Modern Health Sciences: Theory, Methodology and Practice

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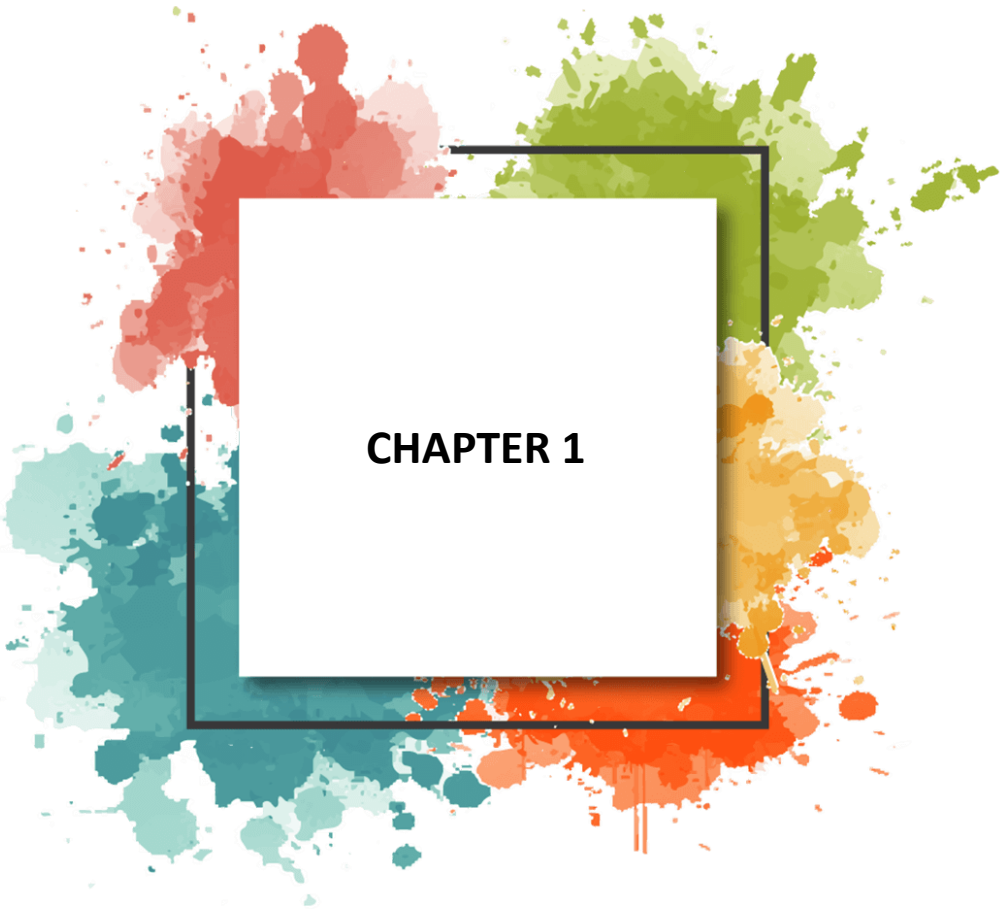
CHAPTER 1

Cystic Echinococcosis: Multidisciplinary Approaches to a Zoonotic Disease

Milad Afşar[1] & Muhammed Yasul[2]

INTRODUCTION

Cystic echinococcosis (CE) is a zoonotic parasitic disease caused by *Echinococcus granulosus*, which leads to significant health and economic losses for both humans and domestic animals. This parasitic disease poses a global public health risk due to its cosmopolitan distribution. Various epidemiological studies conducted in Türkiye indicate that CE has a high prevalence, particularly in the Southeast Anatolia, East Anatolia and Central Anatolia regions where livestock farming is intensive. This situation demonstrates that the disease continues to be a serious problem in Türkiye in terms of both animal and human health (Demir et al., 2014; Babaoğlu, 2015; Yılmaz et al., 2016; Huzaifa and Sharman, 2021).

Echinococcus granulosus has a heteroxenous life cycle and requires two different mammalian hosts. The definitive host is usually carnivorous animals such as dogs, wolves and jackals. The intermediate host is herbivorous mammals such as sheep, goats, cattle and humans. The larval form of *E. granulosus* settles in vital organs such as the liver and lungs of intermediate hosts, forming fluid-filled cystic structures. Small or calcified cysts are generally asymptomatic, while larger cysts can cause disease by exerting pressure on tissues and organs. Ruptured cysts, however, can lead to anaphylaxis and result in death (Deplazes et al., 2017; Akcam et al., 2014; Huzaifa and Sharman, 2021).

Echinococcus granulosus sensu lato (s.l.), previously considered a single species, is now classified as a group of species that differ in terms of host specificity, pathogenicity and potential for transmission to humans. This complex includes the subspecies and genotypes *E. granulosus sensu stricto* (G1–G3), *E. felidis*, *E. equinus* (G4), *E. ortleppi* (G5), *and E. canadensis* (G6/7, G8, G10). The most common and most pathogenic to humans is *E. granulosus s.s*., which circulates cyclically between sheep and dogs. *E. canadensis* is associated with pigs, deer and similar animals, and some genotypes can also cause infection in

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humans; however, the transmission rate to humans of species such as *E. equinus*, *E. ortleppi* and *E. felidis* is quite low. Recent studies have demonstrated that these species can be distinguished at the mitochondrial DNA level and that genetic differences are closely related to host adaptations (Woolsey and Miller, 2021; Babaoğlu et al., 2018; Selçuk et al., 2024).

Numerous different genotypes of *E. granulosus* have been taxonomically identified. These strains, classified as G1-G10 as a result of molecular studies, differ in terms of characteristics such as the parasite's biological behaviour, pathogenicity and host preference (Yıldız et al., 2022; Afshar, 2023). A wide variety of gene regions are used in the identification of *E. granulosus* genotypes. A meta-analysis study reported that the mitochondrial cytochrome c oxidase I (cox1) and NADH dehydrogenase subunit 1 (nad1) genes are most frequently used in genotyping. It has also been reported that the *cox1* and cytochrome b (*cob*) genes are commonly used genes in genotyping (Manterola et al., 2022; Wei et al., 2023).

Morphology

Adult *Echinococcus granulosus* live in the small intestines of domestic and wild carnivores, their definitive hosts, while the larvae settle in the tissues and organs of mammals and humans, primarily the liver and lungs. The adult form consists of a scolex, neck and body. The number of rings on the body varies between 2 and 7, but the parasite usually consists of three rings. The adult form is usually 2-7 mm long, rarely reaching up to 11 mm (Eckert et al., 2001; Altıntaş et al., 2004).

In the adult forms of *Echinococcus* species, there are immature segments immediately following the neck where the reproductive organs have not yet developed, mature segments in the middle that are functional and contain the reproductive organs, and finally proglottid segments that are morphologically the largest and contain the eggs. It takes approximately 3-4 weeks for the adult forms to reach sexual maturity (Özcel et al., 2007b; Yalçınkaya, 2016).

In mature rings, the reproductive organs are developed and observed to have reached twice the width of the body. In the centre of the mature ring, where the female reproductive organs are located, there is an ovary, behind it a vitelline sac, and in front of and behind the genital pore there are testes with 25-80 follicles. The uterus, filled with fertilised eggs, extends from front to back within the pregnant ring and branches out towards the sides (Şenlik and Diker, 2004; Yalçınkaya, 2016).

E. granulosus eggs are dark brown in colour, oval-shaped, uncapable and double-membraned, with a diameter of 20-50 μm and a length of 28-36 μm. Inside the egg is a well-developed oncosphere with six hooks. On the outer part of the egg, there is a layer known as the embryophore, which protects the embryo and provides defence against external factors. The embryophore is a thick layer consisting of keratin-like protein layers, marked by transverse radial lines, which protects the structure of the egg and, consequently, the oncosphere. Eggs usually do not have a capsule, or the capsule is so thin that it breaks down easily while in the uterus. Therefore, the capsule is not usually observed in eggs that are passed in the faeces (Şenlik and Diker, 2004; Ayaz and Tınar, 2006; Özbilgin and Kilimcioğlu, 2007; Avcıoğlu, 2013; Şenlik, 2013).

Echinococcus granulosus eggs are morphologically difficult to distinguish from the eggs of other *Echinococcus* and *Taenia* species, as they bear a resemblance to them. However, *Echinococcus* eggs can be diagnosed using anti-oncospherical monoclonal antibodies developed by Craig and colleagues (Sakamoto, 1981; Smyth, 1994; Şenlik and Diker, 2004).

The larval form of *Echinococcus granulosus*, known as the hydatid cyst, is structurally round in shape and filled with fluid. The outer part of the cyst has a fibrous layer, beneath which lies the cuticular layer. While there is a laminar layer in the middle, the inner part contains a germinal layer with reproductive characteristics. Depending on this layer, protoscoleces, protoscoleces that separate from the germinal layer and move freely, reproductive capsules, and daughter sacs that detach from them and develop freely are formed (Soulsby, 1986; Kassai, 1999; Özbilgin and Kilimcioğlu, 2007).

The fluid contained within a hydatid cyst is also known as rock water and has a clear, colourless, sterile structure. This fluid contains various proteins that carry antigenic properties belonging to the host. Sterile cysts do not contain reproductive capsules, protoscoleces, or female sacs, whereas fertile cysts do contain these structures. Sterile cysts are mostly seen in older animals and cattle, which are less susceptible to infection, while fertile cysts are generally observed in humans and sheep. If the front end of the protoscoleces in the cyst fluid is turned inwards, this indicates that the protoscoleces have an invaginated structure. However, if a protoscoleces emerges from the fluid with its front end turned outwards, this indicates that it has an evaginated structure (Aziz et al., 2011; Siles-Lucas et al., 2017; Mohammed et al., 2018).

Biology

In the life cycle of *Echinococcus granulosus*, when carnivores, which are the definitive hosts, consume the organs of intermediate hosts containing fertile cysts, the protoscoleces evaginate in the stomach of the definitive hosts under the

influence of the pepsin enzyme, pH and bile. The evaginated protoscolex settles between the intestinal villi and rings begin to form around its neck. Once the parasite reaches maturity, eggs are released into the environment when the pregnant ring or rings break down in the intestines and are excreted in the faeces. When these eggs, which are excreted in faeces, are ingested by humans, who are either intermediate hosts or accidental intermediate hosts, the oncospheres that hatch from the eggs pass through the intestinal mucosa and migrate via the blood and lymphatic systems to the lungs and other organs, primarily the liver. The oncosphere that settles in these organs transforms into a hydatid cyst, which is a sac filled with fluid. When the cyst-containing organs, which contain protoscoleces, are eaten by the final hosts, the adult form of the parasites develops in the small intestines of these animals, completing the life cycle (Avcıoğlu, 2013; McManus, 2013; Şenlik, 2013; Gökpınar et al., 2017).

The eggs laid by the last hosts are extremely resistant to environmental conditions and can remain viable in the soil for approximately one year (Eryıldız, 2010; Wen et al., 2019; Ertürk et al., 2021).

Epidemiology

Echinococcus granulosus sensu lato (s.l.) is endemic in many regions of the world, and its distribution varies according to both ecological factors and socio-economic conditions. Its prevalence is increasing, particularly in rural areas where the sheep-dog cycle persists. Parasites pose a threat to public health across a wide geographical area, primarily in Mediterranean countries, Central Asia, South America, China, parts of Africa and the Middle East. Furthermore, in the Mediterranean basin, the prevalence of CE exceeds 50% in southern regions of Italy such as Basilicata and Campania, while in coastal areas of France and Spain, this rate is below 0.1%. This situation clearly demonstrates the impact of factors such as agricultural structure, dog control policies and slaughterhouse inspection between countries (Bosco et al., 2021).

In Asia, Iran, Türkiye and China are the prominent endemic countries. In China, G1 and G3 genotypes are predominant, particularly in the Tibetan Plateau and western provinces; rare genotypes such as G5, G6/7 and G8/10 have also been found (Hua et al., 2022). Similarly, high prevalence is observed in Central Asian countries, with animal movement and traditional livestock farming methods contributing to the continued spread of the disease. Comprehensive meta-analyses conducted in Africa show that the prevalence of CE in humans ranges from 0.3% to 11.0%. High prevalence rates have been reported in both humans and dogs, particularly in Sudan (49.6%) and North African countries (Libya, Egypt, Tunisia). The infection rate in dogs averages 16.9% across the continent, reaching up to 35% in some areas (Karshima et al., 2022).

In South America, CE is prevalent in countries such as Argentina, Uruguay and Peru, where the active sheep-dog cycle plays a critical role in sustaining the disease. Prevalence is particularly high in the Patagonia region. Similarly, in developed countries such as Australia and New Zealand, CE, which was endemic in the past, has been brought under significant control thanks to intensive eradication programmes. Global data reveal that *E. granulosus s.s.* (particularly the G1 genotype) is predominant on almost every continent, while *E. canadensis* (G6–G10) genotypes have been detected primarily in hosts such as pigs, camels and deer in regions including Central Asia, Canada and Africa (Manterola et al., 2022; Shams et al., 2022).

Türkiye is one of the countries where CE is endemic, and it has been reported that the G1 genotype is the most common type in human cases. Systematic meta-analyses conducted in Türkiye have revealed that the G1 genotype is prevalent in samples isolated from humans, and that the G3 and G6/7 variants are also encountered. These findings support the notion that the classic sheep-dog cycle is the primary source of CE in Türkiye. In particular, the feeding of domestic dogs with raw offal in rural areas, weak slaughterhouse controls and low public health awareness contribute to the persistence of the infection. Furthermore, the existence of a large rural population that makes its living from agriculture and livestock farming is one of the key factors contributing to the spread of CE (Afshar, 2023; Akil et al., 2023; Akkaş et al., 2023).

In Türkiye, CE has been included in the list of notifiable infectious diseases since 2005. According to data from the Ministry of Health, an increase in the number of cases has been observed since this date. When CE cases and morbidity/mortality rates among people in Türkiye are examined by province, Van province had an incidence rate of 4.12 (per 100,000) between 2010 and 2014, while this rate rose to 8.70 (per 100,000) between 2015 and 2019, placing it in first place. Van is followed by the provinces of Ağrı, Iğdır and Kırşehir, while the lowest incidence rate of CE was recorded in the province of Zonguldak with 0.13 cases per 100,000 (Topluoğlu, 2020).

Studies conducted on the prevalence of the parasite in dogs, the final host worldwide, have reported rates of 1.2-3.8 per cent in Europe, 12.2-25.3 per cent in the Middle East and Africa, and 4.2-38.0 per cent in Asia, the Far East and Oceania. In studies conducted in Türkiye, *E. granulosus* was detected in 14% of dogs in Ankara, 10.8% in Erzurum, and 4% in Van (Topluoğlu, 2020).

Most studies conducted on CE prevalence in slaughter animals that are intermediate hosts worldwide and in Türkiye have been carried out by examining the internal organs of animals slaughtered in abattoirs. Studies conducted on cattle have reported CE prevalence rates of 0.1–8.1 per cent in Europe, 0.5–49.5 per cent (generally 20 per cent) in Middle Eastern and African countries, and 2.4–

40 per cent (generally below 10 per cent) in Asia, the Far East and Oceania. In Türkiye, it has been detected at rates of 6.8-34.3 per cent in the Eastern Anatolia Region, 3.4 per cent in the Central Anatolia Region, 11.3 per cent in the Black Sea Region, and 4.0 per cent in the Marmara Region. Worldwide, CE prevalence in sheep has been reported as 0.004-65.6% in Europe (lowest in Italy and France, below 1%), and 3.2-14.9% in the Middle East, Africa, Asia, and Far East countries. In our cou in Türkiye, research conducted on sheep has shown that CE prevalence rates are 31.7-46.4 per cent in the Eastern Anatolia Region, 4.9 per cent in the Central Anatolia Region, 6.5 per cent in the Black Sea Region, and 22.9 per cent in the Marmara Region (Topluoğlu, 2020).

Pathogenicity and Clinical Signs

In humans, it is known that necrosis occurs due to the inflammatory reactions caused by the size of the cyst and the organs in which it is located, as well as the fibrous capsule that develops around the cyst, the mononuclear cell infiltration in the affected areas, and the obstruction of blood flow due to the pressure created. The rupture or bursting of growing cysts can lead to toxic shock, allergies and anaphylactic shock, which can be fatal (Eckert and Deplazes, 2004; Sayek, 2004; Ayaz and Tınar, 2006; Özbilgin and Kilimcioğlu, 2007; Avcıoğlu, 2013).

Hydatid cysts most commonly settle in the liver; cysts that settle in the liver parenchyma are called tumoral, while cysts that settle in the bile ducts are called biliary. Symptoms observed in patients include fever, prolonged nausea, vomiting, severe abdominal pain, jaundice due to obstruction of the bile ducts, hepatomegaly, hepatic or inferior vena cava obstruction, and portal hypertension. Additionally, when cysts found in the liver rupture due to trauma or any other cause, anaphylaxis may develop as a result of the cyst fluid coming into contact with the peritoneum (Özbilgin and Kilimcioğlu, 2007; Yalçınkaya, 2016; Ertürk et al., 2021).

Patients may experience symptoms such as Quincke's oedema, urticaria, chest pain, recurrent coughing fits, breathing difficulties, haemoptysis and dyspnoea due to cysts forming in the lungs. If cysts perforated due to trauma in the lungs open into the pleura or peritoneum, anaphylaxis may develop (Özbilgin and Kilimcioğlu, 2007; Yalçınkaya, 2016; Ertürk et al., 2021).

If cysts settle in the kidneys, albuminuria, haematuria and hydatiduria may develop. If they settle in the spleen, swelling, pain and nausea may be observed in the left side of the abdomen. Its settlement in the bones of the front, side and spine areas of the body causes pain and can lead to bone fractures with even the slightest trauma (Özbilgin and Kilimcioğlu, 2007; Yalçınkaya, 2016; Ertürk et al., 2021).

Diagnosis

The most commonly used method for diagnosing hydatid cysts (HC) in humans is ultrasound (USG). In addition, imaging techniques such as radiography, computed tomography (CT) and magnetic resonance imaging (MRI) are also used. However, these methods are generally not preferred in animals, both for economic reasons and due to the difficulties in their application; they are only used in animals of breeding value (Thompson and Lymbery, 1988; Avcıoğlu, 2013; Alemu et al., 2015; Yalçınkaya, 2016; Gonzalez et al., 2018; Topluoğlu, 2020).

Direct diagnosis can be made by microscopic examination of hooks, membranes, protoscoleces or larval sacs found in cyst material removed during surgical operations or as a result of animal necropsy. To definitively diagnose the disease, indirect haemagglutination (IHA), indirect immunofluorescence, latex agglutination, double diffusion immunoelectrophoresis, radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunoelectrotransfer blots (EITB), and immunoblotting, as well as molecular methods (PCR) (Thompson and Lymbery, 1988; Avcıoğlu, 2013; Alemu et al., 2015; Yalçınkaya, 2016; Gonzalez et al., 2018; Topluoğlu, 2020).

Treatment

It is performed using a combination of medical, percutaneous and surgical methods. Until recently, there was no established gold standard method for CE treatment; however, surgical methods were the most commonly preferred treatment option until the 1990s. Nowadays, surgical treatment methods are still prioritised, especially in complicated cases. However, due to complications that may arise during surgical interventions, such as bleeding, infection, trauma to surrounding organs and anaphylaxis, there has been an increase in the use of percutaneous treatment. Considering economic reasons and ease of application, percutaneous treatment is also recommended as a highly reliable method in addition to medical treatment (Brunetti et al., 2009; Stojkovic et al., 2009; Moro and Schantz, 2009; Cebeci et al., 2016; Tirado et al., 2018).

A meta-analysis study on the efficacy of benzimidazole-derived drugs such as albendazole, mebendazole, flubendazole and praziquantel used in CE treatment revealed that these drugs are often insufficient and that 60% of cysts remain viable at the end of the first year and 40% at the end of the second year. Additionally, complications such as hepatic toxicity, anaemia, thrombocytopenia, alopecia, and teratogenicity may also be observed during drug treatment. The guidelines published by the World Health Organisation (WHO) state that albendazole treatment should be used for liver cysts smaller than 5 cm, while for cysts larger than 5 cm, the PAIR method should be used in addition to albendazole

treatment (Brunetti et al., 2009; Stojkovic et al., 2009; Moro and Schantz, 2009; Karabulut et al., 2014; Çaycı and Tihan, 2016; Cebeci et al., 2016; Tirado et al., 2018).

Protection and Control

Controlling this disease, which is frequently seen in humans, will be possible by preventing transmission between sheep and dogs. To this end, measures such as treating dogs with antihelminthic drugs, destroying cyst-infected organs by burning or burying them, preventing dogs from accessing cyst-infected organs without being fed, ensuring that slaughter outside slaughterhouses is carried out in a controlled manner, and raising public awareness about hydatid cyst disease should be taken. As a result of efforts to eradicate CE in developed countries, the disease has been completely eradicated in Tasmania and New Zealand. China (from 18.5% to zero in dogs, from 88.8% to 5.6% in sheep), the Falkland Islands (from 1.7% to 0.1% in dogs, from 59% to 0.16% in sheep), Chile (from 70% to 5% in dogs, from 60% to 25% in sheep), Argentina (from 61% to 5% in dogs, from 61% to 2.9% in sheep), Wales, Spain and Italy (90% in dogs, 50-75% in sheep) (Narrod et al., 2012; Rabinowitz et al., 2013; Craig, 2017; Topluoğlu, 2020). Although the protection and control programmes implemented for CE disease have yielded successful results, the cycle between sheep and dogs living in the same environment continues in Türkiye.

Studies have shown that praziquantel, which is actively used in the treatment of tapeworms, breaks the life cycle in dogs. Drugs such as arecoline hydrobromide, which do not possess helminthicidal properties, are also used effectively in the treatment of canines. However, these drugs do not kill cestodes such as *Echinococcus* and *Taenia* spp., but merely facilitate their expulsion from the intestines. Therefore, they are used primarily to monitor the presence of CE and minimise its impact (Gemmell, 1990; Grove, 1990; Wei et al., 2005; Craig, 2017; Topluoğlu, 2020).

Regarding the control and eradication of CE, vaccine trials (EG95 vaccines) conducted in countries such as New Zealand, Australia, Argentina, China and Romania have reportedly achieved a success rate of 96-98 per cent (Çırak, 2004; Lightowlers, 2006; Morarıu et al., 2010; Avcıoğlu, 2013; Yalçınkaya, 2016).

Cystic Echinococcus Molecular Genotyping

It is known that genetic variability in the *Echinococcus granulosus sl*. species complex (G1-G10) affects host specificity as well as morphological, biochemical and other biological differences. Molecular studies have shown that the G1 genotype is the most common genotype, accounting for approximately 88.44% of global human CE cases, and is primarily associated with transmission via sheep. Other genotypes belonging to *E. canadensis*, such as G6 and G7, also

contribute significantly to human infections, particularly in regions where camels and goats (G6) or pigs (G7) serve as intermediate hosts. Furthermore, the identification of genotype-specific infection patterns is crucial for understanding epidemiology and optimising CE control measures, including vaccine design. For example, while the EG95 vaccine shows high efficacy against G1 strains, its protective capacity against G6 and G7 remains uncertain due to antigenic differences (Rojas et al., 2014).

The most commonly used methods for genotyping *Echinococcus granulosus* are molecular-based and mostly rely on mitochondrial DNA sequences. In particular, the amplification of the *cox1* (cytochrome c oxidase subunit 1) and *nad1* (NADH dehydrogenase subunit 1) genes by polymerase chain reaction (PCR) followed by sequence analysis provides high accuracy in distinguishing between genotypes G1-G10. Additionally, it has been reported that the *cox1* and cytochrome b (*cob*) genes are commonly used genes in genotyping. Furthermore, techniques such as restriction fragment length polymorphism (RFLP) analysis of PCR products and multiplex PCR are used for faster and more cost-effective genotyping. In some studies, nuclear genetic markers such as the ITS1 and ITS2 regions of ribosomal DNA have also been used; however, the resolution of these regions is lower compared to mitochondrial markers. These molecular methods make a significant contribution to determining the distribution of genotypes in different geographical regions and host species; thus, the epidemiology of the parasite is better understood and control strategies can be shaped accordingly (Rojas et al., 2014; Khademvatan et al., 2019; Wei et al., 2023).

The prevalence and current status of genotypes in Türkiye and worldwide

Worldwide and in Türkiye, it has been reported that the G1 and G3 genotypes are widely prevalent and are the primary cause of *Echinococcus granulosus* infections in both humans and animals. Research conducted in various regions of Türkiye to genotype *E. granulosus* revealed that the disease is prevalent in the provinces of Ağrı, Ardahan, Diyarbakır, Elâzığ, Erzurum, Malatya, Kars, Kilis, Siirt and Van, located in the Eastern Anatolia and Southeastern Anatolia regions (Ütük et al., 2008; Şimşek et al., 2011; Şimşek et al., 2011; Ütük et al., 2012; Kinkar et al., 2016; Kinkar et al., 2017; Kinkar et al., 2018; Oğuz et al., 2018; Avcıoğlu et al., 2019; Barazesh et al., 2019; Shahabi et al., 2021; Yıldız et al., 2022; Afshar, 2023), In the Aegean Region, in the provinces of Afyon, Izmir and Manisa, (Snabel et al., 2009; Boğa, 2012), In the Central Anatolia Region, in the provinces of Kırıkkale and Yozgat (Ergin et al., 2010; Gökpınar et al., 2017; Öge et al., 2017), In the Black Sea Region, in the provinces of Samsun, Ordu and Amasya (Ütük and Şimşek, 2008) and in the Thrace Region (Eryildiz and Şakra, 2012) Studies have reported that the G1 and G3 genotypes are prevalent. Recent

research conducted in different regions of Türkiye has reported the presence of the G7 genotype in addition to the G1 and G3 genotypes (Babaoğlu et al., 2018; Kesik et al., 2021; Selçuk et al., 2024).

In addition to the commonly observed G1 and G3 genotypes, the presence of the G7 genotype has been reported in a similar manner in studies conducted in the form of case presentations both worldwide and in Türkiye. According to studies conducted on wild boars, *E. granulosus s.s.* (G1 and G3) and *E. canadensis* (G7) species are predominantly observed. In a hydatid cyst sample obtained from a wild boar in Ukraine, the *E. granulosus* G7 strain was identified through molecular analysis. In a study conducted in France, *E. canadensis* (G6/G7) was identified in wild boars. Genetic analyses conducted on two wild boars in Italy detected *E. granulosus s.s.* (G1). In Spain, *E. canadensis* (G7) and *E. granulosus s.s.* (G1) have also been detected in wild boars. In Türkiye, however, prevalence and genotyping studies in pigs and wild boars have been limited in number (Babaoğlu et al., 2018; Selçuk et al., 2024).

In a study conducted in the Aydın region, the genotypes of a total of 20 *E. granulosus* isolates were determined. The majority of these isolates were identified as Genotype 1 (G1), the sheep strain, while the remainder were identified as G6/7, the pig/camel strain. The genetic data obtained points to results associated with Türkiye's geographical location. The presence of the G1 and G6/7 strains suggests that camels and wild boars may play a role in the spread of cystic echinococcosis in the region and that the risk of transmission to humans has increased. In studies conducted in the provinces of İzmir, Manisa and Siirt, the swine strain (G7) was detected for the first time. In the Thrace Region, G1 and G7 strains were identified in human isolates, marking the first report of the G7 strain in the region (Babaoğlu et al., 2018; Kesik et al., 2021).

It has been reported that the G1 and G3 genotypes identified in different regions of the world belong to different mitochondrial lineages. One of the most important factors shaping this genetic diversity and biogeographic patterns is considered to be domestication and, in particular, the increase in live animal trade between neighbouring countries (Laurimäe et al., 2019; Mehmood et al., 2020; Shahabi et al., 2021).

