies indicate that sub-MIC antibiotic concentrations can selectively promote the growth of resistant bacteria. The "selective window" refers to a phenomenon that has significant implications for the dissemination of antibiotic resistance in environmental contexts (Wang et al., 2025).

Genetic Mobility

The spread of *ermTR* is accelerated by mobile genetic elements, such as plasmids and transposons, which speed up the widespread distribution of resistance characteristics (Varaldo et al., 2009).

Furthermore, MGEs carry genes that not only provide resistance to antimicrobial agents from other classes, but also to metals and biocides (Yang et al., 2024).

The horizontal transfer of erm genes, which include *ermTR*, can occur through processes like conjugation, transformation, and transduction. Genetic mobility enables the spread of resistance traits not only within a species but also between various bacterial species, thus facilitating the swift evolution and widespread dissemination of antibiotic resistance.

6.The unique characteristics of *ermTR*

Differential Resistance Levels

Two key differences set *ermTR* apart from other methylases: (1) the level of erythromycin resistance provided by constitutively expressed *ermTR* is relatively low at 8 mg/L in comparison to >128 mg/L for other methylases, and (2) erythromycin induction does not enhance resistance to erythromycin but does increase resistance to clindamycin (Oryaşın et al., 2020).

Methylation Mechanism

The level of macrolide resistance caused by *ermTR* is lower than the resistance profiles associated with alterations at position 2058 of 23S rRNA (Oryaşın et al., 2020). The distinction between the methylation mechanism or target site of *ermTR* and those of other extensively researched methylases may be significant.

Recent research into the structural composition has shed light on the erm-mediated resistance process. According to a 2021 study by Svetlov et al., dimethylating A2058 does not bring about substantial structural modi-

fications to the ribosome, but rather impacts the binding of macrolides via interactions facilitated by water molecules.

7.Clinical Implications

The unique resistance characteristics of *ermTR* have substantial clinical implications, particularly in the treatment of infections resulting from *S. pyogenes* and other *streptococci*. The dual resistance phenomenon complicates treatment because first-line treatments such as erythromycin and alternatives including clindamycin may not be effective against strains positive for *ermTR* (Pinheiro et al., 2009).

The presence of *ermTR* in *Streptococcus agalactiae* (Group B Streptococcus) can result in clindamycin resistance that can be triggered, thereby increasing the risk of treatment failure in conditions like neonatal sepsis (DiPersio & DiPersio, 2007). The significance of precise detection and identification of *ermTR* in clinical samples is underscored by this point.

The existence of *ermTR* and other *erm* genes in clinical isolates requires prudent antibiotic management and the creation of innovative treatment approaches. Newer macrolides and ketolides, like telithromycin, have been developed in order to overcome several types of *erm*-mediated resistance.

8.Geographical Distribution and Prevalence

Research has demonstrated that the frequency and spread of *ermTR* can differ across different geographical areas. Zhou et al. (2014) found evidence of erythromycin-resistant genes such as *ermTR* in group A beta-hemolytic Streptococci in Chengdu, Southwestern China. Lo et al. (2015) examined the antibiotic resistance patterns and the mechanisms behind erythromycin resistance in erythromycin-resistant group G *Streptococcus dysgalactiae* subspecies equisimilis isolates from central Taiwan.

Regional variations underscore the necessity for localized surveillance and tailored antibiotic stewardship programs (Zakerifar et al., 2023). The spread of *ermTR* and other erm genes is influenced by factors like antibiotic usage patterns, healthcare practices, and environmental conditions.

9.Future Directions

Identification of Methylation Sites

Further investigation is required to precisely determine the methylation site of the methylase encoded by *ermTR* (Oryaşın et al., 2020). This could offer vital information about its distinct mode of operation and mi-

ght lead to the creation of novel methods to counteract *ermTR*-mediated resistance.

Sophisticated techniques including cryo-electron microscopy and high-resolution X-ray crystallography can be utilised to elucidate the precise structural changes resulting from ErmTR methylation. These studies could expand upon the research by Svetlov et al. (2021) and lead to a more comprehensive understanding of the molecular basis of *ermTR*-mediated resistance.

Development of New Strategies

Comprehending the complex processes behind *ermTR*-mediated resistance is vital for creating novel antibiotics and implementing successful treatment plans to counteract the escalating problem of antibiotic resistance. The options may involve designing new medications that can counteract resistance or creating diagnostic equipment for the quick identification of *ermTR*-positive strains (Gygax et al., 2006; Gygax et al., 2007).

Advances in CRISPR-Cas9 and other gene-editing technologies offer promising strategies for combating antibiotic resistance. These approaches could be employed to target and inactivate erm genes or to alter the ribosomal binding sites of antibiotics in order to overcome resistance.

Combination therapies and the development of antibiotic adjuvants which can either inhibit Erm methyltransferases or boost the effectiveness of existing antibiotics against resistant bacterial strains are current research focuses.

10.Conclusion

The *ermTR* gene illustrates the intricacies of antibiotic resistance through a combination of genetic adaptation and environmental responsiveness. Macrolide resistance mechanisms are complicated by the antibiotic's unique properties, specifically its low-level resistance to erythromycin paired with high-level resistance to clindamycin (Oryaşın et al., 2020).

Svetlov et al. (2021) has greatly enhanced our comprehension of the molecular processes driving *erm*-mediated resistance, laying the groundwork for forthcoming drug development initiatives. Research on the part water plays in macrolide binding and the fact that dimethylated ribosomes show no substantial structural changes provides the basis for creating more targeted and logical drug treatments.

Antibiotic resistance remains a major health concern worldwide, underscoring the need for in-depth research into singular resistance me-

chanisms such as that mediated by *ermTR*. Comprehending the distinct properties of *ermTR* and its influence on both macrolide and lincosamide resistance is essential for creating innovative therapeutic approaches. Future studies should concentrate on explaining the exact molecular process of *ermTR*-mediated resistance, especially its varying impacts on macrolides and lincosamides. This discovery could lay the groundwork for the creation of new antibiotics that can counteract this resistance mechanism, possibly addressing both macrolide- and lincosamide-resistant strains at the same time.

The purpose of discovering and summarizing bacterial resistance is to prevent, control, and combat resistance effectively. This review highlights four promising strategies, including chemical modification of antibiotics, the development of antimicrobial peptides, the initiation of bacterial self-destruct programs, and antimicrobial stewardship, to fight against resistance and safeguard global health. By unraveling the intricacies of these adaptive bacterial responses, we can hope to stay one step ahead of the ongoing evolutionary arms race between bacteria and antibiotics.

The fight against antibiotic resistance requires a multifaceted approach, including continued basic research into resistance mechanisms, development of new antibiotics and alternative therapies, improved diagnostic techniques, and responsible antibiotic stewardship. The *ermTR* gene and its counterparts within the *erm* family represent crucial targets in antibiotic resistance research, with ongoing studies expected to provide valuable insights.

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