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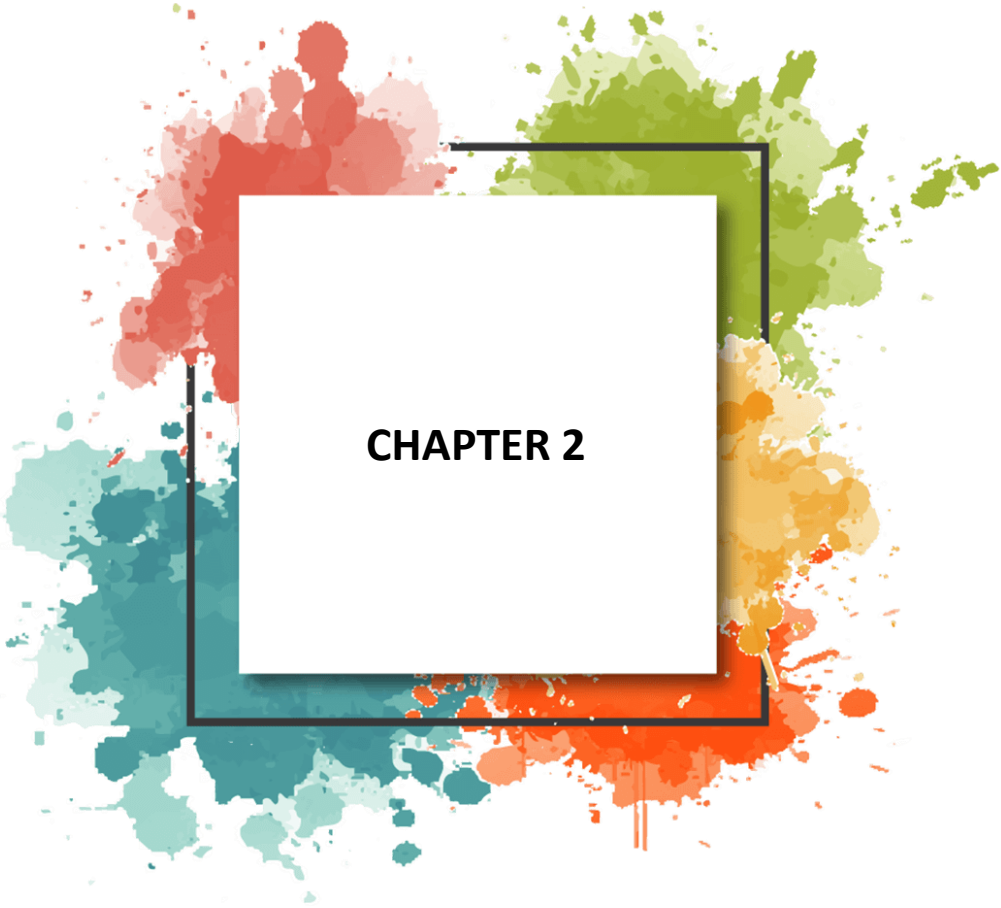
CHAPTER 2

In-Depth Understanding of Cancer Cell Biology and the Tumor Microenvironment

Betul Celik[1] & Seref Bugra Tuncer[2]

1. Cancer Cell Plasticity and Therapeutic Resistance

In recent years, remarkable progress in the field of cancer cell biology and the tumor microenvironment has deepened our understanding of the intricate mechanisms underlying cancer cell plasticity and resistance to therapy. Cancer cell plasticity describes the dynamic capacity of tumor cells to reprogram their phenotype and functional behavior in response to environmental cues and therapeutic interventions (da Silva-Diz, Lorenzo-Sanz, Bernat-Peguera, Lopez-Cerda, & Munoz, 2018). This inherent adaptability not only fuels intratumoral heterogeneity but also plays a pivotal role in the emergence of treatment resistance, highlighting its significance as a central focus in contemporary oncology research.

A prominent feature of cancer cell plasticity is the adaptive response to metabolic stressors such as hypoxia and nutrient deprivation(Paredes, Williams, & San Martin, 2021). Within the tumor microenvironment, these harsh metabolic constraints compel cancer cells to reprogram their metabolic networks to sustain survival. For example, perturbations in glucose metabolism under stress conditions have been associated with the regulatory activity of long non-coding RNAs (lncRNAs), which facilitate metabolic reprogramming essential for cellular endurance in energy-limiting states (Y. T. Tan, Lin, Li et al., 2021; Yoshida, 2015). Likewise, the ability of tumor cells to adapt to purine scarcity illustrates how fluctuations in nutrient availability can foster more aggressive malignant phenotypes, thereby opening avenues for the development of novel targeted therapeutic strategies (J. Yu, Jin, Su et al., 2025).

The dynamic interaction between cancer cells and the tumor microenvironment is equally pivotal in determining therapeutic responses (X. Guo, Song, Liu, Ou, & Guo, 2024). Evidence suggests that persistent stressors, such as the acidic milieu commonly present in solid tumors, foster adaptive mechanisms that enhance tumor cell survival and proliferation (X. Wu, Wang, Hou et al., 2025).

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Such conditions can drive alterations in the expression of key proteins that promote growth and resilience, ultimately contributing to pronounced therapeutic resistance (Koizume & Miyagi, 2025). Moreover, the immune microenvironment exerts a profound influence on treatment outcomes, as adaptive immune resistance enables tumor cells to evade immune surveillance—an emerging focus of therapeutic intervention (Castagnino, Haas, Musante et al., 2025).

Notably, the molecular pathways that underlie therapeutic resistance are becoming increasingly well defined. Mutations in critical oncogenes such as **KRAS** have been shown to activate stress-response programs that enable tumor cells to endure therapeutic pressure (Iovanna, Estaras, Grasso et al., 2025). Likewise, alterations in TP**53** are particularly significant, as these mutations are closely associated with chromosomal instability and can endow cancer cells with a proliferative advantage during chemotherapy (Hertel & Storchova, 2025). In addition, adaptive mechanisms—including the upregulation of intracellular protective factors and modifications in key signaling cascades—further enhance the capacity of tumor cells to withstand treatment (H. Li, Yang, Wang et al., 2025).

Emerging research is increasingly directed toward innovative approaches that either counteract adaptive mechanisms or harness them as therapeutic opportunities. One promising avenue involves targeting the metabolic vulnerabilities of cancer cells, exemplified by the development of enzymatic inhibitors designed to disrupt essential metabolic pathways and thereby potentiate the efficacy of conventional therapies (Hong, Tang, Zhou et al., 2025). In parallel, the integration of circulating tumor cell profiling into clinical practice holds great potential for guiding treatment modifications in real time, enabling more precise and adaptive strategies to overcome therapeutic resistance (Stana, Ben-David, Weissman, & Ram, 2025).

1.1. Cellular state transitions (epithelial–mesenchymal transition, state switching)

Cell state transitions, such as the epithelial–mesenchymal transition (EMT), play a crucial role in cancer progression and treatment resistance (Allgayer, Mahapatra, Mishra et al., 2025). During EMT, cells lose their epithelial characteristics and gain mesenchymal traits, which make them more mobile and invasive (Ribatti, Tamma, & Annese, 2020). Recent research shows that EMT is not a strict on–off switch between epithelial and mesenchymal states. Instead, it is a flexible and reversible process shaped by environmental signals (Sahai, Astsaturov, Cukierman et al., 2020). This plasticity enables cancer cells to shift between states, helping them adapt to microenvironmental stress and therapeutic pressure (Gillen, Riemondy, Amani et al., 2020).

Studies show that inflammatory signaling pathways—especially JAK/STAT in prostate cancer—play a major role in promoting lineage plasticity, which allows tumor cells to evade androgen receptor (AR) inhibition (Chan, Zaidi, Love et al., 2022). In addition, the tumor microenvironment, rich in cancer-associated fibroblasts (CAFs), drives reciprocal signaling that strengthens cellular plasticity, highlighting the importance of targeting CAFs to overcome resistance (Sahai, Astsaturov, Cukierman et al., 2020). Evidence from pediatric ependymomas further indicates that under hypoxic conditions, tumor cells can undergo EMT, demonstrating how the microenvironment actively shapes cellular plasticity and contributes to tumor progression and metastasis (Gillen, Riemondy, Amani et al., 2020).

1.2. Acquisition of stem cell–like properties

The development of stem cell–like traits in tumor cells is a key driver of therapeutic resistance and is closely linked to the cellular state transitions (El-Tanani, Rabbani, Satyam et al., 2025). Cancer stem cells (CSCs) possess distinctive features that allow them to withstand aggressive treatments such as chemotherapy and radiotherapy (Chu, Tian, Ning et al., 2024). In glioblastoma, new findings challenge the classic hierarchical view of CSCs, showing that stem-like properties can arise through epigenetic reprogramming triggered by microenvironmental cues (Guetta-Terrier, Karambizi, Akosman et al., 2023). Increasingly, histone modifications and metabolic reprogramming are being recognized as critical factors shaping the CSC landscape, offering promising avenues for targeted therapies (Y. Xu, Zhang, & Nie, 2025).

Recent studies emphasize that elements of the tumor microenvironment—such as growth factors and cytokines—play a central role in sustaining and expanding cancer stem cell (CSC) populations, thereby reinforcing their resistance to standard therapies (Bunsick, Matsukubo, & Szewczuk, 2023). Comprehensive gene expression analyses have further revealed the dynamic nature of stemness, showing that changes in critical signaling pathways can either enhance or reduce these traits during cancer progression (Das, Bhattacharya, Adhikari et al., 2024). Gaining deeper insight into these mechanisms is essential, as it opens new opportunities for therapies designed to eliminate CSCs and prevent disease relapse.

1.3. Epigenetic reprogramming and phenotypic adaptation

Epigenetic reprogramming is now widely recognized as a key driver of cancer cell adaptability and of the phenotypic diversity that underlies tumor heterogeneity (Sadida, Abdulla, Marzooqi et al., 2024). First described by Hanahan as a hallmark of cancer, this concept highlights how non-mutational epigenetic changes can profoundly shape tumor behavior and influence

therapeutic responses (Hanahan, 2022). Mechanisms such as DNA methylation and histone modifications enable cancer cells to adjust to their microenvironment and evolve under treatment pressure, underscoring their importance in resistance and disease progression (Y. Wang, Liu, Zhang et al., 2024).

Recent research shows that epigenetic reprogramming can drive phenotypic changes that promote resistance to treatment (Walker, Rentia, & Chiappinelli, 2023). In gastrointestinal cancers, for example, therapeutic outcomes are strongly influenced by the interplay between metabolic reprogramming and epigenetic modifications (X. Xu, Peng, Jiang et al., 2023). Understanding these mechanisms not only deepens our knowledge of tumor biology but also points to new treatment strategies, such as targeting specific epigenetic markers or pathways to block the malignant adaptations shaped by the tumor microenvironment (Yamada, Sankoda, & Yamada, 2025). Ongoing studies continue to highlight the critical impact of epigenetic dynamics on cancer progression and therapy resistance.

1.4. Molecular mechanisms of drug resistance

Understanding the molecular basis of drug resistance is essential for improving cancer treatment. One key mechanism involves the activation of autophagy, which tumor cells use to survive chemotherapy and targeted therapies (Lei, Tian, Teng et al., 2023). For example, overexpression of proteins such as ACTR10 has been linked to hepatocellular carcinoma (HCC) progression and resistance to tyrosine kinase inhibitors (TKIs) by enhancing autophagy, underscoring its protective role against anticancer drugs (Luo, Qin, He et al., 2024).

Genetic alterations also play a major role. The ALK L1256F mutation is associated with resistance to several ALK inhibitors, illustrating how single mutations can undermine therapeutic efficacy (Y. Yu, Wang, Wang et al., 2023). Similarly, adaptive changes in pathways such as Notch and Akt contribute to resistance, with studies highlighting the role of Notch signaling in promoting drug resistance in HCC and other cancers (X. Yang, Liu, Liang, & Sun, 2021). Innovative research approaches have provided deeper insights into the mechanisms of drug resistance. In cancers such as glioblastoma, studies highlight how complex signaling networks interact to drive resistance, with particular attention to the blood–brain barrier's role in limiting drug effectiveness (Dymova, Kuligina, & Richter, 2021). A comprehensive perspective is also crucial, as shown in high-grade serous ovarian cancer, where disrupted signaling pathways and distinct molecular markers contribute to treatment failure (R. Fu, Hu, Li et al., 2025). Exploring these mechanisms not only advances our understanding of resistance but also guides the development of new therapeutic strategies to overcome these barriers.

In summary, drug resistance arises from a complex interplay of genetic mutations, adaptive changes in signaling pathways, and the modulatory effects of the tumor microenvironment.

2. Neuronal Signaling and Cancer

Neuronal signaling is increasingly recognized as a key factor in shaping the tumor microenvironment and driving cancer progression (Nguyen, Ngoc, Choi, & Lee, 2023). Communication between the nervous system and tumors influences critical processes such as growth, metastasis, and treatment response (J. Zhao, Cheng, Yang et al., 2025). In particular, neuroimmune interactions and their signaling pathways play a central role in regulating tumor dynamics and determining therapeutic outcomes.

2.1. Interactions between the nervous system and the tumor microenvironment

Recent research has underscored the intricate relationship between neuronal signaling and tumor biology (S. Huang, Zhu, Yu, Huang, & Hu, 2025). Myeloid-derived suppressor cells (MDSCs), for example, can act as intermediaries in the communication between neural signals and cancer cells. However, it is important to note that the study by Liu et al. (M. Liu, Cao, Guo et al., 2022), while detailing the role of MDSCs in ovarian cancer, does not explicitly investigate their connection to neuronal signaling pathways, highlighting an area for future research. This highlights the importance of precision when describing the connections between neural signaling and the tumor microenvironment.

Cancer cells can also exploit neuronal signaling to promote their survival and growth. Wu et al. suggested that neuronal activity influences tumor behavior through pathways such as Wnt and MAPK, which are essential for sustaining cancer stem cell traits (Lin, Wang, Chen et al., 2022). This finding indicates that neural inputs can profoundly shape tumor properties and impact treatment responses.

The presence of nerve fibers within tumors has been linked to poorer prognoses across multiple cancer types. Studies indicate that neurogenic signaling promotes tumor aggressiveness by activating pathways that drive cell proliferation and migration (M. Liu, Cao, Guo et al., 2022). These findings underscore the influence of neuronal interactions on cancer progression and point to the therapeutic potential of targeting these pathways.

Non-coding RNAs are increasingly recognized for their role in mediating the effects of neuronal signaling on tumor cells. For example, while Fu et al. (X. Fu, Zhang, Wang, Xu, & Dong, 2024) discuss synthetic pathways and drug resistance in ovarian cancer, their study does not directly address circRNAs. As such, it may

be more appropriate to reference broader oncological research trends on non-coding RNAs to establish a clearer connection, rather than citing studies with limited relevance.

2.2. Mechanisms of perineural invasion

Perineural invasion (PNI) is a key feature in many cancers, particularly pancreatic ductal adenocarcinoma (PDAC), where tumor cells infiltrate the perineural spaces around nerves (Sun, Jiang, Liao, & Wang, 2024). This phenomenon is strongly linked to poor prognosis and contributes to both local recurrence and distant metastasis. Gaining deeper insight into the mechanisms driving PNI is crucial for developing therapeutic strategies aimed at limiting cancer progression. Recent studies show that PNI is driven by intricate interactions between tumor cells and the neural microenvironment. A key mechanism involves cholinergic signaling: Yang et al. (M. W. Yang, Tao, Jiang et al., 2020) demonstrated that PNI in PDAC alters the immune microenvironment via cholinergic pathways, underscoring the critical role of tumor–nerve communication in promoting PNI. Using genetically engineered mouse models that closely reflect human disease, the study established a direct connection between neural signaling and cancer progression.

Nerve growth factor (NGF) has been shown to increase the neuroinvasive capacity of pancreatic cancer cells. Peng et al. (Peng, Guo, Gan et al., 2022) demonstrated that NGF enhances the Warburg effect, stimulating cell proliferation and elevating the levels of exosomal miRNA-21, a molecule linked to cancer cell invasion and migration. These findings suggest that neurotrophic factors released by tumor cells can act on adjacent nerves, creating a microenvironment that supports PNI.

In addition, cellular and molecular mechanisms are crucial in driving PNI. Li et al. (J. Li, Kang, & Tang, 2021) described how neurotrophins, cytokines, and metabolites collectively promote PNI in PDAC. Their study highlighted the role of Schwann cells, which respond to tumor-derived signals and actively contribute to the invasion process. This close interaction between cancer cells and nerves represents a key pathway for the spread of pancreatic cancer.

Spatial transcriptomics studies, such as those by Lakis et al. (Lakis, Chan, Lyons et al., 2025) have shed light on specific pathways involved in PNI. Their research identified the EPHA2 receptor as a key mediator of growth cone collapse, a process critical for interactions between tumor cells and nerve structures during invasion. By regulating cellular adhesion and migration, EPHA2 exemplifies how tumors can exploit signaling pathways to infiltrate neural tissues.

Recent studies have also highlighted the role of matrix metalloproteinases (MMPs) in driving PNI. Xu et al. (X. Xu, Lu, Chen, Peng, & Ji, 2022) identified a pathway in which MMP1 influences the NT-3/TrkC axis, revealing a novel mechanism that enables tumor cells to invade along nerves. These findings emphasize the importance of proteolytic activity in facilitating tumor migration within neural structures.

In summary, perineural invasion is driven by complex interactions among tumor cells, nerve growth factors, and diverse signaling pathways.

2.3. Role of neurotransmitters in tumor progression

Neurotransmitters are increasingly recognized as important regulators in tumor biology, influencing cancer growth, metastasis, and the tumor microenvironment (TME). Through their interactions with cancer cells and immune components, neurotransmitters help establish a complex network that fosters malignant progression (Xiao, Li, Fang, Yu, & Chen, 2023).

Recent studies have shown that certain neurotransmitters can directly promote tumor aggressiveness. For example, Zhang et al. (2023) identified a gene signature linked to neurotransmitter receptors in colorectal cancer, which may serve as prognostic and therapeutic biomarkers (Zhang, Deng, Yang, Deng, & Li, 2023). Their work demonstrated that aberrant neurotransmitter signaling supports proliferation, migration, invasion, and angiogenesis, while also shaping the behavior of immune and endothelial cells within the TME.

Dopamine and norepinephrine are among the best-studied neurotransmitters in this context. Wang et al. reviewed their roles across different cancers, showing that neurotransmitter receptors regulate key processes such as tumor initiation, growth, and metastasis (X. Wang, Shi, Tian, & Yu, 2025). The presence of these receptors in cancer cells underscores the dual role of neurotransmitters—essential in normal physiology, yet capable of driving malignant transformation. Crosstalk between neurotransmitters and tumor cells can ultimately create cellular responses that favor survival and expansion.

Serotonin (5-HT) also plays diverse roles in tumor biology. Liu et al. (2025) showed that serotonin promotes liver metastasis by inducing neutrophil extracellular trap formation, linking it to immune evasion and metastatic spread (K. Liu, Zhang, Du et al., 2025). In glioblastoma, altered serotonin receptor expression has been associated with tumor growth and invasiveness, suggesting that the interaction between serotonin signaling and microenvironmental factors is central to disease progression (Abedini, Amjadi, Hedayatizadeh-Omran, Lira, & Ahangari, 2023). These findings highlight how neurotransmitter systems can shift beyond their classical neuronal roles to adopt new functions within tumors.

Another emerging concept is **neoneurogenesis**, where tumors stimulate the formation of new nerves that, in turn, release neurotransmitters to support cancer growth. Nguyen et al. (2023) described this feedback loop as a mechanism that not only sustains tumor progression but also enhances the invasive capacity of cancer cells in surrounding tissues (Nguyen, Ngoc, Choi, & Lee, 2023).

Building on these insights, therapeutic opportunities are beginning to emerge. Shi et al. (2022) reported that blocking excessive adrenergic signaling with beta-blockers can mitigate tumor progression in certain cancers (Y. Shi, Luo, Wang et al., 2022). Likewise, Jayachandran et al. (2023) emphasized the potential of modulating neuroactive compounds as a strategy to improve treatment efficacy (Jayachandran, Battaglin, Strelez et al., 2023). Together, these findings highlight neurotransmitter pathways as promising targets for innovative cancer therapies.

In summary, neurotransmitters play multifaceted roles in cancer by directly regulating tumor behavior, shaping the immune microenvironment, and contributing to metastasis. Understanding and targeting these pathways offers a unique opportunity to develop novel therapeutic approaches that can complement existing cancer treatments.

2.4. Therapeutic opportunities in neuro-oncology

Neuro-oncology has advanced considerably in recent years, offering new therapeutic opportunities for the management of brain tumors and related disorders. Progress in immunotherapy, targeted therapies, advanced imaging, artificial intelligence, and rehabilitation strategies underscores the growing potential to improve patient outcomes in this challenging field.

One of the most promising areas is the application of immunotherapy in glioblastoma, one of the most aggressive brain tumors. In a randomized phase III trial, Reardon et al. (2020) compared nivolumab, a programmed cell death protein 1 (PD-1) inhibitor, with bevacizumab, an angiogenesis inhibitor, in patients with recurrent glioblastoma. The trial specifically assessed whether nivolumab could enhance anti-tumor immune responses, and its findings underscored the therapeutic value of leveraging the immune system to combat glioblastoma, particularly in recurrent disease (Reardon, Brandes, Omuro et al., 2020).

In parallel, advances in clinical imaging have opened new avenues for diagnosis and therapy monitoring. Zhou et al. (2022) outlined consensus recommendations for magnetic resonance imaging (MRI) approaches that improve tumor characterization and monitoring. These methods provide clinicians with detailed biochemical information, thereby supporting personalized treatment strategies and enhancing patient management (J. Zhou, Zaiss, Knutsson et al., 2022).

Complementing these advances, rehabilitation strategies tailored to the needs of neuro-oncology patients are also emerging. Capozzi et al. (2023) described the implementation of an oncology rehabilitation triage clinic designed to evaluate and direct patients toward appropriate rehabilitation services. These programs aim to improve functional outcomes for individuals experiencing cognitive, mobility, and coordination challenges. Emphasizing rehabilitation reflects a broader recognition of holistic care in neuro-oncology, extending beyond tumor-directed treatments to quality-of-life improvements (Capozzi, Daun, Francis et al., 2023).

Building on diagnostic and supportive advances, the integration of artificial intelligence (AI) has further expanded therapeutic possibilities. Khalighi et al. (2024) highlighted the transformative role of AI in brain tumor diagnosis, prognosis, and precision treatment. By applying machine learning algorithms to complex datasets, AI can improve clinical decision-making and facilitate the development of more personalized and effective treatment approaches (Khalighi, Reddy, Midya et al., 2024).

At the molecular level, targeting specific pathways has also led to innovative therapeutic strategies. Desgraves et al. (2024) investigated the use of antisense oligonucleotides (ASOs) in glioblastoma and demonstrated that these agents achieve effective CNS penetration with a favorable safety profile. Their results highlight the promise of gene-targeted therapies for high-grade gliomas, particularly in cases where conventional treatments are insufficient (Desgraves, Mendez Valdez, Chandar et al., 2024).

Finally, the expansion of telemedicine during the COVID-19 pandemic has reshaped neuro-oncology care. Feldheim et al. (2023) evaluated remote consultations and showed that telemedicine can improve access to care, especially in underserved regions. While challenges remain in maintaining therapeutic relationships, telemedicine has enhanced patient engagement and is likely to remain a lasting component of neuro-oncology practice (Feldheim, Schmidt, Oster et al., 2023).

In summary, neuro-oncology now encompasses a broad range of therapeutic opportunities that combine innovative treatments with supportive care approaches. Future progress will likely be driven by the integration of immunotherapies, AI-based technologies, molecularly targeted agents, telemedicine, and personalized rehabilitation programs, all of which hold promise for significantly improving patient outcomes.

3. Metabolic Adaptation and Targeting

3.1. The Warburg effect and glycolytic dependence

At the metabolic level, the **Warburg effect** represents a hallmark of tumor biology. It refers to the tendency of cancer cells to rely on aerobic glycolysis rather than oxidative phosphorylation, even when oxygen is sufficient. This metabolic reprogramming enables cancer cells to rapidly generate ATP and accumulate lactate, thereby fueling accelerated growth and proliferation (Park, Pyun, & Park, 2020). Recognizing this phenomenon is critical for understanding cancer metabolism and highlights promising targets for therapeutic intervention.

Building on this concept, cancer cells often display increased glucose uptake and enhanced glycolytic activity compared with normal cells. Li et al. (2024) demonstrated that stabilization of c-Myc promotes glycolysis and metastasis in bladder cancer, illustrating how metabolic reprogramming can drive tumor aggressiveness. Their findings emphasized that the shift toward aerobic glycolysis is central to bladder cancer progression, contributing not only to increased invasiveness but also to the development of drug resistance (M. Li, Yu, Ju et al., 2024).

In parallel, hypoxia-inducible factor 1-alpha (HIF-1α) has been identified as another key regulator of the Warburg effect. Huang et al. (2021) showed that HIF-1α can alter the functionality of transforming growth factor-beta (TGF-β) signaling, thereby driving glucose metabolic reprogramming in non-small cell lung cancer (NSCLC). This finding underscores the importance of hypoxia and its associated signaling pathways in reinforcing glycolytic dependence, highlighting HIF-1α as a promising target for strategies designed to modulate cancer metabolism (Y. Huang, Chen, Lu et al., 2021).

The variable production of lactate and its impact on the tumor microenvironment (TME) further shape cancer progression. Wei et al. (2024) demonstrated that hypoxia enhances metastasis in gastric cancer by alleviating microRNA-mediated suppression of glycolysis. This study illustrates how metabolic shifts driven by hypoxia facilitate cancer dissemination and highlights the intricate regulatory networks underlying the Warburg effect (W. Zhou, Tang, He et al., 2024).

Furthermore, alterations in the expression and regulation of key glycolytic transporters and enzymes can profoundly influence tumor development. Nie et al. (2020) reported that O-GlcNAcylation of phosphoglycerate kinase 1 (PGK1) links glycolysis with the tricarboxylic acid (TCA) cycle, thereby promoting tumor growth. These findings emphasize the interconnectedness of metabolic pathways and their collective role in sustaining cancer cell survival and proliferation (Nie, Ju, Fan et al., 2020).

Expanding on these insights, research has also focused on therapeutic strategies that disrupt the glycolytic reliance of cancer cells. Xu et al. (2022) highlighted the potential of targeting glycolytic pathways in NSCLC, while also noting the challenges inherent to developing metabolic therapies. Glycolysis inhibitors have demonstrated efficacy in several preclinical models, underscoring the need for continued investigation into their clinical applications (J. Q. Xu, Fu, Zhang et al., 2022).

Moreover, studies on the transcriptional regulation of glycolysis-related long noncoding RNAs (lncRNAs) reveal the intricate molecular networks driving the Warburg effect. Huang et al. reviewed how lncRNAs modulate multiple facets of glycolysis, emphasizing their potential as therapeutic targets in cancer metabolism. Targeting these regulatory molecules may open new avenues for controlling tumor growth and overcoming therapeutic resistance (P. Huang, Zhu, Liang et al., 2021).

In summary, the Warburg effect and the resulting dependence on glycolysis are defining features of cancer cell metabolism. Insights into these metabolic adaptations present valuable opportunities for therapeutic intervention, with the potential to enhance treatment outcomes. Moving forward, continued investigation into the interplay between metabolic pathways and cancer biology will be essential for developing innovative and more effective therapeutic strategies.

3.2. Lipid and amino acid metabolism in cancer progression

Lipid and amino acid metabolism are vital to cancer progression, providing both energy and essential building blocks for the rapid proliferation of tumor cells. Dysregulation of these pathways not only fuels tumor aggressiveness but also creates new opportunities for therapeutic targeting.

3.2.1.Lipid Metabolism in Cancer

Lipid metabolism is profoundly reprogrammed in tumors, where cancer cells exploit both lipid synthesis and degradation to satisfy their energy demands and biosynthetic requirements. A hallmark of this reprogramming is enhanced **de novo lipogenesis**, enabling cancer cells to synthesize fatty acids from non-lipid precursors (Reardon, Brandes, Omuro et al., 2020). This metabolic adaptation is especially evident in lung cancers, where lipid metabolic alterations are tightly linked to epidermal growth factor receptor (EGFR) signaling (C. Wang, Lei, Wang et al., 2025). Understanding these shifts in lipid metabolism provides valuable insights and opens avenues for novel therapeutic strategies targeting lipid pathways in lung cancer (Wang et al., 2025).

Furthermore, Zhao et al. (2024) demonstrated that programmed cell death ligand 1 (PD-L1) expression in liver cancer cells can reprogram lipid metabolism through the EGFR/ITGB4/SREBP1c signaling axis (M. Zhao, Yuan, Yang et al., 2024). These findings suggest that metabolic signaling pathways not only drive cancer cell proliferation but also contribute to therapeutic resistance, including reduced sensitivity to agents such as cetuximab.

In breast cancer, Qian et al. (2024) reported that dysregulated fatty acid metabolism plays a pivotal role in tumor progression, with distinct lipidomic profiles correlating with hormone receptor status (Qian, Jin, He, Zhang, & Hu, 2025). These findings highlight the significance of lipid metabolism in breast cancer biology and emphasize its potential as a therapeutic target in disease management.

Moreover, Łaźniewska et al. (2023) described a dynamic interaction between sortilin and syndecan-1 in prostate cancer, showing that energy metabolism in castration-resistant prostate cancer (CRPC) is strongly dependent on lipid sources (Lazniewska, Li, Johnson et al., 2023). This metabolic reliance was linked to enhanced tumor migration and metastasis, underscoring the need for therapeutic strategies that disrupt lipid metabolism as a means to limit cancer progression.

3.2.2. Amino Acid Metabolism in Cancer

Amino acid metabolism also plays a critical role in cancer progression, with tumor cells frequently reprogramming amino acid utilization to sustain growth and survival. One well-documented example is glutamine metabolism, which provides essential support for the tricarboxylic acid (TCA) cycle and multiple biosynthetic pathways. As highlighted by Wang et al. (2022), many cancer cells display a form of “glutamine addiction,” making this pathway an especially attractive target for therapeutic (G. Wang, Qiu, Xing et al., 2022).

Additionally, Kim et al. (2023) reviewed how amino acid availability shapes the tumor microenvironment (TME), emphasizing that metabolic rewiring of amino acids can profoundly influence cancer progression (D. H. Kim, Song, & Yim, 2023). As a result, targeting amino acid metabolism has become an area of growing interest, with the potential to exploit tumor-specific metabolic dependencies for therapeutic benefit.

3.3. Metabolic competition within the tumor microenvironment

Metabolic competition in the tumor microenvironment (TME) plays a crucial role in cancer progression and immune regulation (Q. Wang, Shao, Zhang et al., 2023). Within tumors, cancer cells and immune cells compete for the same nutrients, often depriving immune cells and limiting their effectiveness (R. Shi,

Tang, & Miao, 2020). This nutrient rivalry not only fuels tumor growth but also reduces the success of immunotherapies.

A key focus of research is how tumor cells adapt their metabolism to outcompete immune cells for vital resources. Fatima et al. (2023) noted that cancer cells frequently display heightened glycolysis and other metabolic adaptations, enabling rapid proliferation while depleting essential nutrients such as glucose and amino acids from the TME (Fatima, Abonofal, & Stephen, 2023).

Moreover, elevated lactate production by tumor cells creates an acidic environment that suppresses infiltrating lymphocytes, including T cells and natural killer (NK) cells (J. X. Wang, Choi, Niu et al., 2020). This underscores how tumor metabolism actively shapes the immune landscape and highlights its potential as a therapeutic target. The availability of specific amino acids is also critical for effective immune function. Elia and Haigis (2021) reported that tumor cells often manipulate amino acid metabolism to limit nutrient access, thereby impairing immune activity within the TME (Elia & Haigis, 2021). For instance, depletion of cysteine and glutamine has been associated with T cell dysfunction, allowing tumors to evade immune surveillance. These findings emphasize the therapeutic importance of restoring nutrient availability to enhance immune responses and improve immunotherapy efficacy.

Lipid metabolism further contributes to immune regulation in the TME. Wu et al. (2025) demonstrated that alterations in lipid metabolism foster an immunosuppressive microenvironment (Y. Wu, Song, Su et al., 2025). By modifying lipid availability, cancer cells can shift immune cell populations toward those that support tumor progression, while suppressing effector immune responses.

The crosstalk between tumor and immune cell metabolism adds another layer of complexity. Yang et al. (2024) showed that metabolites released by tumor cells reprogram T cells within the TME, leading to the upregulation of fatty acid oxidation genes and the downregulation of glycolytic (G. Yang, Cheng, Xu et al., 2024). These alterations impair T cell function and weaken anti-tumor immunity. A better understanding of these metabolic dynamics may inform strategies to optimize immunotherapy.

Targeting these metabolic pathways presents promising therapeutic opportunities. Faubert et al. (2020) demonstrated that inhibiting glycolysis in tumor cells can relieve metabolic pressure on immune cells, restoring their anti-tumor activity (Faubert, Solmonson, & DeBerardinis, 2020). In addition, strategies designed to modulate the TME—such as employing metabolic inhibitors or optimizing nutrient availability—have shown potential in preclinical studies to enhance the effectiveness of immune checkpoint inhibitors (Fatima,

Abonofal, & Stephen, 2023). **In summary,** metabolic competition within the TME is a major determinant of both cancer progression and immune suppression. The battle for nutrients between tumor and immune cells frequently compromises immune function, underscoring the need for therapies that directly target these interactions. By addressing the adaptive metabolic programs of both cancer and immune cells, new therapeutic strategies can be developed to strengthen anti-tumor immunity and improve patient outcomes.

3.4. Therapeutic manipulation of metabolic pathways

Growing recognition of the importance of metabolic pathways in cancer progression has spurred the development of innovative therapeutic strategies designed to manipulate these processes. By exploiting metabolic vulnerabilities, researchers aim to improve treatment outcomes and address persistent challenges such as drug resistance. Approaches to metabolic therapy include inhibiting key enzymes, modulating nutrient availability, and leveraging the distinct metabolic features that differentiate cancer cells from normal cells.

3.4.1 Targeting Glycolysis and Lipid Metabolism

One widely studied strategy is the inhibition of glycolytic enzymes, a key approach for targeting the Warburg effect that characterizes many cancer cells. Stine et al. (2021) reviewed several methods for disrupting cancer metabolism, highlighting the promise of glycolytic inhibition in suppressing tumor growth (Stine, Schug, Salvino, & Dang, 2022). Compounds such as 2-deoxyglucose (2-DG) have been investigated for their ability to reduce glucose utilization in tumor cells, thereby inducing apoptosis and enhancing the efficacy of conventional therapies.

In parallel, lipid metabolism has emerged as an important therapeutic target. Wang et al. (2023) demonstrated that altering lipid metabolism can help overcome cancer drug resistance, suggesting that the metabolic shift toward greater dependence on fatty acids rather than glucose may be exploited therapeutically (Z. Wang, Wang, Li et al., 2023). For instance, inhibitors of fatty acid synthase (FASN)—an enzyme frequently overexpressed in multiple cancers—have shown encouraging results in both preclinical and clinical studies. Such interventions highlight the potential of targeting lipid metabolism to impair tumor viability and limit metastasis.

3.4.2.Dysregulation of Amino Acid Metabolism

Amino acids are essential for tumor growth, and many cancer cells reprogram amino acid metabolism to meet their elevated nitrogen requirements for protein synthesis and nucleotide production. Among these, metabolites derived from branched-chain amino acids (BCAAs), such as isoleucine and leucine, have

attracted considerable attention for their roles in cancer progression. Sivanand and Heiden (2020) emphasized that targeting BCAA metabolism could profoundly impact both cancer cell proliferation and the tumor microenvironment (Sivanand & Vander Heiden, 2020). Therapeutic strategies that inhibit enzymes involved in BCAA metabolism may suppress tumor growth while sparing normal cells, offering the potential for more selective and less toxic treatment options.

Moreover, glutamine metabolism has gained significant attention as a promising therapeutic target. Jin et al. (2023) reviewed how glutamine catabolism is essential for sustaining tumor proliferation and survival, particularly in high-grade malignancies. Inhibiting glutaminase—the enzyme that converts glutamine to glutamate—has demonstrated strong potential in preclinical models, resulting in reduced tumor growth and increased sensitivity to conventional therapies (Jin, Byun, Choi, & Park, 2023).

3.4.3. Exploiting Metabolic Competition

The competitive nature of nutrient utilization within the tumor microenvironment further highlights the therapeutic promise of targeting metabolic pathways. Mukha et al. (2021) emphasized that disrupting tumor metabolic adaptations can increase radiosensitivity and enhance the effectiveness of chemotherapy by depriving cancer cells of critical nutrients (Mukha, Kahya, Linge et al., 2021). For example, manipulating glucose or amino acid availability—whether through dietary interventions or pharmacological approaches—can shift the balance of nutrient competition between tumor and immune cells, potentially restoring effective anti-tumor immune responses.

In addition to direct interventions in metabolic pathways, hybrid strategies, which combine metabolic therapies with immunotherapies, are gaining traction. For instance, the study by Zheng et al. discusses how metabolic modifications can alter immune responses, potentially enhancing the effectiveness of checkpoint inhibitors and other immunotherapies in treating tumors (Zheng, Song, & Zhang, 2021). By modifying the metabolic landscape of tumors, it may be possible to enhance the infiltration and activity of T cells, thereby improving therapeutic outcomes.

4. Mechanobiology

4.1. Extracellular matrix stiffness and tumor biology

The interaction between extracellular matrix (ECM) stiffness and tumor biology is a central theme in cancer mechanobiology. The physical properties of the ECM, particularly its rigidity, strongly influence tumor behavior, including growth, invasion, and treatment response.

In solid tumors, the ECM undergoes structural and biochemical remodeling that increases stiffness. This often results from collagen deposition and cross-linking, processes that make the tumor microenvironment more rigid. Such stiffening is closely linked to aggressive cancer behavior. For example, in glioblastoma, greater matrix stiffness has been associated with higher malignancy and worse patient survival (Amereh, Seyfoori, Dallinger et al., 2023; Umesh, Rape, Ulrich, & Kumar, 2014) . These changes are largely driven by tumor-associated fibroblasts and matrix metalloproteinases (MMPs). Elevated levels of MMP2 and MMP9 facilitate tissue remodeling, reinforcing stiffness and promoting a cycle of tumor invasion (Shou, Teo, Li et al., 2023).

At the cellular level, mechanosensitive pathways mediate the effects of ECM stiffness. Integrins, when engaged with a rigid ECM, trigger intracellular signaling that promotes cancer cell proliferation and migration (Deng, Zhao, Kong et al., 2022). Similarly, the transcriptional regulator YAP (Yes-associated protein) is activated under stiff conditions, translocating to the nucleus and upregulating genes that drive proliferation and epithelial–mesenchymal transition (EMT), key steps in metastasis (Ishihara & Haga, 2022; Lv, Wang, Li, & Zhao, 2021). This feedback loop positions ECM rigidity as a powerful regulator of tumor dynamics and therapy resistance (Jiang, Zhang, Wang et al., 2022; Mai, Lin, Lin, Zhao, & Cui, 2024).

Beyond growth, ECM stiffness also contributes to chemoresistance. Studies show that stiffer matrices can reduce the sensitivity of tumor cells to chemotherapy, such as doxorubicin in Breast In addition, tumors in stiff environments often secrete ECM-degrading enzymes to facilitate invasion, demonstrating how cancer adapts to mechanical stress (Piersma, Hayward, & Weaver, 2020).

Recent advances in imaging technologies, including MRI elastography, now allow clinicians to assess ECM stiffness as a potential biomarker. Increased tumor rigidity has been correlated with poor prognosis and aggressive behavior in cancers such as hepatocellular carcinoma and breast cancer (Chen, Wu, Zhang et al., 2024; Zhong, Long, Chen et al., 2023). These insights highlight new opportunities for therapies aimed at modulating ECM stiffness, intending to improve patient outcomes (Jiang, Zhang, Wang et al., 2022).

In conclusion, ECM stiffness is a critical regulator of tumor biology, shaping how cancers grow, invade, and respond to treatment. Its interaction with key signaling pathways underscores the importance of mechanobiology in cancer research and points to new therapeutic strategies that target the tumor's physical environment.

4.2. Mechanotransduction pathways (YAP/TAZ, integrin signaling)

Mechanotransduction pathways, particularly those involving Yes-associated protein (YAP) and the transcriptional coactivator with PDZ-binding motif (TAZ), are central to how cells sense and respond to mechanical stimuli. These pathways are critical in cancer biology because they mediate the influence of extracellular matrix (ECM) properties, such as stiffness, on key cellular behaviors including proliferation, migration, and invasion.

YAP and TAZ act as mechanosensitive signaling molecules that respond to ECM stiffness and other physical cues. Operating downstream of integrin signaling, they convert mechanical inputs into biochemical responses that regulate gene expression. Increased ECM stiffness typically leads to elevated YAP/TAZ activity, which promotes cell proliferation, migration, and other traits that drive cancer progression (H. Li, Raghunathan, Stamer, Ganapathy, & Herberg, 2022; Piccolo, Panciera, Contessotto, & Cordenonsi, 2023). Beyond cancer, YAP/TAZ signaling also plays fundamental roles in embryogenesis, tissue repair, and regeneration.

Integrins serve as essential mechanosensors in this process. By anchoring cells to the ECM, integrins transmit external mechanical signals into intracellular signaling cascades that regulate YAP/TAZ activity (Piccolo, Panciera, Contessotto, & Cordenonsi, 2023). Integrin β1, in particular, has been identified as a key component in mechanotransduction, enabling cells to respond to physical forces while maintaining tissue homeostasis. Activation of integrin-mediated pathways stimulates Rho GTPases and cytoskeletal remodeling, which in turn determine YAP/TAZ nuclear localization and transcriptional activity (Q. Zhou, Lyu, Bertrand et al., 2021).

Emerging research highlights the complex regulatory networks involving YAP/TAZ. For example, stiff ECM environments not only activate YAP/TAZ but also reorganize the cytoskeleton through integrin signaling and Rac1 activation (Y. Guo, Mei, Huang et al., 2022). This suggests a feedback loop in which mechanical cues alter cytoskeletal dynamics, which then amplify YAP/TAZ signaling, further reinforcing tumor-promoting behavior.

The mechanosensory roles of YAP/TAZ also carry significant therapeutic implications. Acting both as sensors of mechanical stress and as transcriptional regulators, YAP/TAZ contribute to tumor progression and have been linked to the development of drug resistance. Consequently, disrupting YAP/TAZ-driven pathways has been proposed as a therapeutic strategy to improve treatment efficacy (Lee, Kang, Shin et al., 2021; Piccolo, Panciera, Contessotto, & Cordenonsi, 2023). The interplay between ECM stiffness, integrin signaling, and

YAP/TAZ activity underscores the importance of mechanotransduction as both a driver of cancer biology and a potential therapeutic target.

In conclusion, YAP/TAZ-mediated mechanotransduction pathways are crucial in translating mechanical cues from the tumor microenvironment into molecular responses that shape cancer cell behavior. By integrating ECM signals through integrins and cytoskeletal regulation, these pathways not only influence tumor growth and metastasis but also affect treatment outcomes. Targeting YAP/TAZ and their associated signaling networks offers promising opportunities for innovative cancer therapies.

4.3. Mechanical forces in tumor progression and metastasis

Mechanical forces are critical regulators of tumor progression and metastasis, shaping cancer cell behavior in multiple ways (Q. Liu, Luo, Ju, & Song, 2020). Within the tumor microenvironment (TME), a range of mechanical stresses—including compressive stress, interstitial fluid pressure, and shear stress—interact to modulate cellular functions essential for malignancy (M. Tan, Song, Zhao, & Du, 2024).

A central mechanism by which mechanical forces drive tumor progression is through their influence on cell–ECM interactions (W. B. Huang, Lai, Long et al., 2025). As tumors expand, they remodel the extracellular matrix (ECM), leading to increased stiffness. This stiffening has been directly associated with enhanced cell proliferation, migration, and invasion, as well as with greater metastatic potential (Ge, Tian, Pei, Tan, & Pei, 2021). ECM mechanics also impact cellular energetics and metabolism, contributing to cancer cell survival and adaptability (O. H. Kim, Tulip, Kang, Chang, & Lee, 2025).

Compressive stress, in particular, has been shown to promote glioblastoma progression through pathways involving mechanosensitive ion channels such as Piezo1 (O. H. Kim, Tulip, Kang, Chang, & Lee, 2025). This signaling enables tumor cells to adapt to pressure-induced stress, enhancing their invasive behavior. Similarly, the physical context of the TME supports metastatic processes: circulating tumor cells (CTCs) that withstand shear forces in the bloodstream and form stable adhesions have higher survival rates and greater metastatic potential (Strelez, Chilakala, Ghaffarian et al., 2021).

Advances in microfluidic technologies have provided new insights into these dynamics by recreating physiological conditions encountered by CTCs in vivo. Such systems have revealed that fluid shear forces play a decisive role in determining whether tumor cells survive and extravasate at distant sites (Strelez, Chilakala, Ghaffarian et al., 2021). In parallel, tumor-derived exosomes reshape the TME at both primary and metastatic sites, further facilitating dissemination (Bai, Wang, Wang et al., 2022).

Mechanical cues also converge on biochemical signaling pathways. Increased mechanical stress can activate YAP/TAZ, which drives transcriptional programs linked to migration, invasion, and survival (Bao, Kong, Ja et al., 2023). Cancer-associated fibroblasts (CAFs) amplify these effects by remodeling the ECM, producing a denser and stiffer microenvironment that fosters tumor progression (Bates, Libring, & Reinhart-King, 2023). The interplay between CAFs, ECM remodeling, and cancer cells illustrates how mechanical forces create a feedback loop that promotes metastasis.

Moreover, tumor cells are subjected to multiple overlapping forces—including compressive stress from the primary tumor and shear stress from circulation—which collectively shape invasive and metastatic behavior (M. Tan, Song, Zhao, & Du, 2024). These mechanical forces act as modulators of biochemical signaling, converting mechanical cues into functional cellular responses that enhance tumor aggressiveness.

In summary, mechanical forces profoundly influence tumor biology by integrating physical and biochemical cues within the TME. By altering cancer cell interactions with the ECM, shaping metabolic states, and activating mechanotransduction pathways, these forces drive tumor growth, invasion, and metastasis. A deeper understanding of these mechanisms offers new therapeutic opportunities, particularly strategies aimed at targeting mechanosensitive pathways or modifying the mechanical properties of the TME to limit cancer progression.

4.4. Targeting mechanobiological pathways in cancer therapy

Targeting mechanobiological pathways has emerged as a promising approach to improving cancer treatment efficacy and overcoming drug resistance (Lei, Tian, Teng et al., 2023). The interplay between mechanical cues, cellular behavior, and tumor progression highlights the therapeutic potential of integrating mechanobiology into oncology. Current strategies focus on components such as extracellular matrix (ECM) stiffness, integrin signaling, and mechanotransduction pathways involving YAP/TAZ to suppress tumor growth and metastasis.

The ECM plays a pivotal role in mechanotransduction, shaping cancer cell behavior throughout tumor progression. As noted by Huang et al. (2021), understanding ECM dysregulation within the tumor microenvironment (TME) enables the identification of therapeutic targets that can modulate tumor aggressiveness (J. Huang, Zhang, Wan et al., 2021). Agents designed to normalize ECM composition or stiffness may interrupt feedback loops that promote proliferation and invasion, thereby enhancing treatment outcomes.

Integrins act as key receptors that transmit mechanical forces into intracellular signals. By activating pathways that converge on YAP/TAZ, integrins promote tumor progression. Slack et al. (2021) discussed the potential of integrin inhibitors as therapeutic agents that disrupt these pathways and reduce metastatic behavior (Slack, Macdonald, Roper, Jenkins, & Hatley, 2022). Several integrin-targeted therapies have already demonstrated efficacy, and ongoing research continues to identify new integrins that could be exploited clinically.

YAP and TAZ are central mediators of mechanotransduction and strongly influence tumor cell motility and invasiveness. Overactivation of YAP/TAZ has been linked to enhanced migration and metastasis (Benedetti, Turco, Gallo et al., 2024). Targeting these molecules—directly or indirectly through pathways such as AMPK—represents a unique therapeutic strategy. Modulators that alter YAP/TAZ localization between the cytoplasm and nucleus further expand treatment options by controlling their transcriptional activity (Shihan, Sharma, Cable et al., 2024).

The development of novel **mechanotherapies** that harness biomechanical properties also shows great promise. Wu et al. (2025) proposed biomechanical regulation of tumor nanotherapeutics as an innovative strategy to disrupt mechanical signaling and enhance treatment response (X. Wu, Fei, Shen et al., 2025). Smart biomaterials capable of modulating the TME may improve the effectiveness of conventional therapies, while force-responsive drug delivery systems could optimize anticancer efficacy and safety (Singuru, Amouzadeh Tabrizi, Bhattacharyya, Ali, & You, 2025). Mechanical stimuli–driven therapies, such as ultrasound and magnetic field–based approaches, are another emerging frontier. These modalities can increase tumor tissue permeability, facilitating drug uptake and targeted delivery (An, Hong, Won et al., 2023). Integration with advanced imaging and nanotechnology further refines their potential, enabling real-time monitoring and adaptive treatment tailored to the mechanical characteristics of individual tumors.

In conclusion, targeting mechanobiological pathways represents a multifaceted and innovative approach to cancer therapy. By focusing on ECM remodeling, integrin inhibition, and YAP/TAZ modulation, as well as emerging biomechanical and physical therapies, researchers are paving the way toward treatments that enhance efficacy, overcome resistance, and fundamentally reshape the therapeutic landscape of oncology.

5. Conclusion and Future Perspectives

Over the past decade, research into cancer metabolism, mechanotransduction, and the tumor microenvironment (TME) has revealed how deeply these processes shape tumor growth, metastasis, and treatment resistance. Key themes include

metabolic reprogramming through glycolysis, lipid and amino acid utilization, competition for nutrients within the TME, and the impact of ECM stiffness and mechanobiological pathways such as integrin–YAP/TAZ signaling. Collectively, these findings underscore that cancer progression is not solely driven by genetic alterations but also by dynamic biochemical and biomechanical interactions.

From a translational perspective, the therapeutic opportunities are profound. Metabolic inhibitors, ECM-modifying agents, integrin antagonists, and YAP/TAZ modulators—especially when combined with immunotherapies or nanomedicine approaches—hold potential to enhance current treatments and overcome drug resistance. Physical therapies, including ultrasound, magnetic fields, and smart biomaterials, further expand the therapeutic arsenal by directly targeting the mechanical vulnerabilities of tumors.

Despite this progress, many open questions remain. How can metabolic and mechanobiological therapies be integrated into existing treatment regimens without excessive toxicity? What biomarkers best predict which patients will respond to these interventions? Can advanced imaging and liquid biopsy techniques provide real-time monitoring of metabolic and mechanical changes within the TME? Addressing these questions will be critical to translating mechanobiology and metabolism research into clinical benefit.

In the future, combining metabolic and mechanobiological targeting strategies with precision medicine approaches may redefine how we manage aggressive tumors. A deeper integration of molecular biology, biophysics, and clinical oncology will be essential to transform these insights into durable therapies that improve survival and quality of life for cancer patients.

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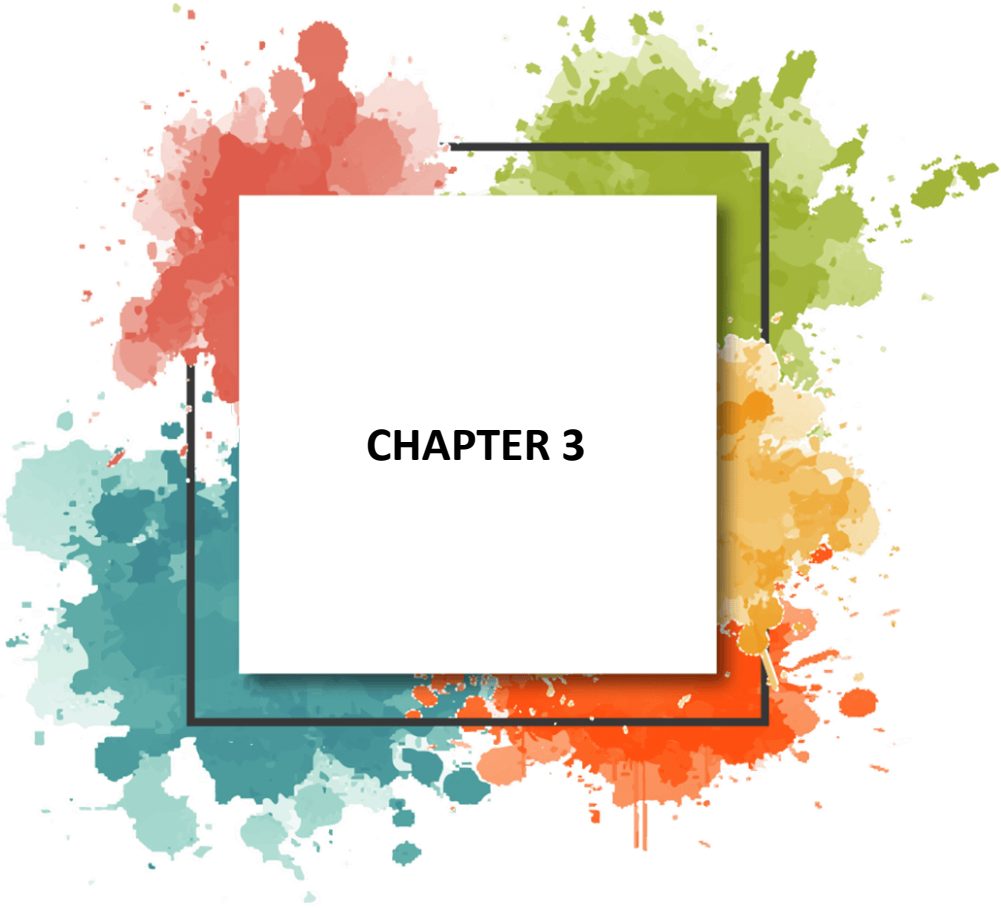
CHAPTER 3

Bispecific T-Cell Engagers (BiTEs): Mechanisms, Clinical Advances, and Future Perspectives

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1. Bispecific T-Cell Engagers (BiTEs)

Over the past six decades, cancer therapy has undergone a paradigm shift. The traditional triad of surgery, radiation, and chemotherapy has been augmented by immunotherapy, which seeks to exploit the immune system's intrinsic capacity to eliminate malignant cells. Within immunotherapy, a diverse array of strategies has been developed to enhance anti-tumor immunity. Among these, immune effector cell retargeting stands out as a promising approach: cytotoxic T, NK cells, and macrophages can be redirected toward tumor cells via specific activating receptors, enabling precise tumor eradication with a level of specificity far beyond that of conventional therapies.

Multiple platforms have been engineered to redirect T-cells toward cancer cells. These range from cell-based therapies, such as chimeric antigen receptor (CAR) T-cell therapies, to a rapidly growing class of antibody-derived molecules that passively bridge effector and target cells. These molecules, known as bispecific antibodies (BsAbs), differ from conventional antibodies in their dual specificity, as they simultaneously bind to two distinct antigens, thereby facilitating direct cell–cell interactions. In recent decades, bispecific antibodies have been extensively investigated for the treatment of hematologic malignancies, with BiTEs emerging as one of the leading formats in clinical development. These molecules are designed to bind a TAA on one arm and a T-cell–specific molecule, most commonly CD3, on the other, thereby facilitating a direct CD3×TAA interaction that redirects T-cells toward tumor cells. This interaction establishes an immunological synapse, activating T-cells to release cytotoxic granules and inflammatory cytokines, thereby inducing tumor cell apoptosis. Because cytotoxicity requires dual binding, BiTEs selectively target TAA-expressing cells, minimizing off-target effects. The concept originated in

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the early 1960s, with the prototype described in 1985. After the FDA approved the prototypical CD3/CD19 BiTE blinatumomab in 2014 for patients with relapsed or B-cell precursor B-ALL, the pace of BiTE research and development in hematologic malignancies increased dramatically.

Today, various BiTE designs are available. The earliest example, blinatumomab, is constructed from two scFvs joined together by a flexible peptide. Shortly afterward, IgG-like BiTEs were developed; for instance, teclistamab has gained approval in the U.S. and Europe for treating multiple myeloma, whereas talquetamab achieved a 70% overall response rate in heavily pretreated multiple myeloma patients during phase 1 studies. Another innovative approach involves immune mobilizing monoclonal T-cell receptors (ImmTACs), which use engineered T-cell receptor (TCR) components to target intracellular antigens. The ImmTAC agent tebentafusp demonstrated a survival advantage in metastatic uveal melanoma over conventional therapy and is now approved in both the U.S. and EU.

Despite these advances, BiTE therapies face significant challenges. While blinatumomab is clinically approved for B-ALL, adverse events such as neurotoxicity and cytokine release syndrome (CRS) remain notable, albeit generally manageable. Moreover, many patients fail to respond, and approximately half of initial responders relapse. This reflects the fact that BiTE-mediated oncolysis constitutes only one step in the cancer–immunity cycle; defects at other stages can limit therapeutic efficacy. Since BiTEs depend on the patient's own T-cell function, factors like T-cell exhaustion or a TME can reduce their therapeutic effectiveness. These barriers may be overcome by immune checkpoint inhibitors (ICIs), offering a rationale for combination strategies to enhance clinical outcomes and extend BiTE efficacy beyond hematologic malignancies to solid tumors.

The transformative potential of T-cell–based immunotherapies was first demonstrated by ICIs, which block inhibitory receptors such as CTLA-4 and PD-1 to unleash anti-tumor T-cell activity. Yet, despite their success in malignancies like melanoma, response rates remain limited due to low tumor immunogenicity—as many TAAs derive from self-proteins inducing central tolerance—and MHC class I downregulation, which hinders T-cell infiltration and generates "cold" tumor phenotypes. Overcoming these barriers remains critical for expanding the clinical utility of BiTEs across cancer types.

BiTEs combine high antigen specificity with potent cytotoxicity, offering a novel approach for cancer immunotherapy. Despite clinical success in hematologic malignancies, limitations include immune evasion mechanisms, tumor microenvironment–mediated suppression, and reduced efficacy in solid tumors. Combination strategies with immune checkpoint inhibitors or other

immunomodulatory agents are under investigation to enhance therapeutic outcomes.

2. Structural Biology of BsAbs and BiTE Formats

2.1. Classification and Overview of BsAbs

BsAbs can be grouped into three primary types according to their binding targets:

One group binds both a tumor-associated antigen (TAA) and an immune-related molecule, while the other targets two immune system components. BiTEs mainly belong to the latter group, as they are engineered to link CD3 on T-cells with a TAA expressed on tumor cells. Bispecific antibodies (BsAbs) represent an advancement over traditional monoclonal antibodies. From a structural perspective, they are generally categorized into two main types: those based on IgG and those built from Fv domains.

2.2. IgG-Based BsAbs

IgG-based bispecific antibodies closely mimic the architecture of natural antibodies. Their assembly usually involves pairing half-antibodies originating from separate monoclonal antibody sources. Advanced engineering methods such as DuoBody and the KiH approach have been developed to facilitate their production.

When selecting IgG subclasses, IgG2 and IgG4 are generally preferred over IgG1 because IgG1 antibodies may mediate elimination of activated T-cells through antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC).

The DuoBody platform (Genmab) generates bispecific antibodies through the swapping of half-antibody molecules between two parental IgGs. Specific mutations in the CH domains promote heterodimer formation by enhancing interactions between non-identical chains.

KiH technology (Roche) promotes heterodimerization by introducing complementary “knob” and “hole” mutations into CH3 domains.

CrossMab technology, derived from KiH, solves the light chain mispairing problem by swapping the CH1 domain of the heavy chain with the constant domain of the light chain (CL).

XmAb platform (Xencor) allows production of BsAbs with Fc domains nearly identical to natural antibodies, enabling robust immune effector recruitment.

Compared to Fv-based constructs, IgG-based BsAbs display longer serum half-lives due to their larger size and decreased renal clearance. The presence of

crystallizable fragment (Fc) domains improves solubility, stability, and immune effector recruitment via ADCC and CDC mechanisms. However, IgG-based BsAbs face challenges, including reduced tumor tissue penetration because of their larger molecular weights and the need for more complex production technologies.

2.3. **Fv-Based BsAbs and the BiTE Platform**

Fv-based BsAbs generally consist of two scFvs connected by a flexible peptide. Their relatively short half-life necessitates continuous infusion, limiting broader clinical use.

The BiTE platform is the prototypical Fv-based BsAb technology. Other platforms include: Single-chain diabodies, DARTs, and TandAbs.

BiTE molecules (Micromet) are constructed by connecting two scFvs with a short peptide linker.

DART molecules (MacroGenics) consist of engineered heterodimeric scFvs with swapped VH domains to improve stability.

TandAbs (Affimed) use four variable domains to bind CD3 and TAAs simultaneously, enabling dual engagement of T-cells and tumor cells.

2.4. Mechanism of Action of BiTEs

Human antibodies typically bind antigens with much higher affinity than T-cell receptors (TCRs). BsAbs exploit this property by simultaneously targeting two distinct antigens—one on immune effector cells and one on tumor cells.

BiTEs link two scFvs: one for CD3ε and one for a tumor antigen.

Unlike physiological TCR signaling, which requires peptide-MHC binding plus co-stimulatory molecules such as CD28, BiTEs bypass MHC restriction and TCR specificity by directly engaging CD3 and TAAs. This allows T-cells to be redirected toward tumors even when MHC expression is downregulated or tumor immunogenicity is low.

When BiTEs engage their targets, T-cells form an artificial immunological synapse, releasing cytotoxic granules (granzymes and perforin) to lyse tumor cells. These T cells also secrete cytokines such as TNF-α, IFN-γ, IL-4, IL-6, and IL-10 proliferate robustly. Remarkably, each T-cell can sequentially kill multiple tumor cells, demonstrating potent cytotoxicity even at very low BiTE concentrations (10–100 picograms per milliliter) and minimal effector-to-target ratios (less than 1:90).

3. BiTE Resistance Mechanisms and Combinatorial Strategies with Checkpoint Inhibitors

3.1. Immune Checkpoints in BiTE Resistance

Effective BiTE therapy requires the presence of endogenous T-cells capable of being redirected toward tumor killing. However, sustained T-cell activation frequently induces immune checkpoint upregulation, particularly PD-1 and CTLA-4, resulting in T-cell exhaustion and immune suppression.

Notably, PD-L1 upregulation has been implicated in blinatumomab resistance: case studies demonstrated PD-L1 expression rising from 2% before therapy to 40% after therapy in non-responders.

One proposed mechanism is that IFN-γ and other proinflammatory cytokines secreted by activated T-cells drive PD-L1 upregulation on tumor cells. Blocking IFN-γ signaling partially reverses this phenomenon. Similarly, blinatumomab has been shown to induce CTLA-4, TIM-3, and LAG-3 expression, further dampening T-cell activity in relapsed or refractory disease.

3.2. Preclinical Evidence for Combination Therapies

Multiple preclinical studies have shown synergistic antitumor activity when checkpoint inhibitors (CPIs) are combined with BiTEs:

CD3xHER2 BiTE: PD-L1 blockade combined with BiTEs yielded complete response rates of 82% in HER2+ mouse models versus 43% with BiTE monotherapy.

CD3xCEA BiTE: In vitro and in vivo studies demonstrated that anti-PD-1/PD-L1 therapy enhances T-cell cytotoxicity and reverses T-cell anergy when administered early.

Other BiTEs: Preclinical data support combining CPIs with BiTEs targeting Trop-2, CEACAM5, GUCY2C, CD33, FLT3, and gpA33, as well as ImmTAC molecules such as NY-ESO-1 ImmTAC in lung cancer models.

CTLA-4 Blockade: Anti-CTLA-4 antibodies moderately enhanced tumor killing and survival in murine models following CD3xEpCAM BiTE treatment.

3.3. Early Clinical Evidence

Promising preclinical findings have led to early-phase clinical trials exploring BiTE–CPI combinations:

Blinatumomab + Nivolumab (anti–PD-1): Phase I data (NCT02879695) showed complete molecular remission in 4 of 5 relapsed/refractory B-ALL patients with manageable safety profiles.

Blinatumomab + Pembrolizumab (anti–PD-1): Early-phase I/II results (NCT03160079) reported complete remission in 2 of 4 evaluable patients without unexpected toxicities.

CD3xCEA BiTE + Atezolizumab (anti–PD-L1): Ongoing Phase I trials (NCT02650713) suggest improved efficacy over monotherapy without additional toxicity concerns.

4. Molecular Mechanisms of BiTEs and Tumor Immune Escape Strategies

4.1. Direct Activation of T-Cells by BiTEs

BiTEs are designed to simultaneously bind TAAs on cancer cells and the CD3 receptor complex on T-cells. By directly linking cytotoxic T lymphocytes to malignant cells, BiTEs bypass the need for conventional antigen presentation and costimulatory signaling pathways, leading to rapid and potent T-cell activation.

4.2. Cytotoxic Effects at the Cellular Level

Once binding occurs, T-cells establish an immunological synapse with the tumor cells, leading to the secretion of cytotoxic molecules like perforin and granzymes. These mediators drive apoptosis in the cancer cells. Moreover, the release of cytokines enhances the overall anti-tumor immune activity, ensuring a prolonged cytotoxic response.

4.3. Tumor Immune Escape Mechanisms in BiTE Therapy

Despite the promising therapeutic efficacy of BiTEs, tumor cells often develop immune evasion strategies. These mechanisms include downregulation of TAAs, upregulation of immune checkpoint molecules such as PD-L1, and recruitment of immunosuppressive cells within the tumor microenvironment. These factors collectively limit T-cell activation and reduce the therapeutic potential of BiTEs in certain malignancies.

4.4. Rationale for Combining BiTEs with Chemotherapy

To overcome tumor immune escape, combination therapies integrating BiTEs with conventional chemotherapeutic agents or immune checkpoint inhibitors are under investigation. Chemotherapy can modulate the tumor microenvironment, increase antigen presentation, and enhance the infiltration and activity of T-cells, thereby synergizing with BiTE-mediated cytotoxicity.

5. BiTE Therapy in Solid Tumors: Current Challenges and Future Perspectives

5.1. Historical Development and Early Lessons

Initial clinical successes of BiTE therapy were primarily observed in hematologic malignancies, such as acute lymphoblastic leukemia. However, translating these outcomes to solid tumors has proven challenging due to the complex tumor microenvironment, physical barriers to T-cell infiltration, and heterogeneity in TAA expression.

5.2. Current Status and Future Directions

Ongoing clinical trials aim to optimize BiTE design, improve tumor penetration, and reduce immunosuppressive signaling. Strategies under exploration include the development of BiTEs with dual or multiple TAAs, combination regimens with checkpoint inhibitors, and the use of cytokine-modulating agents to enhance T-cell function. These advances hold promise for expanding the clinical utility of BiTEs beyond hematologic cancers to a broader range of solid tumors.

6. Blinatumomab: Clinical Profile, Efficacy, and Safety Data of a Prototype BiTE

6.1. Molecular Structure and Pharmacokinetics

Blinatumomab (MT103) is a BiTE composed of two scFv that separately bind CD19 and CD3. This adaptable design enables blinatumomab to efficiently attach to both T-cells and tumor cells. Due to its low molecular weight (~55 kDa), its elimination half-life is short (mean 2.11 hours).

6.2. Clinical Efficacy: Breakthrough Results in ALL and NHL

Blinatumomab has demonstrated efficacy in patients with ALL and NHL. Even at concentrations as low as 0.06 mg/m²/day, it can **engage** cytotoxic T-cells.

Minimal Residual Disease (MRD): Blinatumomab is also effective in eradicating minimal residual disease (MRD) in ALL. MRD refers to residual leukemic cells detectable in patients who are in complete remission by conventional pathological assessments and is associated with poor prognosis. In 2018, blinatumomab was approved for MRD-positive B-ALL, becoming the first therapy with FDA approval specifically for MRD treatment.

6.3. Efficacy Confirmed by Clinical Trial Data

Relapsed/Refractory (R/R) Ph-negative B-ALL: In a Phase clinical study, blinatumomab led to complete remission (CR) in 33% of primary relapsed/refractory (R/R) Ph-negative ALL patients and complete remission with

partial CRh in an additional 10%, for a total remission rate of 43%. For patients who had relapsed after undergoing allo-HSCT, the remission rate reached 45%.

MRD-Positive B-ALL: In another Phase study involving 108 CR Ph-negative B-ALL patients, 85 patients became MRD negative after a single cycle of blinatumomab at 15 μg/m²/day. Early administration during the first CR was linked to improved long-term survival.

Comparison with Chemotherapy: Blinatumomab demonstrates superior outcomes compared with conventional chemotherapy, including higher CR rates and extended overall survival. Its use before allogeneic stem cell transplantation further enhances CR rates, effectively acting as a bridge to transplant.

Ph+ ALL and TKI Resistance: Blinatumomab is an effective option in R/R Ph+ ALL patients resistant to tyrosine kinase inhibitors (TKIs) such as imatinib. In a Phase II study of imatinib-intolerant or refractory Ph+ ALL patients, 36% achieved CR/CRh after two cycles of blinatumomab monotherapy. Dasatinib-blinatumomab combination therapy in newly diagnosed Ph+ ALL achieved a CR rate of 98%.

Non-Hodgkin Lymphoma (NHL): In relapsed/refractory DLBCL, the ORR was 56%. A study identified 60 μg/m²/day as the maximum tolerated dose, achieving an ORR of 69% and a CR/CRu rate of 37%.

7. Administration and Dosing Regimen

Blinatumomab is administered in six-week cycles. The first cycle begins with 9 μg/day in week 1, increasing to 28 μg/day for weeks 2–4, followed by a 2-week break. Subsequent cycles consist of 28 μg/day for 4 weeks, then a 2-week treatment-free interval.

7.1. Adverse Events and Management

Blinatumomab treatment can cause various side effects, most commonly including fever, fatigue, headache, tremor, and leukopenia, particularly during the first cycle. Compared with CAR-T therapy, it carries a lower risk of severe cytokine release syndrome (CRS), which is an important advantage.

CRS results from excessive cytokine production, especially IL-6, and can present with fever, rash, nausea, and vomiting. Severe cases are typically managed with tocilizumab, while preventive measures such as dexamethasone premedication and gradual dose escalation help reduce the likelihood of serious reactions.

Neurological Adverse Events: Neurological symptoms may occur due to activated T-cells adhering to cerebral vessels and crossing into cerebrospinal fluid. Symptoms are generally reversible upon discontinuation. Pre-treatment

with steroids and close clinical monitoring are critical to prevent severe neurological events.

7.2. Forward-Looking Combination Strategies: TKIs and Costimulation

7.2.1. Combination with Tyrosine Kinase Inhibitors (TKIs)

Studies indicate a mechanistic synergy between TKIs and BiTEs. For instance, ibrutinib can lower immunosuppressive PD-1 and CTLA-4 levels on T-cells in CLL, enhancing T-cell survival and the cytotoxic effects of blinatumomab.

The blinatumomab-dasatinib combination, used as first-line therapy in newly diagnosed adult Ph+ ALL, achieved 88% disease-free survival at a median follow-up of 18 months. Even in patients with the TKI-resistance driver ABL1 Thr315Ile mutation, a regimen substituting dasatinib with ponatinib (active against this mutation) achieved high complete molecular response rates (87% in newly diagnosed, 79% in R/R patients), avoiding chemotherapy and allogeneic stem cell transplantation entirely.

7.2.2 Enhancement via Costimulation (Signal 2)

Full T-cell activation requires, in addition to signal 1 (TCR/CD3-antigen interaction), the activation of costimulatory pathways such as CD28 and 4-1BB (signal 2). Targeting these pathways offers an attractive method to enhance BiTE-directed cytotoxic T lymphocyte (CTL) activity.

BsAb Pairs: Combining a CD3xTAA BsAb (signal 1) with a CD28xTAA BsAb (signal 2) significantly increases T-cell proliferation and tumor-dependent cytotoxicity. Notably, targeted CD28 costimulation demonstrates a safe profile without inducing systemic cytokine release.

“Repurposing” PD-L1 as a Target: An innovative strategy uses PD-L1, an immunosuppressive signal, as a TAA. Combining a CD3xCD19 BsAb with a CD28xPD-L1 BsAb redirects CTLs to selectively target PD-L1-overexpressing B-ALL cells, transforming an immunosuppressive signal into a targetable cytotoxic cue.

Trispecific Antibodies: CD3xCD28xTAA trispecific antibodies combine both signal 1 and signal 2 in a single molecule. For example, a CD3xCD28xCD38 trispecific antibody has demonstrated efficacy against myeloma cells.

8.BiTE Applications in Solid Tumors – Challenges and Emerging Opportunities

8.1. Gastrointestinal Tumors

Carcinoembryonic antigen (CEA) is a glycoprotein expressed in normal epithelial tissues of the colon, stomach, esophagus, tongue, cervix, and prostate. In many cancers—particularly gastrointestinal adenocarcinomas—CEA expression becomes markedly upregulated and is no longer restricted to the apical or luminal cell surfaces.

MEDI-565 (AMG 211) is a BiTE specifically targeting CEA. In a phase I clinical trial utilizing intermittent IV infusion, 28% of patients achieved SD, although no partial or complete responses were reported. Adverse events included diarrhea, vomiting, fever, and CRS. Short serum half-life and high immunogenicity—with anti-drug antibodies developing in 49% of patients—were major limitations. Another human study demonstrated tumor-specific localization of radiolabeled MEDI-565 to CEA-expressing tissues, confirming target engagement; however, immunogenicity again emerged as a major challenge, emphasizing the need for humanized scFv formats.

8.2. Prostate Tumors

Prostate-specific membrane antigen (PSMA) is a transmembrane glycoprotein with highly restricted expression in normal prostate tissue but marked overexpression in prostate cancers, where it contributes to tumor progression.

Pasotuxizumab (AMG 212) was the first PSMA-targeted BiTE tested in humans. In a phase I study of 47 patients with castration-resistant prostate cancer, subcutaneous dosing was associated with universal development of anti-drug antibodies, whereas continuous IV infusion in 16 patients resulted in dose-dependent antitumor responses, including reductions in serum PSA levels. Notably, 12.5% of patients achieved long-term responses lasting 14–19 months, with one patient showing complete regression of soft tissue metastases and major regression of bone lesions. These findings provided the first clinical evidence that BiTEs could elicit meaningful antitumor activity in solid tumors with manageable safety profiles.

Acapatamab (AMG 160), a half-life-extended (HLE) BiTE, is currently being studied both as a monotherapy and alongside immune checkpoint inhibitors like pembrolizumab or AMG 404. Early results are promising, showing notable reductions in PSA levels in a substantial portion of patients.

8.3. Lung Tumors

DLL3, a Notch pathway ligand that is abnormally expressed in SCLC but largely absent in normal lung tissue, is emerging as a potential therapeutic target.

Tarlatamab (AMG 757), an HLE BiTE targeting DLL3, is being evaluated as monotherapy and in ICI combinations. In an ongoing phase I trial, among 38 evaluable SCLC patients, 16% achieved confirmed partial responses (PR), 3% had unconfirmed PR, and 29% achieved SD. CRS occurred in 43% of patients but was predominantly grade 1–2, reversible, and rarely recurred after the first treatment cycle. These favorable safety and efficacy signals have led to the initiation of a phase II trial.

8.4. Malignant Gliomas

The EGFRvIII mutation, detected in one-third of glioblastomas, drives tumor proliferation and represents a unique, tumor-specific epitope.

Etevritamab (AMG 596), an EGFRvIII-directed BiTE, was tested in a phase I trial involving 14 evaluable patients. Although adverse events occurred in all patients and half experienced grade ≥3 toxicities (mainly headache and decreased consciousness), no treatment discontinuations occurred. Sustained PR was achieved in 12.5% of patients and SD in 25%. Despite program discontinuation due to portfolio reprioritization, these results demonstrated that EGFRvIII BiTEs can be both tolerable and clinically active in glioblastomas.

9. BiTE and BsAb Applications in Other Hematologic Malignancies

9.1. Acute Myeloid Leukemia (AML)

AML is the most common acute leukemia in adults, with relapsed/refractory (R/R) disease having cure rates below 10%.

CD123/CD3 (IL-3Rα): Overexpressed in AML and associated with adverse prognosis.

Flotetuzumab (MGD006), a DART molecule, achieved 26.7% CR/CRh and 30% overall response rates in primary induction failure or early relapse AML, requiring continuous infusion.

Vibecotamab (XmAb14045), engineered with extended half-life, demonstrated 23% CR in R/R AML but with high CRS incidence (77%).

CD33/CD3: Expressed in ~88% of AML cases.

AMG 330 targets CD33 and immunosuppressive CD33+ MDSCs, enhancing antitumor immunity.

AMV564, a bivalent TandAb (two CD3 and two CD33 binding domains), showed extended half-life and MDSC depletion, restoring immune surveillance.

Additional targets include FLT3, CLEC12A/CLL-1 (MCLA-117), and WT1, all showing promising preclinical or early clinical activity.

9.2. Multiple Myeloma (MM)

MM is driven by malignant plasma cell proliferation and remains largely incurable despite therapeutic advances.

BCMA/CD3:

AMG 420 achieved 70% overall response and 50% CR rates at 400 μg/day in R/R MM but required continuous infusion.

AMG 701, a half-life–extended BiTE, enables intermittent dosing and shows strong synergy with PD-1 blockade in preclinical studies.

GPRC5D/CD3: Talquetamab (DuoBody platform) demonstrated robust preclinical activity and is in clinical testing.

CD38/CD3: AMG 424 and Bi38-3 efficiently killed CD38+ MM cells while sparing most normal CD38+ T cells.

FCRL5/CD3: Anti-FcRH5/CD3 TDBs demonstrated potent preclinical activity with long half-life supporting intermittent dosing schedules.

9.3. Non-Hodgkin and Hodgkin Lymphomas

CD20/CD3:

REGN1979 achieved 100% ORR in R/R follicular lymphoma; efficacy in DLBCL increased with dose escalation.

Mosunetuzumab induced 64.1% ORR in indolent and 34.7% in aggressive NHL.

CD20-TCB (RG6026) with a 2:1 binding format showed higher potency and extended half-life.

CD47-based BsAbs: Blocking the "don’t eat me" signal via CD20–CD47SL BsAbs outperformed anti-CD20 or anti-CD47 monotherapies in survival endpoints.

Hodgkin lymphoma: AFM13, a CD30×CD16A NK cell engager (BiKE), demonstrated 61.5% ORR in R/R HL patients.

9.4. Myelodysplastic Syndromes (MDS)

MDS represents a heterogeneous group of clonal stem cell disorders with limited therapeutic options.

CD123: Overexpressed in MDS bone marrow, making it a key target; Flotetuzumab is under clinical evaluation.

CD33: AMV564 depleted CD33+ cells and restored immune homeostasis in MDS, showing dose-dependent activity in early studies.

10. Major Limitations and Innovative Strategies

On-target, off-tumor toxicity remains the key challenge for solid tumor BiTEs. Discovery of truly tumor-specific antigens is critical.

Prodrug (masked) BiTEs, engineered to be activated only within the tumor microenvironment (TME) under acidic pH or protease-rich conditions, can markedly reduce systemic toxicity. For example, masked CD3–EGFR BsAbs demonstrated a 60-fold increase in maximum tolerated dose in preclinical models.

Oncolytic viruses (OVs) engineered to deliver BiTEs directly into tumors represent a novel strategy to localize immune activation and minimize systemic toxicity. Preclinical studies using CD3xEpCAM, CD3xCD20, and CD3xPD-L1 BiTE-armed OVs have shown potent antitumor activity.

TME immunosuppression by ECM barriers, Tregs, and MDSCs limits BiTE efficacy. Combination approaches with ICIs, anti-VEGF agents, or metabolic modulators (e.g., IDO-1 inhibitors) have demonstrated synergistic activity; notably, anti-VEGF + BiTE outperformed anti–PD-1 + BiTE in some preclinical models.

CRS and neurotoxicity remain universal concerns. Proactive strategies include IL-6, TNF-α, or IL-1β blockade, stepwise dosing, subcutaneous delivery, and tuning CD3 binding affinity.

11. Future Perspectives

Bispecific T-cell engagers (BiTEs) represent a rapidly expanding frontier in cancer immunotherapy, with the potential to transform treatment paradigms despite their deceptively minimalist architecture. Comprising two unique scFvs connected via a flexible linker, BiTEs simultaneously bind CD3 on cytotoxic T-cells and a TAA on malignant cells. By binding to both T-cells and tumor cells at the same time, this interaction activates T-cells and leads to the destruction of the tumor cells. The necessity for dual-antigen recognition minimizes the risk of off-target T-cell activation and helps overcome T-cell anergy. The remarkable

potency and versatility of these molecules have been clearly demonstrated in both preclinical models and clinical trials.

Blinatumomab, with its transformative impact in B-cell malignancies, stands as the most mature example of this platform. Yet, the BiTE technology is advancing toward a broader spectrum of applications, including solid tumors. Early clinical investigations targeting PSMA (prostate cancer), DLL3 (small-cell lung cancer), and EGFRvIII (glioblastoma) indicate that BiTEs could provide meaningful activity beyond hematologic malignancies. Modern BiTE constructs are continuously engineered to overcome limitations encountered by first-generation molecules, such as Fc-mediated toxicity and immunogenicity. Innovations—including half-life–extended (HLE) BiTEs, TandAbs, DARTs, and trispecific antibodies—enhance binding affinity, molecular flexibility, and pharmacokinetics, improving practical clinical utility.

Despite these advances, significant challenges remain, particularly for solid tumors:

Target selection and toxicity: On-target, off-tumor toxicity continues to limit therapeutic windows. The identification of truly tumor-specific antigens such as CLDN18.2 and GPRC5D, along with advanced engineering strategies like "masked" prodrug BiTEs, offers promising solutions.

Suppressive tumor microenvironment (TME): Immunosuppressive cells (Tregs, MDSCs), checkpoint pathways, and physical barriers (e.g., ECM) can significantly curtail BiTE efficacy.

CRS and neurotoxicity: These remain dose-limiting adverse effects across all BiTE therapies. Proactive strategies—including stepwise dose escalation, preemptive tocilizumab use, and optimization of CD3 binding affinity—are critical for safe administration.

Overcoming these obstacles and fully realizing BiTE potential will rely heavily on rational combination therapies, leveraging the synergistic potential of BiTEs with other immunotherapeutic and targeted agents:

ICIs: Can mitigate T-cell, PD-L1 upregulation induced by BiTEs, sustaining T-cell activity within the TME.

Tyrosine kinase inhibitors (TKIs): Combinations with dasatinib or ponatinib, particularly in Ph+ ALL, demonstrate high remission rates without the need for conventional chemotherapy.

Costimulatory agonists (e.g., CD28, 4-1BB): Provide "signal 2," enhancing T-cell activation and persistence.

Oncolytic viruses (OVs): Deliver BiTEs directly to the tumor, amplifying local immune responses while minimizing systemic toxicity.

Chemotherapy and anti-angiogenic agents: Reduce tumor burden, enhance immunogenicity, and remodel the immunosuppressive TME.

Future clinical studies will increasingly focus on combination regimens, not only comparing BiTE monotherapies with combinatorial approaches but also systematically evaluating different combination strategies to identify the most effective and safe therapeutic "cocktails."

12. Concluding Remarks

BiTEs occupy a unique and indispensable position in the cancer immunotherapy landscape, combining "off-the-shelf" availability, potent efficacy, and relative simplicity compared to personalized CAR-T therapies. They have already achieved groundbreaking success in hematologic malignancies and continue to evolve to address the challenges of solid tumors. Through deep scientific investigation, innovative molecular engineering, and strategic combinatorial approaches, the BiTE platform is poised to deliver more effective, safer, and widely accessible therapies to a broader patient population. This dynamic and rapidly advancing field is set to reshape oncology treatment paradigms, heralding a new era in the fight against cancer.

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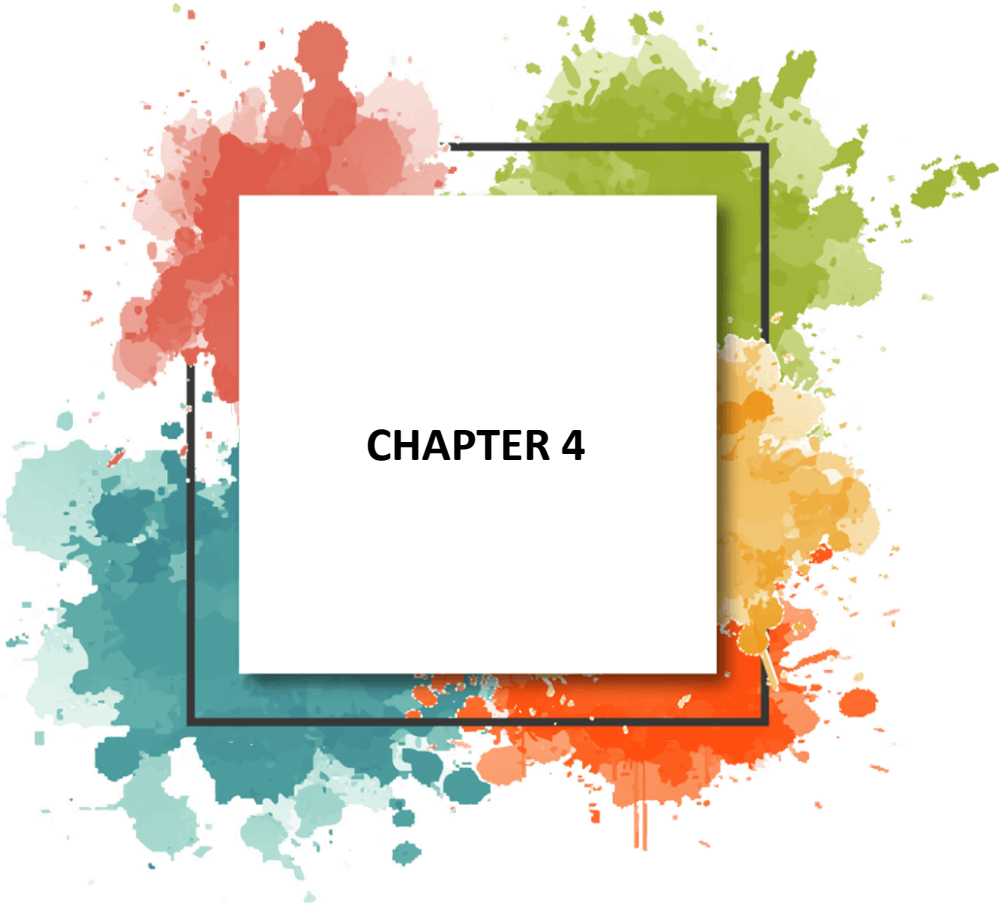
NCT02879695 (Blinatumomab + Nivolumab)

NCT03160079 (Blinatumomab + Pembrolizumab)

NCT02650713 (CEA BiTE + Atezolizumab)

NCT03399799 (Talquetamab)

NCT04590326 (MUC16 BiTE combination)


CHAPTER 4

Anatomy and Common Clinical Pathologies of Patella

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Embryology and Development

The patella develops from a continuous band of fibrous connective tissue in the mesenchymal interzone along the surface of the knee joint at the distal femoral border. Cartilage formation of this fibrous band begins around the 9th week of pregnancy, forming the quadriceps tendon superiorly and the patellar ligament inferiorly. By the 14th week, the patella becomes fully cartilaginous. The medial and lateral patellar facets are initially equal in size. However, the lateral facet becomes larger than the medial facet by the 23rd week of gestation.

Primary ossification usually does not occur until age 5 or 6. However, radiographic evidence of ossification may be present by age 2 or 3. Initially, numerous small foci of ossification appear. These spread toward the edges of the bone, which will later coalesce to form adult bone. Periosteum forms early on the anterior patellar surface. However, the other patellar edges retain chondro-osseous interfaces that persist throughout adolescence. This makes them susceptible to avulsion fractures until skeletal maturity [1, 2].

Anatomic Structure

The patella is the largest sesamoid bone in the human body. It is located anterior to the knee joint, within the tendon of the quadriceps femoris muscle [2], and articulates with the femoral condyle to form the patellofemoral joint, a major component of the knee joint [3]. The patellofemoral joint is part of the knee joint, the most complex and largest joint in the body, and works together with the tibiofemoral joint [4]. The upper part of the patella facing downwards is called the apex patella, and the lower part facing upwards is called the basis patella and is shaped like an inverted triangle [5]. The patella base is rough to accommodate the vastus intermedius and rectus femoris muscles. The patella has superior, medial, and lateral borders, as well as anterior and posterior surfaces. The medial and lateral edges are rough and rounded for the attachment of the vastus medialis

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and lateralis muscles [1]. The top dimensions of the patella are on average 4 - 4.5 cm in length, 2 - 2.5 cm in thickness and 5 - 5.5 cm in width [6].

The anterior surface of the patella is marked by a series of rough vertical ridges formed by the fibers of the quadriceps tendon. This portion is slightly convex and divided into three parts. The rough upper third, corresponding to the base of the patella, receives the insertion of the quadriceps tendon. The superficial portion of this tendon continues over the anterior surface to form the deep fascia that attaches to the bone. The middle third contains vascular openings and vertical lines. The lower third terminates in a pointed apex surrounded by the patellar ligament [1]. The anterior surface is covered with a thin layer of periosteum and acts as a protective barrier for the knee joint [3].

The posterior surface contains an oval, broad, and smooth facet covered with articular cartilage [1, 7, 8]. The posterior surface of the patella is divided into several facets, and a large vertical ridge divides this surface into medial and lateral halves [6] (Fig.1). This vertical ridge coincides with the trochlear groove between the condyles on the anterior surface of the distal femur. The inferior portion, representing 25% of the patella height, is non-articular. This area, filled with vascular openings whose vessels pass through the densely adherent infrapatellar fat pad, forms the apex of the patella [7]. The upper and articulating part of the posterior surface is completely covered with articular cartilage and constitutes 75% of the patella height. The middle part of this articular cartilage is 4 to 5 mm thick and is the thickest part of the body [1, 7, 8]. This thick cartilage is thought to disperse the large joint reaction forces generated during forceful contractions of the quadriceps muscle [6].

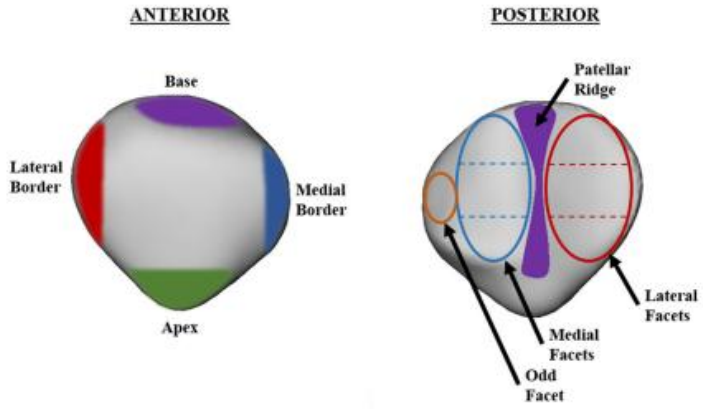

Fig. 1. Anterior view: regions of the patella; posterior view: facets of the articular surface and patellar ridge [9]

Articular Surface

Articular cartilage, which is hyaline cartilage, is a highly specialized tissue consisting of a dense extracellular matrix with a sparse distribution of chondrocytes. It is composed primarily of water, proteoglycans, collagen, and, to a lesser extent, other noncollagenous proteins and glycoproteins. The combination of these components provides a smooth, gliding surface for low-friction articulation between the patella and the trochlear groove of the femur [10]. The articular cartilage of the patella is the thickest in the body, reflecting the magnitude of the stresses to which it is subjected. In a patella unaffected by pathology or erosion, its natural thickness can be half its width [11].

The articulating portion of the patella, approximately oval in shape, is divided into lateral and medial facets by a vertical ridge [12]. The medial and lateral facets of the patella articulate with the corresponding medial and lateral condyles of the femur. This interaction forms the patellofemoral joint of the knee [11]. The median process is located on the longitudinal axis of the patella and is approximately equally prominent throughout.

Wibeeg developed a classification system for different patellar facet sizes by comparing the configuration of the lateral and medial facets on the undersurface of the patella. In Type I, which occurs with a prevalence of 10%, the lateral and medial facets are concave and approximately equal in size. In type II, the medial facet of the patella is flat or slightly convex and significantly smaller than the lateral facet. Type III, seen in 25% of all cases, has a smaller medial facet than the lateral facet, but unlike type II, it has a convex structure. These facets are important for the functional anatomy of the patellofemoral joint, which is a diarthrodial plane joint [12, 13].

The medial facet is divided into two subdivisions by the medial facet itself and a much smaller facet, the ODD facet (Fig. 1), along the medial border of the patella. The ODD facet is separated from the rest of the medial facet by a vertical ridge. It is described as a "secondary ridge" because it is less prominent than the median ridge and develops after birth in response to loading. The secondary ridge runs obliquely and is more prominent distally. This ridge conforms to the curve of the lateral border of the medial condyle when the knee is fully flexed, while the median ridge conforms to the straight medial border of the lateral condyle. There is considerable anatomic variation in the prominence of a secondary ridge. The surface configuration of the articular surface is determined not only by the underlying subchondral bone but also by variation in the thickness of the patellar cartilage itself [7].

The lateral facet is concave in both the transverse and vertical planes. Some studies have identified three transverse segments on the articular surface in adults,

defined by the presence of two transverse processes on the lateral and medial facets at the junction of each third [1]. These processes are thought to isolate three segments of different functional importance as the lower, middle, and upper thirds of the patella gradually come into contact with the femur in flexion (in that order) [7]. Other studies have reported the existence of a relatively fixed process separating the middle and lower thirds, more commonly found on the lateral facet [7, 14, 15].

The upper third of the patella serves as the attachment point for the vastus intermedius and rectus femoris muscles. The quadriceps tendons converge at their distal attachment points and form the deep fascia lata, which passes superficially over the anterior surface of the patella and attaches to the lateral condyle of the tibia [2].

The patellar ligament, commonly referred to as the patellar tendon or ligamentum patellae, is the continuation of the quadriceps femoris muscle tendon from the patellar apex to the tibial tuberosity. It forms part of the extensor mechanism of the lower extremity and stabilizes the patella. The patellar ligament is clinically important because of its association with patellar tendinopathy, sometimes called jumper's knee, and its frequent use as an autograft in anterior cruciate ligament reconstruction [16]. The patella serves as an attachment point for both the quadriceps tendon and the patellar ligament [2].

Microscopically, the patella consists of dense cortical bone on its outer surface, which provides resistance and durability against tensile forces. Cancellous bone with a trabecular structure, which absorbs and distributes compressive loads, is located on the inner surface of the patella. The posterior surface has one of the thickest cartilage layers in the body, reflecting its role in load distribution and joint stability [17].

Soft Tissue

Due to the shallowness and incongruity between the patella and trochlea, the stability of the patellofemoral joint depends on static and dynamic soft tissue structures. The patellar tendon, joint capsule, and ligaments provide static stability. The medial structures become important in minimizing lateral translation, with the primary structure of lateral restriction being the medial patellofemoral ligament. This ligament extends from the adductor tubercle to the medial border of the patella [6] (Fig. 2). Desio et al. describe the medial patellofemoral ligament as a structure that provides 60% total restriction at 20 degrees of knee flexion. Secondary restriction includes the medial meniscopatellar ligament, which originates from the anterior surface of the menisci and inserts into the lower third of the patella and the medial retinaculum.

The superficial fibers are interwoven with the medial collateral ligament and medial patellar tendon [18].

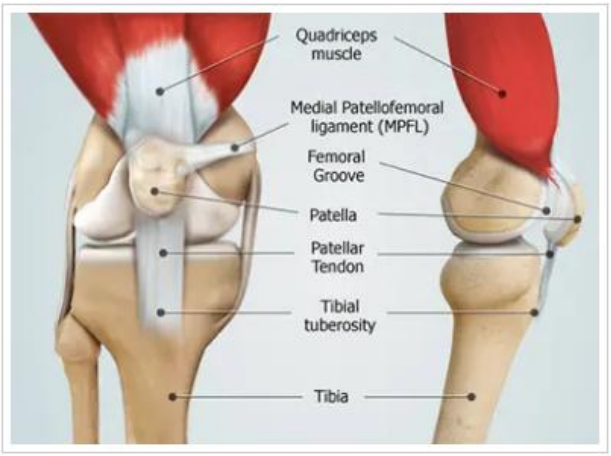

Fig. 2. Medial patellofemoral ligament (MPFL) [19]

On the lateral side of the patellofemoral joint, structures that aid in stability are the lateral patellofemoral ligament, lateral retinaculum, joint capsule, and iliotibial band. The lateral retinaculum consists of a thinner superficial layer extending from the iliotibial band to the patella and quadriceps extension, and a thicker deep layer that interdigitates with the vastus lateralis, patellofemoral ligament, and patellotibial ligament. The joint must rest on the medial and lateral retinaculum and joint capsule at angles of flexion less than 20-30 degrees because bony stability is minimal or nonexistent. Dynamically, the contractile nature of the quadriceps muscle, pes anserinus muscle group, and biceps femoris muscle helps maintain patellar alignment. The importance of the vastus medialis oblique muscle has been discussed extensively in the literature [20, 21]. The vastus medialis oblique muscle attaches to the medial portion of the patella, the medial patellofemoral ligament, and the adductor magnus tendon. Its more oblique alignment compared to the vastus medialis longus muscle provides a mechanical advantage for increasing medial stabilization forces on the patella. The rectus femoris muscle inserts on the superior anterior aspect of the patella. The vastus intermedius muscle inserts posteriorly at the base of the patella. The vastus lateralis muscle, along with the iliotibial band and superficial oblique retinaculum, provides lateral dynamic reinforcement. Tightness in the iliotibial band can cause the patella to shift and/or tilt laterally. Inferiorly, the patella is stabilized by the patellar tendon and its attachment to the tibial tubercle [6].

Patellofemoral joint reaction force (PFJRF)

Patellofemoral joint reaction force is the compression force acting on the joint and depends on the knee joint angle and muscle tension. The actual stress applied to the patellofemoral joint is determined by dividing the patellofemoral joint reaction force by the patellofemoral joint contact area, and is called joint stress, measured as force per unit area. The larger the contact area between the patella and the femur, the less stress is exerted on the joint tissue. A high patellofemoral joint reaction force combined with a small contact area results in high patellofemoral joint stress and can damage the articular cartilage.

As the point of contact between the trochlea and patella changes throughout the range of motion, joint forces change due to the corresponding changes in the leverage system. In non-weightbearing situations, the contact area between the patella and trochlea increases when the knee is flexed from 0 to 90 degrees. Therefore, as knee flexion increases, less patellofemoral stress is created. Minimizing patellofemoral joint stress is widely accepted. Open-chain exercises should be performed between 90 and 30 degrees of knee flexion. With the foot stationary, the patellofemoral joint reaction force increases from 90 degrees to 45 degrees and then decreases as the knee approaches full extension. Patellofemoral joint reaction force and patellofemoral joint stress can be very high even during the simplest activities of daily living. Studies have shown forces of up to 1.3 times body weight during level walking, 5.6 times during running, 3.3 times during stair climbing, and 7.8 times during deep knee bending or squatting [6].

Function

The patella's function is multifaceted. Its primary purpose is to act as a mechanical pulley for the quadriceps muscle, altering the direction of extension forces throughout the knee's range of motion. Its contribution increases with progressive extension. The patella is critical in the last 30 degrees of knee extension. In full knee extension, the patella provides 31% of the total knee extension torque, but only 13% between 90 and 120 degrees of flexion. The patella also serves as a bony shield for the anterior trochlea. Its interposition between the quadriceps tendon and the femur prevents excessive friction between the tendon and the femoral condyles [6, 22].

Static and dynamic patellar alignment clarifies the etiologies of patellofemoral pain. Static patellar alignment depends on the shape of the patella, the depth of the femoral sulcus, and the height of the lateral femoral condylar wall. Gross patellar alignment is usually assessed in the supine position with the knee in full extension. In this position, the patella is at its most mobile because contact between the femur and patella is minimal. In a fully extended knee, the patella lies above the trochlea and between the two condyles, occasionally shifting

slightly laterally. When the knee is slightly flexed to 30 degrees in full extension, the patella settles at or proximal to the joint line. In this position, the ratio of the patellar ligament length to the patella height should be approximately 1.0. A ratio significantly less or greater than 1.0 may indicate patella baja or patella alta, respectively. Individuals with patella alta are at higher risk of patellar subluxation.

Each edge of the patella should be equidistant from the femur. Anterior or posterior tilt describes the position of the inferior pole of the patella in the sagittal plane. Inferior tilt occurs when the inferior pole is tilted downward, and superior tilt occurs when the inferior pole is tilted upward. Inferior tilt can compress and irritate the patellar fat pad deep to the patellar ligament, causing pain. Lateral tilt occurs when the lateral border of the patella is tilted downward in the transverse plane. Medial tilt refers to a position in which the medial border of the patella is depressed in the transverse plane. Lateral tilt may cause patellofemoral compression syndrome. Patellar rotation is defined as the direction of rotation of the inferior pole of the patella. Lateral or medial rotation of the patella may suggest underlying tibial torsion.

The extensibility of the connective tissue around the patella, the contraction of the active quadriceps muscle, and the geometry of the patella and trochlear groove are factors that affect dynamic movement [6]. During tibiofemoral movement, the patella functions as a gliding joint and can move in multiple planes. During knee extension, the quadriceps muscle contracts, pulling the patella upward, resulting in superior glide. During knee flexion, inferior glide occurs. Medial and lateral glide refer to the movement of the patella toward the medial and lateral sides, respectively. During normal patellar movement, a slight medial or lateral glide occurs. However, when the knee is fully extended, the patella is positioned slightly laterally due to external tibial rotation. The articular surface of the patella changes as the knee completes its range of motion. When the knee is flexed, the patellar contact point moves downward and posteriorly along the femoral condyle and more proximally on the patella. Initially, during flexion, the lateral facet of the patella is the first to contact the uppermost portion of the lateral femoral condyle. However, when the knee is flexed to 30 degrees, the contact surface is evenly distributed on both sides of the patella and femoral condyles. The patella's contact surface expands with knee flexion. It is approximately 2.0 cm at 30 degrees of flexion and increases to approximately 6.0 cm at 90 degrees of flexion. This helps prevent the harmful effects of repeated exposure to high compressive loads by distributing joint forces over a larger surface area. The superior surface of the patella abuts an area of the femoral groove above the femoral notch when the knee is flexed to 90 degrees. When the knee is in deep flexion, the patella bridges the intercondylar notch, and contact

occurs only at its lateral and medial edges. In full flexion, the only point of contact is between the unilateral aspect of the patella and the lateral surface of the medial femoral condyle [2].

Patellar blood supply

All arterial blood flow to the patella originates from the peripatellar vascular anastomotic ring formed by the five genicular arteries (supreme, superolateral, superomedial, inferomedial, inferolateral) and the anterior tibial recurrent artery (Fig. 3). The lateral and medial portions of the ring lie within the retinaculum, extend along the patellar border, and anastomose with the superior and inferior vessels. The lower portion of the ring lies behind the patellar tendon and is formed by the transverse infrapatellar branch, which lies within the infrapatellar fat pad.

Two vascular systems, dorsal and radial, arise from this peripatellar vascular ring to provide intraosseous blood flow to the patella. The dorsal system branches from the prepatellar anastomotic network on the anterior surface and enters the anterior cortex via the midpatellar vessels. The radial system enters the patellar border via the peripatellar vessels, which pierce the retinaculum medially and laterally, and the polar vessels, which lie at the distal pole of the patella, posterior to the patellar tendon and anterior to the articular cartilage [23].

A recent study using qualitative and quantitative MRI findings reported that the predominant arterial supply penetrates the patella from the distal pole, predominantly inferomedially [24]. The importance of preserving the peripatellar anastomotic ring, especially the lower patellar network, in preserving the vascular supply of the patella has also been reported in previous studies [23, 25].

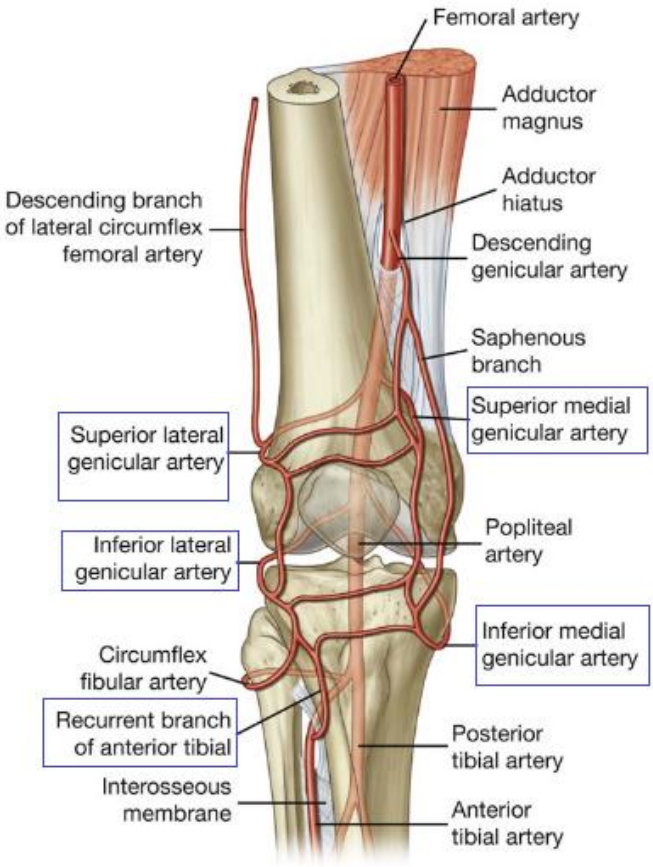

Fig. 3. Patellar blood supply [26]

Nerves

The anterior cutaneous innervation of the knee is provided by the L2-L5 nerve roots. The femoral, genitofemoral, obturator, and saphenous nerves provide anteromedial innervation to the knee. The lateral femoral and lateral sural cutaneous nerves innervate the anterolateral region. No nerve root endings were identified in the patella, the knee joint itself, or the femoral sulcus [1].

Common Clinical Pathologies of the Patella

Patellar Instability

Patellar instability is the pathological separation (subluxation or complete dislocation) of the patella from the patellofemoral joint. It is usually caused by multiple factors, such as chronic ligamentous laxity, acute trauma, bone malalignment, connective tissue disorder, or anatomic pathology [27]. These

imbalances lead to chronic instability and secondary flattening of the lateral aspect of the femoral trochlea. The patella shifts laterally or dislocates completely during flexion, or it returns to its medial position as flexion progresses [2]. Over time, patients with patellar instability may experience severe pain, limitations in basic functions, and long-term arthritis [27].

Patellar instability can be treated nonsurgically with immobilization and weight-bearing restrictions. Physical therapy is usually sufficient to correct mechanical imbalances. However, dislocations are often accompanied by tissue damage. Because the patella may become less stable after healing, recurrence of dislocations is common. Multiple patellar dislocations require surgery to correct the underlying condition. Arthroscopic reconstruction of the patellar ligaments is often performed [2].

Chondromalacia Patellae

Chondromalacia patellae, also known as runner's knee, is a common cause of anterior knee pain among young people, especially young women who participate in sports. Chondromalacia patellae is characterized by anterior knee pain associated with visible changes in the patellar cartilage. It is sometimes used as a general term to describe patellofemoral pain, with or without a recognized cartilage abnormality. The health of the patella is determined by the integrity and thickness of the overlying hyaline cartilage. The normal appearance of the hyaline cartilage of the patella is bluish-white, shiny, smooth, and flexible. The initial pathological changes in chondromalacia patellae include a dull or slightly yellowish-white cartilage and, in the early stages, a soft, swollen, and edematous lesion. The lesion is characteristically located in the middle or just distal to the medial patellar facet, typically measuring approximately 1.25 cm or more in diameter. In advanced stages, the cartilage exhibits fibrillation, fissures, fragmentation, or erosion.

Direct trauma to the patella, increased cartilage sensitivity (congenital, post-arthrotomy/cast rehabilitation period), bony anatomic variations (patellofemoral instability, osteochondral ridge, congenitally flattened lateral femoral condyle, etc.), abnormal patellar kinematics (valgus knees, patella alta, excessively laterally placed tibial tubercle, etc.), and occupational exposures (military, sports training sites, and jobs requiring excessive kneeling and squatting) play a role in the etiology of chondromalacia patellae. Among the causes of chondromalacia patellae, subluxation is probably the most common and, because it is a frank patellar dislocation, the most frequently overlooked. Treatment is either conservative or surgical [28].

Trochlear Dysplasia

Trochlear dysplasia is defined by a sulcus angle greater than 150 degrees [12]. It is a common cause of recurrent patellar instability. It refers to anatomic defects in the femoral trochlear that interfere with the normal movement of the patella. These defects include decreased medial femoral condyle height, decreased trochlear depth, increased sulcus angle, and decreased lateral trochlear facet depth, which may be flat or convex.

Trochlear dysplasia can be identified radiographically by the crossover sign, defined as the junction of the deepest part of the femoral groove with the most prominent part of the lateral femoral trochlear facet. In a normal trochlea, the two lines remain distinct from the origin of the trochlea. The crossover sign is seen in 85% of individuals with recurrent patellar instability and 96% of individuals with objective patellar dislocation. Trochlear dysplasia, like patellar instability, is treated conservatively, and recurrent dislocations are treated surgically. Surgical options include medial patellofemoral ligament reconstruction, tibial tubercle osteotomy, and tracheloplasty [1, 2, 12].

Patella Alta

The patella alta is a patella that is abnormally high relative to the femur, femoral trochlea, or tibia, requiring increased knee flexion angles to engage the trochlea due to decreased bony stability (Fig. 4). An abnormally high patella may not adequately engage the proximal trochlea groove during both extension and early knee flexion, making it a potential risk factor for patellar instability. A patella alta is present in 30% of patients with recurrent patellar dislocations. Patellofemoral pain is also seen in other disorders, such as knee extensor disorders, Sinding-Larsen-Johansson disease, chondromalacia, Osgood-Schlatter disease, osteoarthritis, and patellar tendinopathy. This makes patella alta a significant predisposing factor for patellar malalignment and patellofemoral-related complaints. However, patella alta can also be a normal variant of an individual's knee anatomy and is well tolerated when not associated with other instability factors [29].

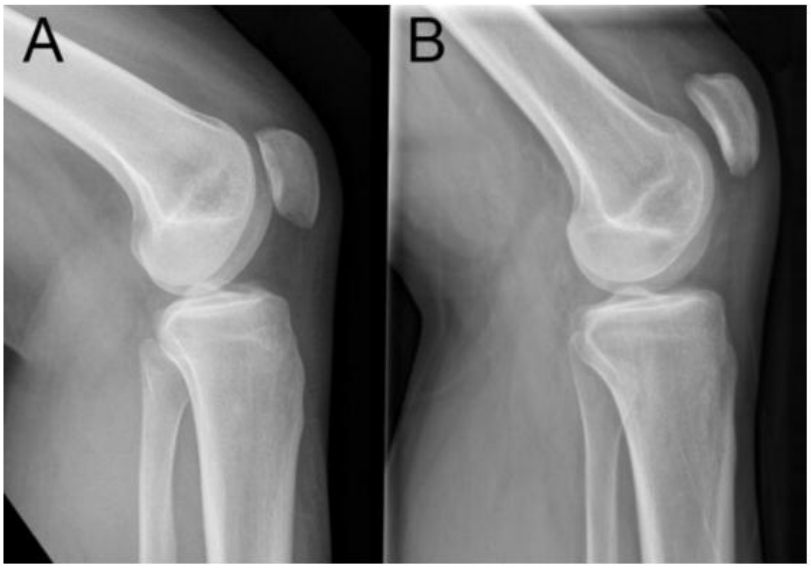

Fig. 4. A: Normal riding patella; B: patella alta [30]

The Insall-Salvati index is the ratio of the length of the patellar ligament to the greatest diagonal length of the patella on a lateral flexed knee radiograph. This index is frequently used to diagnose patella alta. An Insall-Salvati value greater than 1.2 is sufficient for the diagnosis of patella alta. Patella alta can be treated conservatively by manual shifting to change the resting height of the patella before knee extension. Taping is another alternative to correct the positional malalignment of the patella. Surgically, patella alta can be treated with a tibial tuberosity osteotomy, which shifts the attachment of the patellar ligament downward on the tibia [2, 29]. Surgical correction of patellofemoral instability in patients with patella alta leads to better functional outcomes [31].

Patella Baja

Patella baja, or patella infera (low-sloping patella), is characterized by a distal position of the patella in the femoral trochlea, a decreased distance between the inferior pole of the patella and the articular surface of the tibia, and/or a permanent shortening of the patellar tendon. Patella baja can also be defined by an Insall-Salvati ratio of 0.8. In patients with patella infera, the patella always contacts the trochlea in extension. A normal patella does not contact the trochlea. A low-sloping patella is most common in the postoperative or posttraumatic knee due to decreased tensile forces of the quadriceps muscle. Patellar fractures, patellar ligament tear and repair, and high tibial osteotomy are associated etiologies for patella baja. However, it has also been described as a complication

after total knee arthroplasty. Adverse effects of subluxation of the patella infera have been reported in the literature. These include joint stiffness, anterior knee pain, decreased lever arm and extensor lag, changes in joint mechanics, and decreased range of motion [1]. Treatment of symptomatic patella baja usually requires surgical intervention to proximalize the patella. There is no gold standard treatment for patella baja. However, surgical techniques such as tibial tubercle transfer and patellar tendon lengthening with autografts or allografts have been used to increase patellar height [1, 2, 12, 32].

Bipartite Patella

Bipartite patella is congenital and results from abnormalities in the ossification process of the patella. This process begins between the ages of 3 and 5 and consists of multiple foci that eventually coalesce. In bipartite patella, the fusion of these ossification centers fails, resulting in the formation of an accessory fragment connected by a fibrocartilaginous region. The most commonly used classification of bipartite patella, developed by Saupe, classifies the lesion according to the location of the accessory fragment. Type 1 (5%) occurs at the inferior patellar pole, type 2 (20%) at the lateral border of the patella, and type 3 (75%) at the superior lateral pole.

Because bipartite patella is usually asymptomatic, it is often discovered incidentally on knee radiographs. The prevalence in the adult population ranges from 0.6% to 2%. Approximately half of these cases are bilateral. It occurs most frequently in male patients and is up to nine times more common than in female patients. However, 2% of these patients have painful bipartite patella. Symptoms may occur following direct trauma or overuse injury that disrupts the fibrocartilaginous region between the primary and accessory patellar fragments. This condition can cause abnormal movement, friction, and swelling. These patients typically present with anterior knee pain localized around the accessory fragment and exacerbated by knee extension.

Initial treatment for painful bipartite patella is conservative. Nonsurgical treatment is successful in most cases within 2 to 6 months. If pain persists, surgical treatment should be considered. Surgical options may be arthroscopic or open. Options include excision of the accessory fragment, lateral retinacular release to reduce lateral and proximal traction on the patella (thereby promoting bony union and reducing pain), or release of the vastus lateralis only at the tendon attachment to the fragment (thereby reducing traction forces on the patella), and open reduction and internal fixation of the accessory fragment [33, 34].

Duplication of the patella

Asymptomatic congenital bipartite patella is not uncommon. However, true patellar duplication is rare, and only isolated cases have been described. A traumatic double patella usually results from extensive ossification resulting from tendoperiosteal avulsion of the inferior pole, a condition referred to as a "sleeve fracture" [35].

Patellofemoral Artrit

Unicompartmental arthritis of the knee generally refers not only to tibiofemoral arthritis but also to disorders of the patella and cartilage. Isolated patellofemoral arthritis, in particular, is a relatively common condition that is receiving increasing research on treatment options. Approximately half of patients diagnosed with degenerative knee arthritis develop patellofemoral arthritis due to cartilage loss in the patella and trochlear groove. This condition is multifactorial and is usually caused by cumulative damage to the joint from repetitive use or excessive force. Genetic factors also play a role in its development [2]. Although patellofemoral arthritis doesn't have characteristic symptoms, anterior knee pain is the most common complaint among patients. The pain is aggravated by climbing stairs, climbing hills, rising from a sitting position, kneeling, or squatting, and is associated with a rubbing or crackling crepitus. Some patients complain of knee stiffness or pseudo-locking due to "kissing" lesions that form between the patella and trochlear groove when the exposed bones rub against each other [36].

Most of these cases can be treated nonsurgically. Interventions include nonsteroidal anti-inflammatory drugs, weight loss, low-impact exercise, physical therapy, and cortisone injections. Viscosupplementation is another method that involves injecting hyaluronic acid into the joint to improve synovial fluid quality. If conservative treatment fails, surgical options include joint-preserving arthroscopic releases, microfractures, cartilage repair procedures, and tibial tuberosity osteotomy [37].

In addition, various anatomic variants of the patella are also known. For example, a hypoplastic patella is called "patella parva", and a hyperplastic patella is called "patella magna", which are dimensional abnormalities. A "hunter's hat" patella is one in which the lateral facet covers almost the entire patellar articular surface. Pebble-shaped and crescent-shaped patellas have also been described [12].

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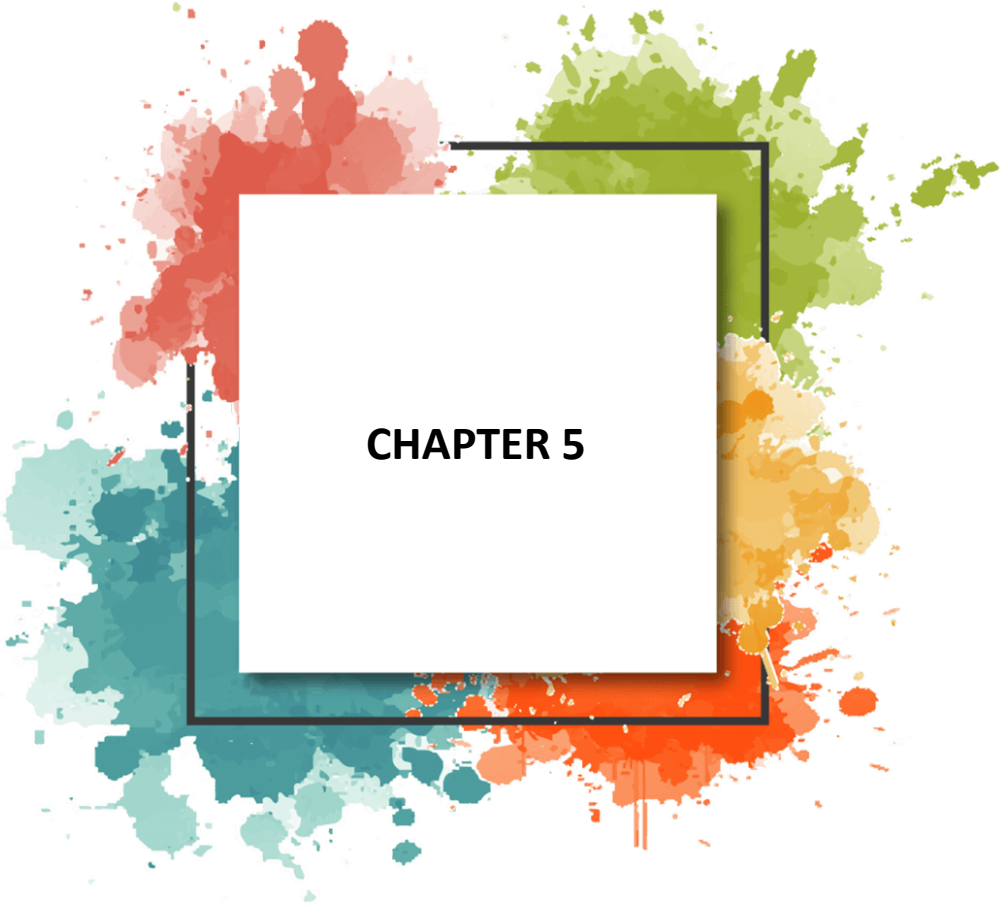
CHAPTER 5

The Importance of Proteomic Technologies in Infectious Diseases of Farm Animals

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1. Introduction

Proteomics is the analysis of all proteins encoded by the genome under specific conditions and in a specific tissue, in other words, the analysis of the proteome. The aim of proteomics studies is to identify the proteins present in a sample and to quantitatively determine changes in protein expression arising from various pathological conditions. Proteomics is widely used in the discovery of biological markers, vaccines and drugs (Savino et al., 2012).

Technological advances in proteomics have expanded the dynamic detection range for low-abundance proteins, enabling the detection of disease-specific proteins for use as potential biomarkers in veterinary medicine (Ceciliani et al., 2014).

Proteomics has been used in elucidating the pathogenesis of various diseases, in investigating host-pathogen interactions, and in improving disease diagnosis through the identification of biomarkers (Katsafadou et al., 2015).

This section aims to provide a comprehensive review of important bacterial infections observed in farm animals using omics technologies, proteomics, and related technologies, through proteomic approaches. Furthermore, potential biomarkers discovered in diseases through proteomic approaches and recommendations for future research will be discussed. This section aims to guide researchers in the diagnosis of infectious diseases in farm animals and in the field of preventive medicine using proteomic approaches.

2. Omic Technologies

The examination and analysis of large amounts of data representing the entire structure and function of a specific biological system at a certain level, defined as 'omics,' has opened up significant new avenues in biological systems research methods (Dai & Shen, 2022). The term 'Omics' refers to technologies that examine the roles, actions and relationships between the various molecules that make up an organism cells (Lérias et al., 2014).

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Omics technologies are a type of science and technology that utilise high-throughput technology to obtain large amounts of data at the molecular level and analyse and interpret this data using bioinformatics and computational methods. They are used to obtain genomic alterations, transcriptomic disruptions and additions, proteomic interactions, post-transcriptional modifications, and disease-related metabolic or immune markers, including genomics, transcriptomics, proteomics, metabolomics, and other technologies (Liu et al., 2024).

Since the establishment of DNA microarray technology, the first high-throughput technology, omics research technologies have developed at a rapid pace. Omics technologies have been used to capture static genomic alterations, temporal transcriptomic disruptions and alternative splicing, as well as spatiotemporal proteomic dynamics and post-translational modifications (PTMs). Furthermore, omics technologies have been extended to analyse disease-related hallmarks such as molecular interactions, the metabolome, and the immunome (Chakraborty et al., 2018; Schena et al., 1995).

2.1. Genomics

Genomics is a scientific field that studies the entire genetic information of an organism and focuses on analysing the structure, function, organisation, and evolution of genomes. Research methods in this field are divided into sections such as genome sequencing, gene expression, structural genomics, functional genomics, and comparative genomics. The field's technological applications are also extensive and encompass techniques such as DNA microarrays, NanoString, real-time quantitative PCR (qPCR), optical mapping, and next-generation sequencing (NGS) (Liu et al., 2024).

Genomics enables the comparison of genetic information from different organisms, the investigation of similarities between organisms at an evolutionary level, and the acquisition of knowledge about the types and numbers of proteins produced by organisms and their functions (Başaran et al., 2010).

2.2. Metabolomics

Metabolomics is the detection, quantification and identification of small-molecule metabolites (peptides, sugars, ketones, etc.) arising from lipids, carbohydrates, vitamins, hormones and other cellular components in tissues, cells and physiological fluids within a specific time frame, using high-throughput technologies (Goodacre, 2013).

2.3. Transcriptomics

Transcriptomics is a subdiscipline that simultaneously examines mRNA transcripts formed by transcription from the cell genome and extracts expression profiles. Microarrays and DNA chips are commonly used to extract these profiles.

The expression profiles extracted are called the transcriptome. The transcriptome is defined as a term used for all gene transcripts (RNA) in a cell or tissue at a given time (Subramanian et al., 2005).

2.4. Proteomics

Proteomics is an analytical technique designed to analyse the expression, structure, function and interactions of proteins in cells, tissues, body fluids and organs under various conditions. It complements genomic and post-transcriptomic methods (Duong & Lee, 2023).

While genomics deepens our understanding of disease mechanisms and drug responses, proteomics has become an indispensable field of research due to its unique role in directly executing life activities within cells. The dynamic changes and biodiversity of proteins increase the complexity of proteomic analyses, posing challenges to the advancement of proteomics (Liu et al., 2024).

Proteomics seeks to systematically characterise and quantify the entire proteome and elucidate the biological functions of proteins. Proteomics is widely used in the discovery of biological markers, vaccines and drugs (Savino et al., 2012).

Proteomics refers to the large-scale study of protein expression, protein-protein interactions, or post-translational modifications. This technique can reveal the dynamics of the cellular response to changes within tissues and their microenvironments. Therefore, it is possible to detect changes in protein expression, interaction, or modification resulting from different physiological states or pathological conditions occurring in that tissue of the organism in question. The creation of large proteomic data sets can be used to demonstrate the interdependence of various cellular processes that are important in normal cell growth or in the cellular response to abnormal conditions or disease states (Gingras et al., 2007; Katsafadou et al., 2015).

Proteomic technologies are divided into three main groups: comparative, structural, and functional proteomics.

Comparative proteomics involves the qualitative and quantitative analysis of protein expression under two or more different conditions. The study, identification, and comparison of proteins affected by exposure to specific internal or external factors within the cell (such as disease states, drug use, chemical or physical stimuli) falls within this field of proteomics (Nenni et al., 2020).

Structural proteomics focuses on the three-dimensional structures of proteins. The structures, functions, and interactions of specific and non-specific proteins within all organelles, in the cytosol, and in body fluids outside the cell are being

elucidated. By elucidating the structure of enzymes, drug molecules with high affinity are being designed. Structural proteomics plays an important role in the design and discovery of new drug molecules (Jung & Lee, 2004; Renfrey & Featherstone, 2002).

Functional proteomics investigates the functions that proteins possess either individually or in combination with other proteins. Environmental factors, drugs, and endogenous chemical factors cause protein modification, altering the structure of proteins and their function in that region. Protein modification studies are conducted through functional proteomics studies (Bíliková et al., 2009; Monti et al., 2009).

3. Technologies Used in Proteomic Studies

Proteomics is widely used in the detection of biological markers and in the development of drugs and vaccines. Various proteomic technologies are employed to achieve these objectives. Traditional proteomic methods such as enzyme-linked immunosorbent assay (ELISA), chemiluminescent immunoassay (CLIA) and immunohistochemistry (IHC) have certain disadvantages in complex protein analyses (Liu et al., 2024).

CLIA detects target proteins in liquids without the need to separate samples by labelling known antibodies or antigens. This method is highly sensitive and can provide rapid results, but it can be affected by reaction conditions and the microenvironment (Hayrapetyan et al., 2023).

IHC accurately detects and visualises specific antigens in tissues by labelling the binding of antibodies to specific target antigens without the need to digest and separate the sample. Although IHC can precisely identify target proteins, it has low reproducibility due to its limited use in analysing multiple targets simultaneously and the availability of specific antibodies (Mebratie & Dagnaw, 2024; Ramos-Vara, 2005).

The proteomics of highly complex samples encompasses not only traditional techniques based on mass spectrometry (MS) and antibody/antigen arrays, but also innovative technologies based on aptamers. The fundamental methods used in mass spectrometry are specially designed sample pre-treatment and liquid chromatography (LC), peptide ionisation, and tandem mass spectrometry scanning to obtain differential protein organisation. The advantage of MS is its ability to analyse total proteins and their modified forms, making it the preferred method in the early stages of biomarker discovery. However, the complexity of the workflow limits its further development, and optimising and simplifying this process is currently the main direction of progress (Ding et al., 2022).

Aptamers are single-stranded DNA, RNA, or peptide molecules that can bind with high affinity and specificity to various target molecules such as proteins, small molecules, cells, or viruses. Aptamer-based assays utilise the high affinity and specificity of aptamers bound to binders and fluorescent markers to identify relevant proteins. These proteins are then purified, eluted, and analysed using aptamer screening assays to obtain relevant protein analysis data (Liu et al., 2024).

X-ray crystallography, X-ray diffraction or nuclear magnetic resonance techniques can be used to directly determine the three-dimensional structure of a protein (MacArthur & Thornton, 1993; Ratnaparkhi et al., 1998).

Electrophoretic and chromatographic techniques are fundamental techniques for separating complex samples in proteomic studies. An advantage of using electrophoretic and chromatographic techniques is the separation of complex protein-peptide mixtures based on their physicochemical properties (Lescuyer et al., 2004).

Traditional techniques for protein purification are chromatography-based approaches. Methods such as ELISA and Western blot can be used for the analysis of specific proteins. These techniques may be limited to the analysis of a few individual proteins and may be insufficient for defining protein expression levels. SDS-PAGE, 2-DE, and 2-DIGE techniques are used for the separation of complex protein samples. Protein microarrays and chips are methods used for high-throughput and rapid expression analysis. Various proteomic approaches, such as MS, have been developed to analyse complex proteins with higher sensitivity. Additionally, Edman degradation has been developed to determine the amino acid sequence of specific proteins (Aslam et al., 2017).

Stable isotope labelling of cell culture samples in vivo (SILAC), in vivo labelling of tail regions enabling affinity isolation of proteins with isotopes (ICAT), and isotopobaric labelling for relative and absolute quantification (ITRAQ) are new quantification techniques that have emerged as a result of developments in proteomics (Cordwell, 2006).

Table 1. Techniques used in proteomic technologies

Traditional Techniques	Advanced Techniques	Quantitative Techniques	High Output Techniques	Predictive Techniques
Chromatographic techniques	Protein microarray	ICAT	X-ray crystallography	Bioinformatics analyses
ELISA	Gel-based approaches	SILAC	NMR spectroscopy	
Western Blot	Mass Spectrometry	iTRAQ		
	Edman sequence	MudPIT SPR		

4. Proteomic Studies in Animals

Proteomics is recognised as an important area of research in diagnosis and prognosis, particularly due to its potential for detecting biomarkers in humans and animals at early stages or in low quantities. Therefore, proteomics is one of the most suitable research approaches for monitoring the health status of farm animals. The biological fluids of most animals, such as milk, serum, plasma, follicular fluid, urine, saliva, tears, seminal fluid, and cerebrospinal fluid, contain a wide variety of protein concentrations that enable the characterisation of the entire proteome complex (Eckersall & Whitfield, 2011).

In animal science, proteomic approaches have been used to demonstrate changes in protein concentrations resulting from transplantation, metabolic diseases, or nutritional deficiencies. Proteome research is used in a range of areas specific to animal life, including growth, immune status, protein composition in various biological fluids, reproductive health, stress assessment, certain animal pathologies and parasitic infections (Eckersall & Whitfield, 2011)

Proteomics has focused on changes in proteome composition due to its potential to identify biomarkers for specific disorders (disease, metabolic disorder) in animal health. Proteomics has become a fundamental research approach for animal breeding and animal monitoring by contributing to the improvement of animal health, welfare and performance (Boschetti et al., 2019).

5. Proteomic Studies in Microbial Diseases

Proteomics is playing an increasingly important role in identifying infectious agents, understanding pathogenesis, and diagnosing diseases. More detailed and

comprehensive information about the proteome of any harmful agent has become possible through the combination of MS with proteomic technologies. In particular, MALDI-TOF MS has been proven to be quite useful in identifying and distinguishing bacterial pathogens. Proteomics also assists in identifying secreted proteins and their virulence-related functions (Zubair et al., 2022).

Proteomics has contributed not only to the discovery of pathogen virulence components, but also to research into the structure of pathogens, pathogenesis, disease diagnosis, and vaccine development or design (Chen et al., 2017). Proteins derived from bacteria and viruses act as virulent agents in the transmission of diseases in humans and animals. Membrane proteins, cell surface proteins, and secreted proteins are among the most important, as they play a significant role in pathogenicity and have been extensively studied using proteomic techniques. These proteins play a crucial role in the onset and progression of disease. Proteomic methods have advanced considerably over the past decade, enabling the search for these critical proteins and the investigation of their structures, molecular functions, and roles in diseases. Proteomics has been useful in identifying microorganisms that cause various diseases and their structures (Zubair et al., 2022).

The term immunoproteomics refers to the study of the protein targets of the immune system using high-throughput proteomics technologies. Immunoproteomics methods enable the understanding of how diseases develop and progress, and facilitate the identification of disease biomarkers and potential vaccine candidate molecules (Fulton & Twine, 2013).

5.1. Mastitis

Mastitis is an inflammation of the mammary glands, most commonly caused by bacterial infections (Hillerton & Berry, 2005). Mastitis causes significant costs and is becoming an increasingly serious problem for the dairy industry in many countries (Seegers et al., 2003).

Hogarth et al. (2004) investigated changes in milk protein composition during clinical mastitis using a proteomic approach to identify new diagnostic biomarkers for mastitis. Whey samples obtained from cows with clinical mastitis and healthy cows were compared using 2-DE and MALDI-TOF MS. Whey from cows with mastitis showed increased concentrations of serum-derived proteins, including serotransferrin and albumin, compared to normal cows, while concentrations of the major whey proteins α-lactalbumin and β-lactoglobulin were decreased.

Ibeagha-Awemu et al. (2010) determined the proteomic profiles of whey samples obtained from healthy cattle and cattle infected with *Staphylococcus aureus* and *Escherichia coli* using LC-MS/MS and bioinformatic analyses. They

stated that whey from *E. coli* and *S. aureus* mastitis showed a broader proteomic profile.

Kim et al. (2011) conducted a proteomic study on *S. aureus* mastitis in cattle and found that serum IL-8, IFN-γ and TGF-β1 responses differed in dairy cows infected with different *S. aureus* strains.

Alonso et al. (2012) used MALDI-TOF MS, 2-DE and WB methods to perform proteomic characterisation of serum and whey from mastitis-affected and healthy animals. As a result of the analysis, a total of 62 protein spots were identified using mass spectrometry and immunological methods. Thus, serum and whey proteins can be easily identified on gels using 2-DE.

Turk et al. (2012) utilised milk proteomes to identify distinct protein expressions that could be beneficial for a better understanding of the pathophysiology of mastitis and for the early diagnosis of the disease. Their study demonstrated that the proteomic markers they identified could be useful for detecting mastitis in its subclinical stage and for better understanding the pathophysiological mechanisms involved in the onset of the disease.

Mansor et al. (2013) demonstrated in a study using capillary electrophoresis and mass spectrometry to identify mastitis biomarkers that milk peptide biomarkers could be used in mastitis diagnosis and enable differentiation between different bacterial agents.

Franco et al. (2021) aimed to evaluate changes in the saliva and serum proteome of cows with mastitis using proteomic approaches. In saliva samples from cows with mastitis, 63 proteins showed significant differences, while in serum samples, 29 proteins showed significant differences.

5.2. Brucellosis

Brucellosis is one of the most common zoonotic diseases affecting animals and humans. In animals, it is characterised by abortion, stillbirth and reduced milk yield, causing significant economic losses.

In order to develop a safer and more effective vaccine and to identify new candidate proteins in the *Brucella abortus* cell membrane, Connolly et al. (2006) used immunoproteomic approaches to identify a total of 163 proteins.

Al Dahouk et al. (2006) aimed to perform the proteomic characterisation of various antigen preparations of the diagnostic reference strain *B. abortus* 1119-3 and to identify suitable immunogenic proteins for serological tests. Two-dimensional immunoblotting of bacteria that cross-react with *Brucella* in agglutination tests demonstrated that protein cross-reactions were negligible.

SELDI-TOF MS was also shown to clearly distinguish bacteria that cross-react with *B. abortus*.

Ko and colleagues (2012) aimed to isolate and characterise the immunodominant insoluble proteins of *B. abortus* from the sera of cattle infected with *B. abortus* and *Yersinia enterocolitica* and from the sera of uninfected cattle. The study identified 18 immunogenic proteins that could be useful resources for minimising cross-reactions in brucellosis diagnosis and for developing vaccines against *Brucella* infections.

Pajuaba et al. (2012) investigated the immunoproteomics of *B. abortus* using proteomic approaches to distinguish infected cattle from vaccinated cattle in brucellosis. In the proteomic characterisation, 56 antigenic sites were identified. The study demonstrated that immunoproteomics could be used to develop specific immunological tests for distinguishing between vaccinated and naturally infected animals.

Zhao et al. (2012) used an immunoproteomic approach to identify new candidate immunogenic proteins from *Brucella melitensis* M5 for distinguishing safe, effective, and vaccinated animals from infected animals. A total of 88 immunogenic protein spots were detected by WB analysis, and 61 of these were identified by MS analysis.

Lee et al. (2014) aimed to identify immunogenic proteins to detect *B. abortus* infection based on the time course responses of brucellosis disease, and it was demonstrated that the identified immunogenic proteins could be helpful in the diagnosis of the disease.

Kim et al. (2014) aimed to identify new candidate antigens that could be valuable in the diagnosis of brucellosis from *B. abortus RB51*, a mutant strain lacking the LPS component, and identified 11 immunoreactive proteins. These immunogenic protein sites have been shown to be usable as alternative antigens for brucellosis and to be helpful in reducing cross-reactions.

Faria et al. (2020) analysed the immunoproteomic profile of *B. abortus 2308* using pooled sera from S19-vaccinated, RB51-vaccinated, naturally infected *B. abortus*, and unvaccinated seronegative animals via the 2D WB method. The results of this study demonstrated that the combined use of MDH and SOD proteins in LPS-free protein-based serological diagnosis could be successful both for detecting bovine brucellosis and for distinguishing vaccinated animals from naturally infected animals.

Tian et al. (2020) used infected animals to evaluate antibody production against the OMP16, BP26, BLS, BCSP31, virB12, SodC and GroEL proteins and

investigated their applications in brucellosis diagnosis. The study demonstrated that BP26 and BLS are the two most immunogenic proteins.

Kornspan et al. (2021) used MALDI-TOF MS to identify protein biomarkers for the differentiation of *B. melitensis Rev 1* vaccine strains from *B. melitensis* field strains.

Paci et al. (2021) aimed to identify new immunogenic antigens using proteomic approaches to develop new serological tests for the diagnosis of *B. melitensis*, and 12 potential candidate protein antigens were identified in the study.

5.3. Tuberculosis

Bovine tuberculosis (bTB) is a chronic infectious disease caused by *Mycobacterium bovis*, affecting cattle, other domestic and wild animals, and humans. This disease causes economic losses in livestock farming and poses a health risk to communities consuming animal-derived products.

Cho et al. (2009) purified antigenic proteins and applied them to the bTB ELISA, identifying them through proteomic analysis. The culture filtrate protein of *M. bovis* was fractionated by column chromatography, and its antigenicity was examined by immunoblotting. Antigenic proteins were identified by mass spectrometry using peptide fingerprinting.

Gao et al. (2019) aimed to compare the differences in protein profiles between nested PCR-positive and nested PCR-negative infected cattle and to screen for biomarkers that would facilitate this, with the goal of early and accurate detection. The sera and PPD-stimulated plasma of infected and uninfected cattle were investigated using iTRAQ and LC-MS/MS. This study demonstrated that PPD-B-stimulated IL-8 and CRP tests could be used to detect bTB and differentiate between nested PCR-positive and nested PCR-negative infected cattle.

In a proteomic study based on 2-DE and LC/MS-MS performed with *Mycobacterium tuberculosis* H37Ra and H37Rv strains, it has been reported that the mmsA and pntAa proteins may be potential biomarkers (Kai-Cheen & Lay-Harn, 2018).

5.4. Paratuberculosis

Paratuberculosis is a disease in ruminants characterised by chronic granulomatous enteritis, lymphadenitis and weight loss, caused by *Mycobacterium avium subsp. paratuberculosis (MAP)*.

Zhong et al. (2011) used SELDI-TOF MS to identify candidate biomarkers in sheep serum. SELDI-TOF MS was used to monitor changes in protein profiles over time during an experimental infection by analysing serum samples collected

at 4, 8, and 13 months of infection. Nine proteomic structures associated with the disease were observed.

Pisanu et al. (2017) investigated proteomic changes in the ileum of sheep infected with paratuberculosis. Using proteomic methods, a total of 2,889 proteins were expressed, 341 of which were at high levels in animals infected with paratuberculosis. Twenty-eight MAP proteins were identified in the study.

5.5. Respiratory System Infections

Zhao et al. (2012) used MALDI-TOF MS to identify immunogenic proteins from isolates of *Mycoplasma capricolum subsp. capripneumoniae.* DE identified 20 immunogenic proteins from whole cells, while 9 were identified from membrane proteins. These results indicated that membrane proteins are the primary immunogenic proteins in *Mycoplasma capricolum subsp. capripneumoniae*. These proteins may have potential for the development of improved diagnostic tests and vaccines.

Corona et al. (2013) used immunoproteomic approaches to identify immunodominant proteins from *Mycoplasma mycoides subsp. capri* isolates. As a result, a total of 27 immunoreactive spots corresponding to 13 different proteins were identified using nanoLC-ESI-MS/MS. These proteins were shown to be biomarkers for the serological diagnosis of infectious agalactia.

Cacciotto et al. (2021) aimed to identify surface antigens of *Mycoplasma agalactiae* using an immunoproteomic approach. Immunodominant antigens (such as MAG_1000 and MAG_2220) were identified using MALDI-TOF-MS. The panel of immunodominant conserved antigens identified in this study will contribute to the development of diagnostic tools and effective vaccines with higher sensitivity and specificity across all natural infection stages.

A MALDI-TOF MS based proteomic study for the detection of *Mycoplasma bovis* biomarkers reported that the lipoprotein MbovP579 was a sensitive and specific antigen for the detection of antibodies in sera from both *M. bovis* infected and vaccinated cattle (Khan et al., 2016).

Ayalew et al. (2010) identified 55 proteins with immunogenic properties in the outer membrane of *M. haemolytica* using 2-D PAGE immunoblot analysis, MALDI-TOF MS and LC MS/MS, and suggested that these proteins could be used in the production of new vaccines in the future.

Boehmer et al. (2011) used nano-LC MS/MS on bronchoalveolar lavage samples from animals with experimental *M. haemolytica* infection. They identified several proteins in the lavage found only in this bacterium. These included molecules such as antimicrobial peptides, acute-phase proteins, and protease inhibitors.

5.6. Other Infections

Serotyping of *Salmonella enterica* by MALDI-TOF MS using 12 biomarker proteins (S8, L15, L17, L21, L25, S7, SodA, peptidylprolyl cis-trans isomerase C, Gns, YibT, YaiA, and YciF) has been presented (Fukuyama et al., 2019).

In a proteomic study conducted to identify the biomarkers of *Bacillus anthracis*, the causative agent of anthrax, four different proteins were reported to be suitable, thus highlighting their value for the more efficient and specific detection of B. anthracis spores (Chenau et al., 2014)

A study conducted with the *Leptospira spp.* LipL32 protein has revealed the proteomic characterisation of this protein, suggesting that it could enable the identification of potential antigen candidates in the future, as well as the rapid and reliable detection of pathogenic *Leptospira* species (Kumaran et al., 2017).

Six biomarkers were identified in a proteomic study based on protein microarray and immunoblotting performed to detect immunogenic proteins of *Campylobacter jejuni* (Liu et al., 2019).

Coxiella burnetti, the causative agent of Q fever, has been identified as a potential candidate biomarker through proteomic studies (Gerlach et al., 2017).

6. Conclusion

There is a need for better biomarkers in veterinary medicine for the diagnosis and prognosis of diseases. Compared to human medicine, proteomics studies in veterinary medicine still lag behind. As technology becomes more applicable in studies designed to investigate and explain the pathology of diseases in veterinary medicine, it is clear that valuable information about the molecular mechanisms of relevant animal diseases is being produced and will continue to be produced in the future.

Proteomics plays an important role in the identification and differentiation of bacterial infections, as well as in understanding their pathophysiology and diagnosis. Researchers have been able to detect infections more effectively by using different proteomic methods together and have been able to identify and characterise proteins that play a role in pathogenicity. In the long term, proteomics is expected to contribute to increasing general knowledge about microbial diseases. In the future, as technological advances increase, the efficiency of methodologies will also increase, contributing to the provision of new information.

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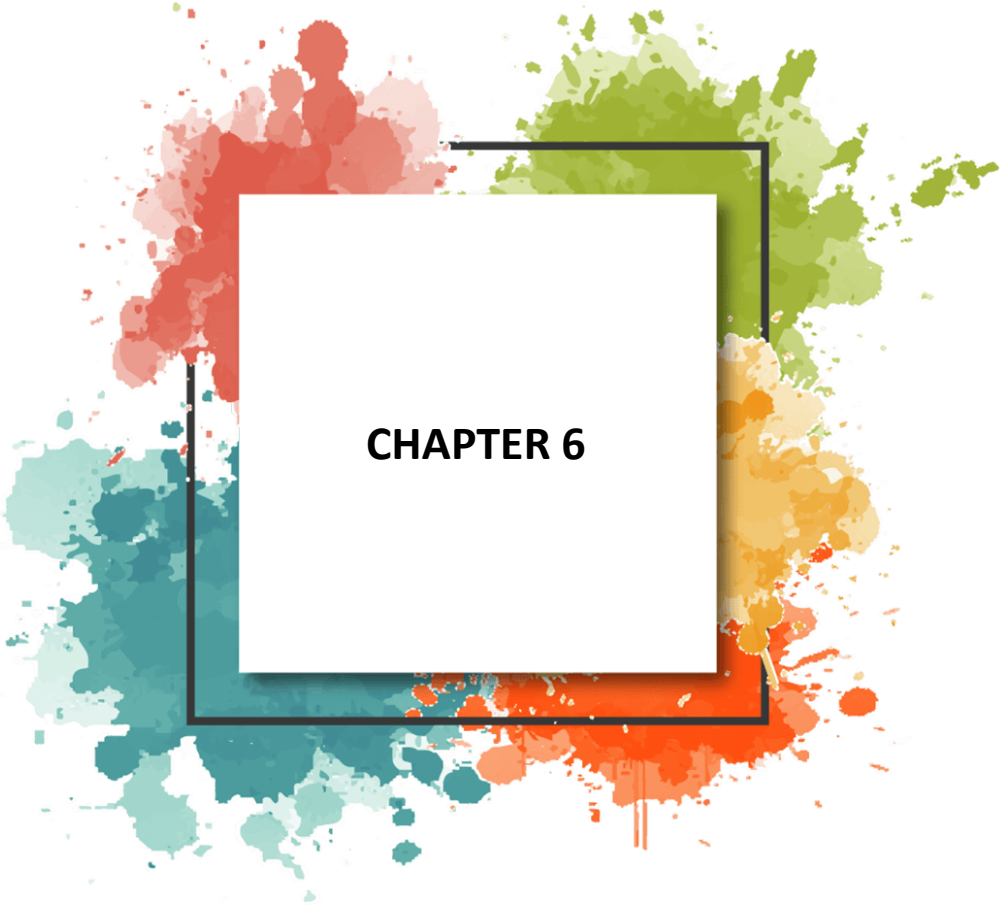
CHAPTER 6

Use of Herbal Extracts and Essential Oils in Broiler Chickens

Muhammet Gören[1]

1. INTRODUCTION

Feed additives are defined as substances preferred to increase the feed utilisation rate, improve the yield and quality of animal products, protect the health of animals and reduce the cost of the product (Kahraman 2009). The continuous development of the poultry sector has led to diversification in feed additives used to increase productivity. The use of antibiotics as feed additives has led to an increase in their preference as performance-enhancing products, particularly in the poultry sector. However, the excessive use of antibiotics in this way has caused various problems (Bayırbağ 2007). Excessive consumption of antibiotics used for treatment in humans, growth and treatment in animals has resulted in deterioration in microflora as a result of the development of antibiotic resistance against harmful bacteria. Subsequently, some antibiotics used in poultry rations were banned in the EU in 1999. Later, in 2006, it banned the use of all antibiotics as feed additives in all farm animals. As a result of these situations, the need for alternative additives has emerged. Organic acids, probiotics, prebiotics, plant extracts and essential oils have been tried to be evaluated as alternatives (Gül et al 2012). The most important benefit of these feed additives is their stabilisation in the digestive system. As a result of the antimicrobial effect, feed utilisation and disease control are the main factors that provide efficiency in animal production. Recently, plant extracts and essential oils that can be preferred instead of antibiotics as growth factors have been obtained (Kahraman 2009). Studies on these aromatic plants and their extracts and essential oils have focused on these feed additives due to their antioxidant, cholesterol-lowering, cancer-preventing effects (Yıldırım and Erener 2010).

2. FEED ADDİTİVES

The use of feed additives has important purposes and features. Controlling pathogenic bacteria such as salmonella and coli, on the other hand, regulating the intestinal microflora with digestive microorganisms are the prominent ones (Gül et al 2012). Apart from this, they are used to increase product quality, contribute to animal health, and make growth and development faster and more efficient. In

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addition to Hutjens's 4R principles, which are responsive, responsive, researchable, and having results, it is important to be reliable, repeatable, and relative (Hutjens 1991). Considering these features, feed additives should not adversely affect animal health, should not leave residues in animal tissues and products, should have known toxic limits, should not pollute the environment, and should be economical when used as a performance enhancer in animals. There are many types of feed additives in terms of their functions, effects and purposes. These can be listed as follows: Feed additives related to feed technology are denaturants, antioxidants, preservatives, binders, emulsifiers, coagulants, stabilisers, thickeners, anti-caking agents, radionuclear contamination inhibitors, acidity regulators, antifungals and silage additives. Colorants and flavorings are sensory feed additives. Energy providers, trace elements, vitamins, urea, amino acids, and other NPN compounds are considered nutritional feed additives. Biotechnological additives that increase feed utilization include probiotics, prebiotics, enzymes, organic acids, essential oils and plant extracts (phytobiotics), immune system regulators, molting agents, fly control agents in fertilizers, and anticoccidials (Ergün et al. 2002).

3. HERBAL EXTRACTS AND ESSENTİAL OİLS

The presence of the products to be used for feed additives in feeds and products to be obtained and the control of the effects of these products on human health are important. The preference for natural and organic products in animal production leads to uncertainties for feed additives. However, new ideas are being tried to eliminate the negative effects of antibiotic use by using alternative feed additives (Kahraman 2009). One of these ideas is the use of natural feed additives such as plant extracts and essential oils as performance enhancers for poultry. They have antioxidant, antibacterial, antioxidant and antibacterial effects on the feed utilisation of animals, overcoming stress conditions easily (Köksal and Küçükersan 2012). In this sense, the evaluation of active substances in plants and their essential oils in terms of animal health has come to the fore again. Therefore, feed additives of this nature are safe in terms of chemical structure and are utilised in the food industry and in many sectors (Simsek et al 2005). With the increasing preference for antibiotics in poultry farming, plant extracts have become prominent as alternative additives (Çetin and Yıldız 2004). The use of plant extracts in poultry diets has been observed to have positive effects on feed conversion, carcass, feed conversion and mortality (Jamroz and Kamel 2002, Çabuk et al. 2003).

4. GENERAL PROPERTİES OF HERBAL EXTRACTS AND ESSENTİAL OİLS

There are about 300 plant families in nature and 1/3 of them contain essential oil. The odour and good effects of aromatic plants are caused by the oils they contain. These oils are oily compounds that are fluid at room temperature, have a strong odor, and can evaporate. Essential oils, on the other hand, have a very complex and variable structure. Aromatic plants are shaped during the flowering period. There are approximately 9000 plant species in Turkey, 3000 of which are used for medicinal purposes and as spices. These plants, used in different ways, are effective with the active ingredients they contain (Adıyaman and Ayhan 2010).

5. EFFECTS OF ESSENTİAL OİLS

5.1. Antioxidant Effects

Antioxidants are used to stop and promote the oxidation of fats and fatty acids. Synthetic antioxidants such as butyratet hyroxyanol, butyratet hyroxytoluene were previously used as additives. Among phytogenic plants, thyme, rosemary and thymol come to the fore. Thyme, rosemary, thymol in phytogenic plants come to the fore. Antioxidants contribute with their properties of inhibiting free radicals, reducing agents and oxygen formation. In this way, it protects the permeability of the cell membrane with lipid peroxidation in the feed. In the study conducted in poultry meat, it is seen that thymol and carvacrol antioxidant effects are similar to synthetic antioxidants, increase antioxidant enzyme activity, prevent fat oxidation, and positively affect the activity of digestive enzymes, immune system and liver values. (Tekce and Gül 2016).

5.2. Antimicrobial Effects

The remarkable aspect of essential oils is their antimicrobial activity. Since these oils are complexes containing different components, their degree of effect varies according to the type and amount of the factor they contain. Its mechanism of action is thought to be related to its lipophilicity and chemical complexes (Bayaz 2013). Essential oils have an effect on microorganisms such as Gram (-) and Gram (+) bacteria. Like carvacrol and thymol and cinnamaldehyde, it has antimicrobial effect on Escherichia coli O157 and Salmonella typhimurium. Active substances such as carvacrol and thymol contribute by breaking down the bacterial membrane, and terpenoids and phenylpropanoids contribute by piercing the bacterial wall with their lipophilic properties (Bayaz 2013).

It is seen that there are additive, antagonistic and synergistic effects between the components of essential oils. In one study, the combined effects of thymol and carvacrol on Staphylococcus aureus and Pseudomonas aeruginosa were superior (Lambert et al 2001).

Cinnamaldehyde in cinnamon has been observed to inhibit bacteria such as Clostridium perfringens, Bacteroides fragilis, Bifidobacterium longum and Lactobacillus acidophilus at certain levels. Lichen, myrtle and clove extracts have been shown to have effects on bacteria such as 'Bacillus megaterium, Bacillus subtilis, Bacillus brevis, Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae, Pseudomonas aeruginosa, Listeria monocytogenes and Staphylococcus aureus' (Helander et al. 1998).

Some plant extracts showed fungistatic, fungicidal effect on molds. Antifungal compounds vary according to essential oils in terms of quantity and distribution. Antiviral activities of essential oils were also observed (Bayaz 2013).

5.3. Antiparasitic Effects

The effects of thyme oil and lasalocid, which are the antiparasitic effects of essential oils, on Eimeria tenella were examined and the effect of Eimeria oocysts was observed. However, it has been found to be less effective than antiparasitic drugs. On the other hand, carvacrol, thymol, isopulegol, and eugenol were observed to be effective against Eimeria oocysts in vitro. A mixture of cinnamon, lemon, and garlic was found to be effective against Tetratrichomonas gallinorum and Histomonas mellagritis (Tekce and Gül 2016).

5.4.Mutagenic Effect

Irreversible base changes in genetic material caused by a chemical agent or occurring spontaneously are called mutagenic effects. The recent increase in mutations and cancers has increased the need for research on anticarcinogenic and antimutagenic agents. Although not all mutagenic agents cause cancer, mutation analyses are among the prominent studies on cancer due to the possible parallel link between mutagenicity and carcinogenicity. Because of their reliability, the essential oils of Mentha spicata L. (curly mint) and Pinus sylvestris L. (Scots pine) are preferred in many places as additives in sectors such as food, beverages, detergents, cosmetics, soaps, and as pest repellents in agriculture. These oils have been observed to have a mutagenic effect on the vinegar fly and immune-enhancing lymphocytes. Furthermore, the active ingredients carvacrol,

thymol, and cinnamaldehyde have been reported to be weak mutagenic in small doses (Bayaz 2013).

5.5. Antimutagenic Effect

Antimutagens, which have the effect of inactivating the mutagen, changing and stopping the mutation in genes, are examined in two parts as desmutagens and bioantimutagens. Desmutagens are substances that block the entry of mutagens into cells. One study found that marjoram essential oil had no mutagenic effects against some Salmonella strains. However, different studies have shown that carvacrol, the active ingredient of marjoram, produces a desmutagenic effect as a result of stopping carvacrol by other components in this oil, which mutates at the rate tested (De Martino et al 2009).

Bioantimutagens are substances that inhibit mutation by incorporating the mutagen into the DNA structure, disrupting DNA replication and repair mechanisms. Bioantimutagens work by increasing the production of DNA polymerases I and III and by inhibiting DNA repair mechanisms that may be faulty (Özbek 2006). In a study, the antimutagenicity of sage essential oil was also investigated in E. coli with and without UV and it was found that the monoterpenes in it affect DNA repair by regulating it (Vuković-Gačić et al 2006).

SOS chromotest was performed on E. coli with myrtle essential oil and it was found that this oil had antigenotoxic effect on Aflatoxin B1 and nifuroxazide (Hayder et al 2004). The essential oil of Izmir thyme was investigated by Ames Salmonella/microsome test and found to be antimutagenic (Ipek et al 2005).

Herbal extracts affect feed consumption and flavour with direct effect on taste and smell. Extracts of aromatic plants such as garlic and mustard had a positive effect on digestion. In addition to these effects, they have also been found to have sedative, antimicrobial, fungicidal, and antioxidative effects, depending on the chemicals they contain. One of the important properties of extracts such as rosemary and thyme is their antioxidant activity (Yeşilbağ 2007).

6. Main Aromatic Plants from which Essential Oil is Obtained and Their Properties

6.1. Oregano

Thyme used as seed has digestive stimulant and antiseptic, antimicrobial, anticoccidial, antifungal and antioxidant effects (Güler and Dalkılıç 2005). Carvacrol and thymol, which are mostly antibacterial compounds, are present in thyme essential oil. Carvacrol, which is flavouring and appetite enhancer, causes pathogenic microorganisms to die by breaking down their stoplasmic membranes

(Parlat et al 2005). "The microorganisms it is effective on are E. coli, S.typhimurium, C.perfingens, S.aureus, P.aeruginosa, E.aerogenes, P.vulgaris, C.albicans, C.tropicalis, P.membranea, B.subtilis, Enterococus fesialis, Penicilium digitatum, Listeria monocytogenes, Aeromonas caviae, Lucilia. merciata, A.flavus, A. Pariticus, A.Ocraceus, Fusarium, Ochratoxin A, E.tenella (Güler and Dalkılıç 2005)."

6.2. Sage

This plant, the active ingredient of which is Cineol, usually used in leaves, has digestive stimulant and antiseptic, antioxidant and antidiabetic effects. "They are effective: E.coli, S.typhimurium, P.aeruginosa, P.digitatum."

6.3. Bay

This plant, whose active ingredient is cineole, whose leaves are generally used, has appetite-stimulating, digestive, antiseptic, bactericidal, and bacteriostatic effects. The microorganisms it is effective against are: C. botulinum, S. typhimurium, C. albicans, E. coli, B. cereus, and L. Monosytogenes.

6.4. Clove

The active ingredient Eugenol, whose leaves, flowers and seeds are generally used, has appetizing, digestive stimulant, antiseptic, antimicrobial, bactericidal and bacteriostatic, antifungal and antioxidant effects. Microorganisms it is effective against: A.flavus, L.monocytogenes, Basillus cereus.

6.5. Coriander

The plant, whose active ingredient is linalool, is generally used in the leaves and seeds; it has appetite-enhancing and digestive-stimulant, antidiabetic, antifungal, antioxidant, hypolipidemic, antimicrobial, hypocholesterolemic, and anticonvulsant effects. "The microorganisms it is effective on are: Gram-positive and Gram-negative bacteria, S. cerevisiae, and E. coli."

6.6. Mint

Menthol, the active ingredient of the plant, whose leaves are generally used, is an appetite enhancer, digestive stimulant, antiseptic and antifungal. "Microorganisms it is effective against: A.flavus, A. Pariticus, A. Ocraceus, Fusarium, Aspergillus ochreceus, Ochratoxin A (Güler and Dalkılıç 2005)." In order to support growth in broiler chickens, virginiamycin, mint (Mentha piperita) ethanol extract, ethanol extract in drinking water in some groups, carcass

yield was positively affected positively and abdominal fat ratio was negatively affected compared to other groups (Çetin 2016).

6.7. Coconut

The active ingredient Sabinene, whose seed is generally used, is digestive stimulant, antidiarrhoeal and antimicrobial. Microorganisms it is effective against: L. monosytogenes, Basillus cereus.

6.8. Cinnamon

This plant, whose bark is generally used and contains the active ingredient Cinnmaldehyde, is effective in increasing appetite, stimulating digestion, antibacterial, antifungal. "Effective microorganisms: E.coli, S.typhimurium, A.parasiticus, A.flavus, A. Pariticus, A. Ocraceus, Fusarium, L.monocytogenes, Basillus cereus."

6.9. Cumin

The plant, whose active ingredient is Cuminaldehyde, is generally used as a seed, and has digestive stimulant, bronchodilator, ulcer prevention and antibacterial effects. Effective microorganisms: B. subtilis, E. coli, P. aeruginosa (Çabuk et al 2003).

6.10. Anise

The plant, whose active ingredient is Anothole, whose seeds are generally used, has digestive stimulant, antiparasitic, antibacterial and antifungal effects. The microorganisms it is effective on are: C. tropicalis, P. membrane, S. cerevisiae, A. flavus, A. parasiticus, A. ochrsceus, and fusarium.

6.11. Parsley

Apiol, the active ingredient used mainly in its leaves, has an appetite-stimulating, digestive, and antiseptic effect. Effective against: K. apicula, R. glutinis (Çabuk et al. 2003).

6.12. Rosemary

Cineol, a substance generally used in its leaves, has digestive stimulant, antiseptic, antimicrobial, and antioxidant effects. Effective microorganisms: B.cereus, S.aureus, Listeria monocytogenes, S.mutants, P.digitatum, Pseodomonas fluorescens, Carnobacterium piscicola, Lactobacillus curvatus.

6.13.Blackpepper

Piperine, the active ingredient commonly used as a fruit, has digestive, antimicrobial, anti-inflammatory, antifungal, larvicidal, and antidiabetic effects. It is effective against: C. botunilum, S. aureus, and Aspergillus flavus

6.14. Horseradish

The plant, whose root is generally used, has an appetising and digestive stimulant effect with the active ingredient called Allylisothiocyanate. Effective microorganisms: S.aureus (Çabuk et al 2003).

6.15. Mustard

The plant, the active ingredient of which is Allylisothiocyanate, is an appetite stimulant, digestive stimulant and antioxidant. Effective microorganisms: S.aureus.

The plant, which contains the active ingredient Allicin, is generally used in its onion and has digestive, antiseptic, anticholesterolemic, antifungal, and antioxidant properties. The microorganisms it is effective against are: S. typhimurium, E. coli, B. cereus, L. plantarum, B. subtilis, Aspergillus niger, and Aspergillus flavus.

6.17. Basil

Its leaves are generally used. It has antimicrobial, antifungal, antioxidant, and anti-stress effects. The microorganisms it is effective against are: L. monocytogenes, A. flavus, A. pariticus, A. ocraceus, and Fusarium.

6.18. Ginger

It has digestive stimulant, blood circulation stimulant, antimicrobial, antioxidant, antidiabetic and antifungal effects with its active ingredient Zingorole, whose rhizome is generally used. Effective microorganisms: Helicobacter pylori, Rhizoctonia solani.

6.19. Celery

Phtalides, whose leaves are generally used, has digestive stimulant, appetite stimulant, nematicidal, antifungal and antioxidant effects. Microorganisms it is effective on: A. flavus, A. pariticus (Güler and Dalkılıç 2005).

6.20. Black cumin

One of the alternative essential oils is found in black cumin. The plant and its seeds contain alkaloids, fixed and volatile oils, and many other active ingredients.

It has been observed that the use of black cumin seeds in poultry rations increased egg laying performance, and the addition of extract to quail feeds increased productivity and positively affected egg shell weight, shell thickness, albumin height and egg yolk values (Erhan et al. 2009).

7. Studies Using Herbal Extracts and Essential Oils

In order to see the effects of menthol and carvacrol essential oil on development, carcass yield and digestion in broiler chickens, it was observed that the live weight and feed conversion rate were better in the carvacrol group. The control and carvacrol groups had better carcass yields than the menthol group. The other groups had reduced abdominal fat compared to the carvacrol group. These conditions show that carvacrol has a more positive effect on growth performance in broiler chickens than the menthol group, although it has no effect compared to the control group (Erener et al. 2005).

An evaluation of the oxidative deterioration of meat products obtained by adding rosemary and its essential oil to feed showed a strong antioxidant effect. Furthermore, the study revealed that the oxidation rate in chicken carcasses was reduced, resulting in longer shelf life (Yeşilbağ et al. 2009).

It has been stated that rosemary is increasingly preferred in organic chicken farming due to its effects on feed consumption, live weight and feed utilization in laying and broiler chickens (Griggs and Jacob 2005).

It has been reported that grape seed has a positive effect on meat quality (colour, pH) and is used as an antioxidant without any negative effect on performance in broiler chickens (Turan and Öztürk 2010).

Due to its strong effects on bacteria, it was observed that rosemary extract inhibited the progression of pathogens such as E.Coli, Salmonella, Clostridium and had a positive effect on live weight, feed intake and feed conversion performance (Ross et al 2001).

The use of garlic and cumin powder in broiler rations decreased abdominal fat. Garlic added to the ration had a positive effect on meat quality and flavour and kept oxidation in meat at lower levels during shelf life (Onibi et al 2009).

Essential oils of bay laurel, thyme, clove, and oregano have been shown to be effective against E. coli. The bacteriostatic and bactericidal effects of bay laurel and thyme were highest, followed by bay laurel and clove (Burt and Reinders 2003).

It has been determined that substances such as "carnosol, carnosic acid, rasmanol, epirasmonol, isorasmonol, rasmaridiphenol, rasmaidal and miltiron"

from rosemary plant have antioxidant properties and among these, carnosol, carnosic acid and rasmanol have high levels in terms of antioxidant effect (Ho et al 2000).

Improvements in feed efficiency and live weight were observed in broiler chickens when pepper, cinnamon, and thyme extracts were added to the groups. It has been suggested that these additives could be used instead of antibiotics (Jamroz and Kamel 2002).

Supplementation of a mixture of garlic, anise, cinnamon, rosemary and thyme extracts to broiler chickens was observed to increase live weight and reduce mortality compared to other groups, and it was observed that it had an effect on E. coli in the digestive system (Tucker 2002).

In the study in which black cumin, thyme, ginger, fennel, rosemary and their extracts were used in broiler chickens, it was observed that feed intake, live weight gain and liver weight were the highest in the group with thyme extract, the lowest enterobacter count and carcass yield were the highest in fennel extract, and liver weight was lower in thyme and rosemary (Avci 2004).

It has been determined that the live weight gain in broiler chickens was better in rations containing garlic and mixtures of herbal extracts such as garlic, horseradish, juniper, milk thistle, thyme and yarrow (Lewis et al. 2003).

In broiler chickens, the addition of avilamycin, thyme, cinnamon and pepper essential oil extracts and sage tea, thyme, rosemary essential oil extracts and antibiotics and plant extracts positively affected dry matter digestion in the digestive system, but had no effect on crude protein digestion and digestive organ weights (Hernandez et al 2004). It was determined that the use of different amounts of thyme and thyme essential oil in broiler chickens caused a decrease in daily feed consumption, while thyme essential oil had a positive effect on feed conversion and did not affect carcass characteristics (Halle 2001).

Flavomycin, thymol and garlic enzyme and non-enzyme interactions decreased the small intestine weight of broiler chickens fed with wheat-dominant mixtures and increased the small intestine length in the control and garlic groups. Decreases in the total number of aerobic bacteria and E. coli in the small intestine were observed in those using thymol and garlic (Sarica et al. 2005).

With the addition of coral lodge extract to the broiler ration, no change was observed in performance parameters such as broiler carcass yield, but increases in feed consumption and feed utilization were observed (Gemici 2008).

In the study on intestinal microflora in broiler chickens, herbal extract supplementation had an effect on the number of total anaerobic, lactic acid, coliform and C. perfringens bacteria in the intestines of broiler chickens. An increase in lactic acid bacteria and a decrease in others were observed with the additive used. A tendency to decrease ileum pH values was observed with the use of herbal extracts (Vidanarachchi et al 2006).

Yucca schidigera, Oreganum vulgare, Thymus vulgaris, Syzygium aromaticum, Zingiber officinale were used to determine the possibilities of using herbal extracts and propolis as growth factors instead of antibiotics. The group with Z.officinale supplementation had a positive effect on live weight gain and feed conversion ratio, and the number of intestinal lactic acid bacteria increased. As a result of this experiment, S.aromaticum (clove) and Z.officinale (ginger) herbal extracts were found to be superior. The use of Z. officinale positively affected the performance and intestinal villi length of broiler chickens. It has been shown that Z. officinale essential oil and propolis, as herbal extracts, can be used instead of antibiotics (Tekeli 2007).

Better live weight and feed consumption were observed in the group fed the diet containing different amounts of Aloe vera extract. Furthermore, the Aloe vera extract group had the longest intestinal length and crypt width compared to the antibiotic group (Darabighane et al. 2011).

The effects of plant extracts and an antibiotic on performance, immunity, blood values and gut in broiler diets were investigated. It was determined that especially garlic extract improved the feed utilisation rate when compared with virginiamycin. In addition, garlic extract has been found to significantly reduce serum cholesterol levels (Rahimi et al 2011).

In different studies, it was observed that the addition of thyme essential oil instead of antibiotics to broiler diets had a positive effect on daily live weight gain (Hertrampf 2001, Jamroz and Kamel 2002).

Addition of yucca extract to broiler diets at different levels significantly increased the feed consumption of broilers (Kutlu 1999, Özkaya and Kaya 2005).

In the effects of probiotic, organic acid and vegetable essential oils on broiler chickens, feed intake increased better in the essential oil supplemented group (Alçiçek et al 2004).

In studies investigating the effects of plant extracts on feed utilisation, it was observed that plant extracts improved feed consumption (Avci 2004, Tekeli 2007).

Although live and organ weights were not affected by the essential oil mixture added to the ration, it was observed that daily feed intake decreased and feed conversion ratio improved positively (Cabuk et al 2006).

In a study with similar results in terms of feed intake and feed conversion ratio, it was observed that black cumin oil positively affected live weight, feed conversion and carcass yield (Çelik and Şahin 2015).

In poultry, weight gain occurs as a result of thickening of the intestines as a result of the increase in pathogenic microorganisms in the intestines due to temperature stress. "In one study, essential oils were found to be effective against bacteria such as Listeria monocytogenes, Salmonella typhimurium, Escherichia coli, Bacillus cereus and Staphylococcus aureus" (Karslı and Dönmez 2007).

Thyme, laurel, fennel, sage tea, myrtle Thyme, laurel, fennel, sage tea, myrtle leaves and orange peel oil were used as feed additives due to their positive effect on feed utilisation, increasing the edible parts in the body, and improving the performance of essential oils of plants (Küçükyılmaz et al 2012).

It was observed that dry thyme or mint leaves had a positive effect on live weight gain in broiler chickens. However, it was found that abdominal fat ratio increased (Ocak et al 2008).

It was observed that the addition of purslane extract to the diet increased live weight and decreased feed conversion, but positively affected intestinal microflora (Zhao et al 2013). They found that the extract of green parts of purslane contains high amounts of phenolic compounds and vitamin C and has the ability to bind free O2 (Konca et al. 2015).

It was found that the feed conversion ratio of Siberian ginseng addition to the mix of commercial broiler chickens was higher than the control group and it positively affected the immunity of broiler chickens (Sohn et al 2008).

8. CONCLUSION

Herbal extracts and essential oils are substances that should be emphasised and researched especially because they are feed additives that can be used instead of antibiotics. Many studies have been carried out and its use in both animal and human health will be made more efficient. Studies show that essential oils and herbal extracts have an important place as feed additives. There are studies showing that the use of herbal extracts in compound feed affects the feed utilisation rate (Erhan 2015). Especially in the poultry sector, it has been reported that live weight is positively affected by the addition of herbal extracts in broiler chick feeds (Alçiçek et al 2004, Avci 2004). Since it can be used for preventive, performance enhancing and improving the quality of animal products, more effective results should be tried to be obtained by focusing on more studies.

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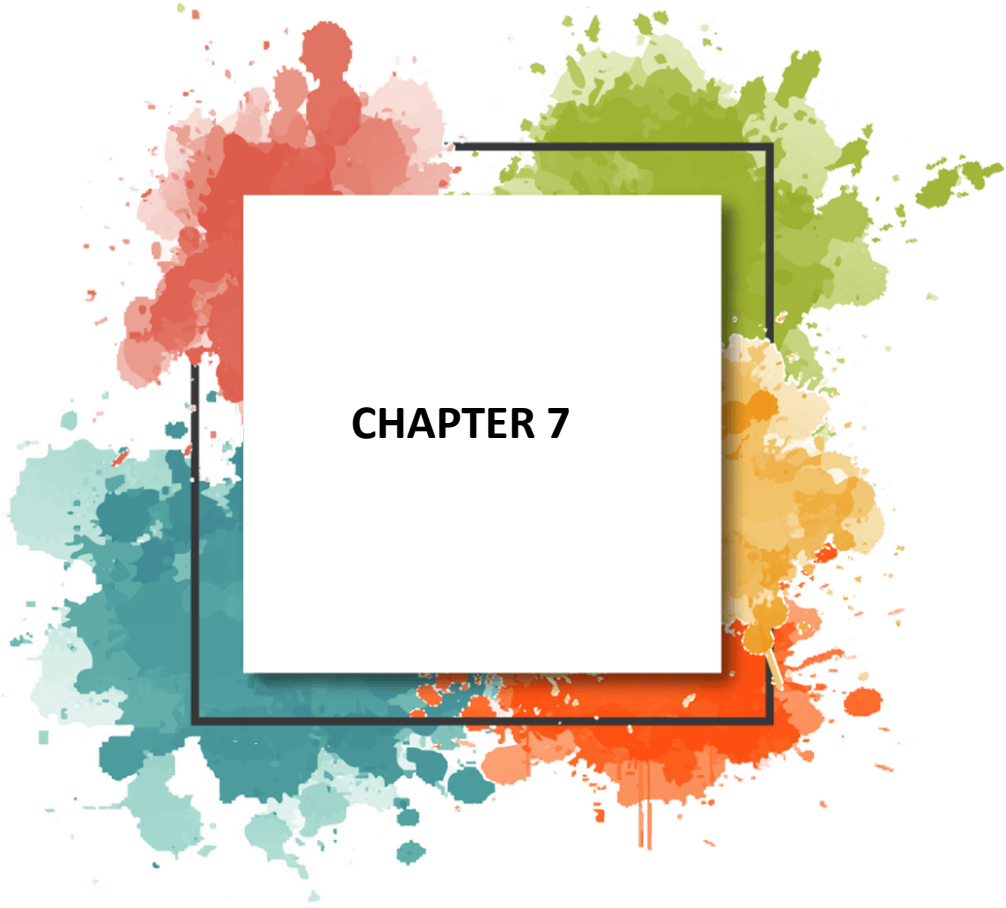
CHAPTER 7

Lactobacillus and Recombinant DNA Technology: Molecular Approaches and Biotechnological Applications

Ayşe Şebnem Özalp Erenler[1]

1. Introduction

The genus *Lactobacillus* consists of Gram-positive, facultatively anaerobic, catalase-negative, and generally rod-shaped bacteria. These microorganisms naturally inhabit diverse ecosystems, particularly the gastrointestinal, oral, and vaginal microbiota of humans and animals (Zheng et al., 2020). Their long-standing use in fermented food production and probiotic formulations has rendered them an important biotechnological resource in both the food industry and healthcare (Marco et al., 2021).

Recombinant DNA technology, which enables the manipulation of an organism's genetic material under laboratory conditions, represents a fundamental approach in genetic engineering. This technology encompasses several key stages, including the isolation of target genes, their cloning into suitable vectors, transfer into host cells, and the subsequent acquisition of the desired phenotype. Due to their well-established safety profile (Generally Recognized as Safe, GRAS status) and beneficial interactions with the human immune system, *Lactobacillus* species have attracted considerable attention as host systems in recombinant DNA applications (Plavec & Berlec, 2019).

Genetically engineered *Lactobacillus* strains have been exploited for the production of therapeutic proteins, antigens, and metabolites, as well as for the delivery of these biomolecules to the mucosal immune system (Spangler et al., 2021). In recent years, the integration of next-generation genome-editing tools, such as CRISPR-Cas systems, into *Lactobacillus* genome engineering has enabled significant advancements in both efficiency and target specificity (Mu et al., 2022).

Thus, the integration of recombinant DNA technologies with the genus *Lactobacillus* extends the conventional role of probiotics, paving the way for a broad range of biotechnological applications, including vaccine delivery vehicles, live biotherapeutics, and metabolic engineering platforms.

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2. Genetic Characteristics of *Lactobacillus*

The genus *Lactobacillus* represents a taxonomically broad and genetically diverse group. In 2020, a comprehensive taxonomic revision reclassified 261 species previously included under this genus into 25 distinct genera based on phylogenetic relatedness (Zheng et al., 2020). This reclassification marked a critical milestone in understanding the genetic organization, ecological niches, and metabolic capacities of *Lactobacillus* species.

2.1. Genome Structure and Size

The genome size of *Lactobacillus* species ranges from approximately 1.8 to 3.3 Mb, with a G+C content typically between 32% and 53% (Kant et al., 2011). This relatively low G+C content can influence strategic decisions in genetic engineering applications, such as promoter selection, codon optimization, and vector design (Plavec & Berlec, 2019). Many species harbor plasmids that encode essential traits, including carbohydrate metabolism, stress tolerance, antibiotic resistance genes, and conjugative transfer systems. The presence of plasmids is advantageous for recombinant DNA technology, as these naturally stable extrachromosomal DNA elements provide suitable platforms for target gene expression. Some *Lactobacillus* strains harbor multiple plasmids, thereby increasing the potential for multigene carriage (Davray et al., 2020).

2.2. Genetic Diversity and Adaptation

Lactobacillus genomes have evolved to enable high adaptability to environmental conditions. Species possess specialized gene clusters adapted to their ecological niches, such as sugar utilization genes, acid tolerance genes, and antimicrobial peptide production genes . For instance, species inhabiting the gastrointestinal tract carry bile salt hydrolase (*bsh*) genes, which confer resistance to bile salts (de Wos et al., 2011).

2.3. Natural Transformation and Genetic Manipulation Potential

Although natural transformation in Lactobacillus is generally limited, certain strains have demonstrated the ability to uptake DNA under in vitro conditions. Among the approaches for recombinant DNA delivery, electroporation is the most commonly employed method, while conjugation and protoplast fusion have been historically used but with more limited efficiency. Interspecies genetic barriers, particularly restriction–modification systems, often restrict the stable integration of foreign DNA. To overcome these challenges, CRISPR-Cas-based genome editing strategies have recently been developed, providing enhanced accuracy and efficiency in gene modification of food-grade lactobacilli (van Pijkeren & Barrangou, 2017).

2.4. Endogenous Promoters and Expression Systems

Strong endogenous promoters within *Lactobacillus* genomes are widely used for heterologous gene expression. The Pldh promoter (lactate dehydrogenase) drives strong constitutive expression, while promoters such as PgroESL (chaperone operon) are stress-responsive but can also be exploited for high-level expression. In addition, inducible promoters, for example the nisin-controlled PnisA system, are applied to regulate target protein expression in response to environmental signals (Rud et al., 2006).

2.5. Signal Peptides and Protein Secretion

For the secretion or cell-surface localization of recombinant proteins, *Lactobacillus* species utilize endogenous signal peptides, such as usp45. Surface display systems can enhance interactions between probiotics and the mucosal immune system, whereas secreted proteins may exert therapeutic effects at mucosal sites. Consequently, signal peptide engineering has become an important approach in the development of *Lactobacillus*-based biotherapeutics (Plavec & Berlec, 2019).

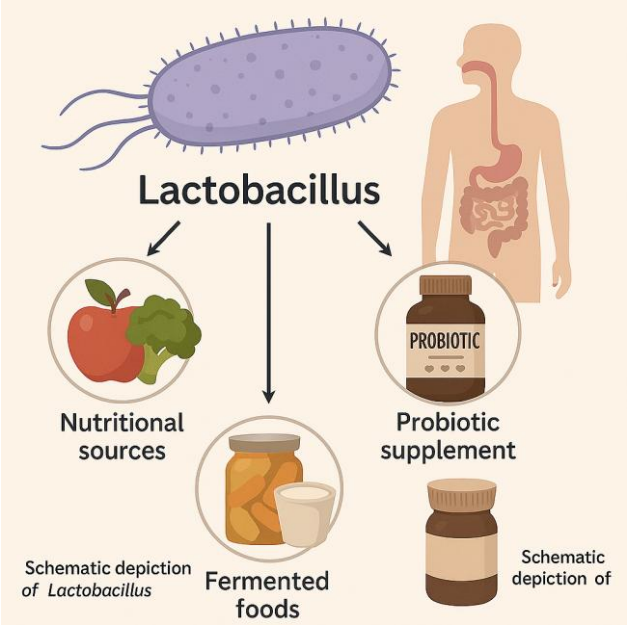

Figure 1. Nutritional sources of *Lactobacillus*, its relation to probiotic supplements and fermented foods.

3. Fundamentals of Recombinant DNA Technology

Recombinant DNA technology encompasses the process of isolating a specific genetic sequence from an organism, inserting it into a suitable carrier (vector),

and transferring it into a target host cell. This method constitutes one of the cornerstones of modern biotechnology, with broad applications in both industrial and medical fields (Watson et al., 2013).

3.1. Basic Stages

The recombinant DNA process generally consists of four major steps:

1. **Isolation of Target DNA:** The relevant gene or gene region is isolated from genomic DNA or a cDNA library. At this stage, polymerase chain reaction (PCR) is frequently employed (Watson et al., 2013).
2. **Cloning into a Vector:** The isolated DNA is cut with suitable restriction endonucleases and ligated into the vector using ligase enzymes. Vector selection depends on the host cell type and the intended mode of protein expression (Sambrook et al., 2001).
3. **Transfer into the Host Cell:** The vector is introduced into the host organism. While *Escherichia coli* is commonly used as a model bacterium, *Lactobacillus* species have gained significance as hosts due to their GRAS (Generally Recognized as Safe) status (Marco et al., 2021).
4. **Expression and Product Analysis:** The target gene undergoes transcription and translation within the host cell to produce the desired protein. The product is subsequently purified using biochemical methods, followed by functional assays (Plavec & Berlec, 2019).

3.2. Tools and Methods

- **Restriction Endonucleases:** Enzymes that recognize and cleave specific DNA sequences. During cloning, both the target DNA and the vector are digested with the same restriction enzyme to generate compatible ends (Watson et al., 2013).
- **Ligases:** Enzymes that join DNA fragments through phosphodiester bonds. T4 DNA ligase is the most frequently used enzyme in cloning procedures (Watson et al., 2013).
- **PCR and Reverse Transcription PCR (RT-PCR):** Techniques used for amplifying DNA or mRNA-derived genes. In low G+C content bacteria such as *Lactobacillus*, codon optimization is often employed to ensure efficient amplification of the target gene (Cabarello et al., 2025).

- **Vector Systems:** Plasmids developed for *Lactobacillus* are typically designed to be high-copy, stable, and easily transformable. In some systems, endogenous promoters (e.g., *Pldh*, *PgroESL*) are employed to achieve high-level expression of the target gene (van Pijkeren et al., 2017).

3.3. Lactobacillus-Specific Approaches

The use of *Lactobacillus* species in recombinant DNA technology presents unique challenges and advantages.

Advantages:

- Safe application in food and healthcare owing to GRAS status.
- Adhesion to human mucosa and favorable interactions with the immune system (Marco et al., 2021) .

Challenges:

- Degradation of foreign DNA due to restriction-modification systems.
- Variation in natural transformation capacity across species .

To overcome these obstacles, CRISPR-Cas9-based systems have recently been implemented, allowing highly precise modifications of target genomic regions (Cabarello et al., 2025). Furthermore, specialized signal peptide sequences have been developed for applications such as surface antigen display and secretion of therapeutic proteins (Plavec & Berlec, 2019).

4. Recombinant Protein Production with *Lactobacillus*

4.1. Vector Systems

Recombinant protein production represents one of the fastest-growing areas of modern biotechnology. Through this technology, enzymes, vaccine antigens, therapeutic peptides, and biologics can be efficiently produced using microbial systems. Traditionally, *Escherichia coli* and yeast species have been employed for these purposes; however, in recent years, bacteria of the genus *Lactobacillus* have gained prominence due to their safety, interaction capacity with the mucosal immune system, and GRAS status .

The probiotic properties of *Lactobacillus* render it an ideal system not only for protein production but also for the direct therapeutic application of the expressed protein. For example, when used as antigen carriers in mucosal vaccines, they enhance both antigen stability and immune targeting.

Vectors developed for *Lactobacillus* are typically designed to be food-grade and high-copy-number. Due to food safety requirements, metabolic markers (e.g.,

thyA, *lacZ*, *alr*) are preferred over antibiotic resistance genes (Levit et al., 2022). Vectors are generally categorized into two main groups:

- **Replicative (plasmid-based) vectors:** Provide high copy number and ease of transformation, making them suitable for transient expression.
- **Integrative vectors:** Integrate the target gene directly into the chromosome, ensuring genetic stability. This approach is preferred in industrial applications requiring long-term production.

In advanced systems, replicative vectors have been combined with CRISPR-Cas9 to enhance gene targeting and editing capacity (van Pijkeren & Britton, 2012). Furthermore, the choice of replicon used in a vector directly influences transformation efficiency in specific *Lactobacillus* species .

4.2. Promoter Selection

The choice of promoter in *Lactobacillus* determines both the level and timing of gene expression:

- **Constitutive promoters (*Pldh*, *PgroESL*):** Provide continuous expression regardless of the growth phase, commonly used in industrial enzyme production.
- **Inducible promoters (*PnisA*, nisin-inducible systems):** Allow production of the target protein only in the presence of specific inducers, which is particularly important for minimizing adverse effects on cell growth during the expression of toxic proteins .

In recent years, promoters responsive to environmental conditions such as pH or temperature have been developed, enabling more dynamic and energy-efficient production processes (van Pijkeren & Britton, 2012).

4.3. Signal Peptides and Protein Stability

The efficiency of recombinant protein secretion and intracellular stability is critical for the success of therapeutic and industrial applications.

Signal peptide engineering:

- The *usp45* signal peptide, derived from *Lactococcus lactis*, is the most widely used secretion signal in *Lactobacillus*.
- Hybrid signal peptides can be designed according to protein structure, enhancing secretion efficiency by more than 50% (van Pijkeren et al., 2017).

Strategies to improve protein stability:

- Deletion or inhibition of protease genes (e.g., *prtP* mutants).
- Incorporation of stabilization domains that facilitate proper folding.
- Optimization of pH, temperature, and growth conditions These approaches help minimize the loss of biological activity, particularly in mucosal vaccines and therapeutic protein applications (van Pijkeren et al., 2017).

4.4. Application Areas

Vaccine Carriers

Mucosal vaccine platforms are based on the principle of delivering antigens via oral or nasal administration of *Lactobacillus*. These systems can induce strong mucosal IgA and systemic IgG responses. Recombinant strains have been tested in preclinical and clinical studies against pathogens such as HPV, HIV, and influenza.

Therapeutic Protein Production

Recombinant *Lactobacillus* strains have been engineered for local production of immunomodulatory cytokines (e.g., IL-10, TGF-β) for chronic inflammatory diseases, metabolic hormones such as GLP-1 for glycemic control in type 2 diabetes, and antimicrobials including bacteriocins and lysozyme to inhibit pathogen colonization.

Industrial Enzyme Production

Enzymes such as lactase, protease, and amylase, widely used in the food industry, can be produced safely and cost-effectively using *Lactobacillus*. Recombinant strains can even be engineered to produce multiple enzymes simultaneously (Levit et al., 2022).

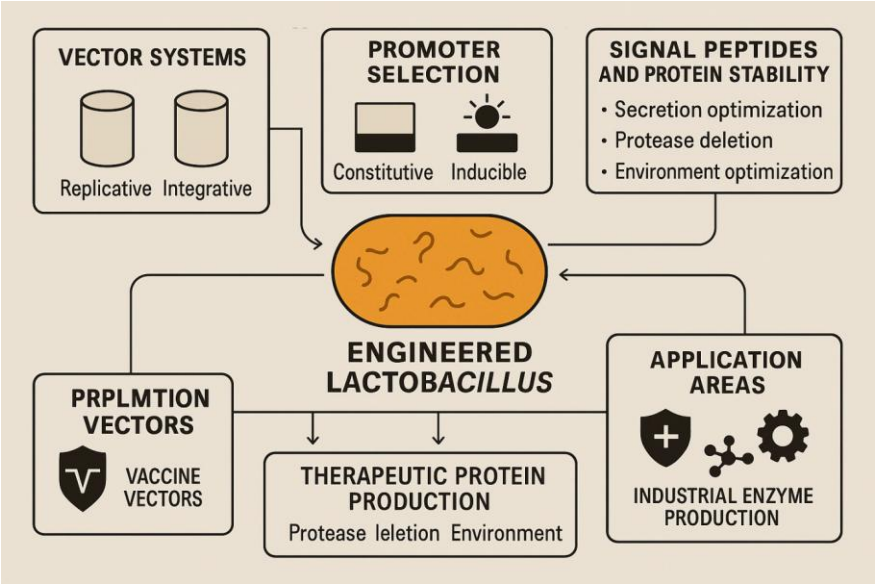

Figure 2. Schematic representation of molecular tools and application areas in *Lactobacillus*: vector systems, promoters, signal peptide engineering, protein stabilization, and therapeutic/industrial applications.

5. Biotechnological and Clinical Applications of *Lactobacillus*-Based Recombinant DNA Technology

5.1. Introduction

Lactobacillus species have gained significant importance in biotechnology and clinical research due to their natural presence in the human microbiota, their GRAS (Generally Recognized As Safe) status, and their compatibility with genetic manipulation techniques. Through recombinant DNA technology, the insertion or modification of specific genes within their genomes enables these bacteria to produce therapeutic proteins, antigens, bioactive metabolites, and industrial enzymes. Consequently, *Lactobacillus* strains function both as live biotherapeutics and as biotechnological production platforms. One of the most notable advantages of recombinant *Lactobacillus* systems is their ability to deliver antigens or therapeutic molecules directly to mucosal surfaces. This feature presents great potential for oral or nasal vaccine development, treatment of gastrointestinal diseases, and targeted drug delivery systems. Moreover, these systems provide cost-effective production without the need for expensive fermenter infrastructures (Levit et al., 2022).

5.2. Clinical Applications

5.2.1. Mucosal Vaccine Carriers

Mucosal vaccination provides protection by eliciting immune responses at mucosal sites, which are the primary entry points of pathogens. Recombinant *Lactobacillus* can trigger such responses either through antigen surface display or secretion. Preclinical studies with strains expressing viral antigens, such as the HPV L1 capsid protein or influenza hemagglutinin (HA), have demonstrated the ability to elicit strong mucosal IgA and systemic IgG responses, offering protection against infection and highlighting their potential as safe and easily administrable vaccine platforms.

5.2.2. Therapeutic Protein and Peptide Production

Recombinant *Lactobacillus* and *Lactococcus* strains have been engineered to produce therapeutic proteins and peptides locally at the site of application, thereby minimizing systemic side effects. For example, strains engineered to secrete IL-10 have shown significant reductions in mucosal inflammation in models of chronic intestinal disease. Similarly, antimicrobial peptides expressed by recombinant strains have been reported to reduce pathogen burden, paving the way for targeted microbial therapies that could serve as alternatives to antibiotics (Levit et al., 2022).

5.2.3. Applications in Oncology

In oncology, *Lactobacillus*-based vectors show promise for carrying tumor antigens and activating immune responses. For example, *Lactobacillus plantarum* expressing the cell-wall anchored NY-ESO-1 tumor antigen elicited antigen-specific humoral and T-cell responses in mouse models following oral immunization (Mobergslien et al., 2015). These systems are particularly relevant for tumors located at mucosal surfaces, such as colorectal and cervical cancers, because direct antigen presentation at these sites may reduce the need for strong adjuvants yet enhance immune targeting specificity.

5.3. Biotechnological Applications

5.3.1. Functional Food Production

Recombinant *Lactobacillus* can enhance food products by producing vitamins, bioactive peptides, and antioxidants. For example, engineered *Lactococcus lactis* strains with enhanced folate biosynthesis pathways increased folate levels in fermented dairy products threefold. Additionally, *Lactobacillus brevis* strains producing gamma-aminobutyric acid (GABA) have been applied in functional yogurt production, demonstrating potential antihypertensive effects (Sybesma et al., 2003).

5.3.2. Industrial Enzyme Production

Enzymes such as amylase, protease, and lactase—widely used in the food industry—can be produced using *Lactobacillus*-based systems. These enzymes may be secreted directly into fermentation products or purified for use in industrial processes. For instance, *Lactobacillus plantarum* engineered to express β-galactosidase has been successfully applied in the production of lactose-free dairy products (van Pijkeren & Britton, 2012).

5.3.3. Biosensor Development

Lactobacillus strains equipped with synthetic biology tools can detect environmental or metabolic changes and produce reporter proteins. For example, strains engineered to express GFP in response to intestinal pH fluctuations have been developed, highlighting their potential for real-time monitoring of microbiota dynamics (Rottinghaus et al., 2020).

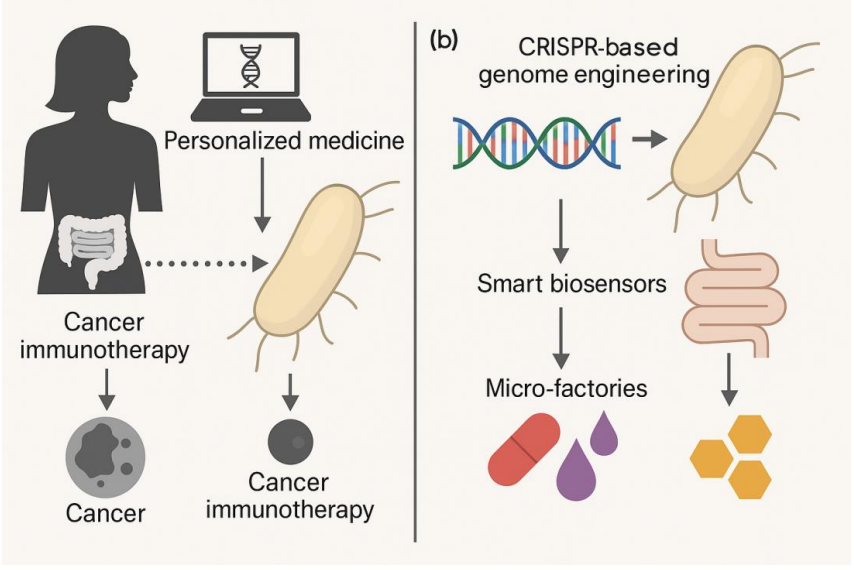

Figure 3. Future applications of *Lactobacillus*-based biotechnologies: (a) personalized medicine and cancer immunotherapy, (b) CRISPR-based genome engineering for smart biosensors, and therapeutic/industrial "micro-factory" systems.

5.4. Advantages and Limitations

Advantages:

- GRAS status
- Ecological compatibility with the human microbiota

- Dual functionality as both a biotherapeutic and a production platform
- Suitability for oral/nasal delivery

Limitations:

- Lengthy clinical approval processes
- Sensitivity of antigen/protein stability to environmental conditions
- Risks of genetic instability and plasmid loss

5.5. Future Perspectives

In the coming years, Lactobacillus-based systems are expected to enter several innovative fields. **Personalized medicine** approaches may allow the design of therapeutic strains tailored to a patient's microbiota and immune profile, providing targeted treatment options beyond conventional probiotics. In **oncology**, recombinant strains expressing tumor antigens or modulating the tumor microenvironment could support mucosal cancer therapies and enhance responses to existing immunotherapies. The integration of **CRISPR-based genome engineering** will further accelerate precise modifications, enabling the production of antimicrobial peptides, bioactive metabolites, or diagnostic markers. At the same time, advances in **synthetic biology** will pave the way for Lactobacillus to act as **living biosensors**, monitoring host conditions such as pH or inflammation and responding dynamically with therapeutic outputs. Finally, the **micro-factory concept** envisions these bacteria as sustainable producers of vitamins, antioxidants, and pharmaceuticals, reducing costs and providing on-site therapy directly within the gut ecosystem. Together, these perspectives highlight the transformative potential of Lactobacillus in preventive healthcare, chronic disease management, and industrial biotechnology.

Acknowledgments

For studies on the use of Lactobacillus in recombinant research, we gratefully acknowledge the support of the Scientific Research Projects (BAP) Unit of Malatya Turgut Özal University under project no. 23G04, entitled '*Investigation of the Effect of* Vitreoscilla *Hemoglobin on the Growth and Reproductive Characteristics of* Lactobacillus *Strains".*

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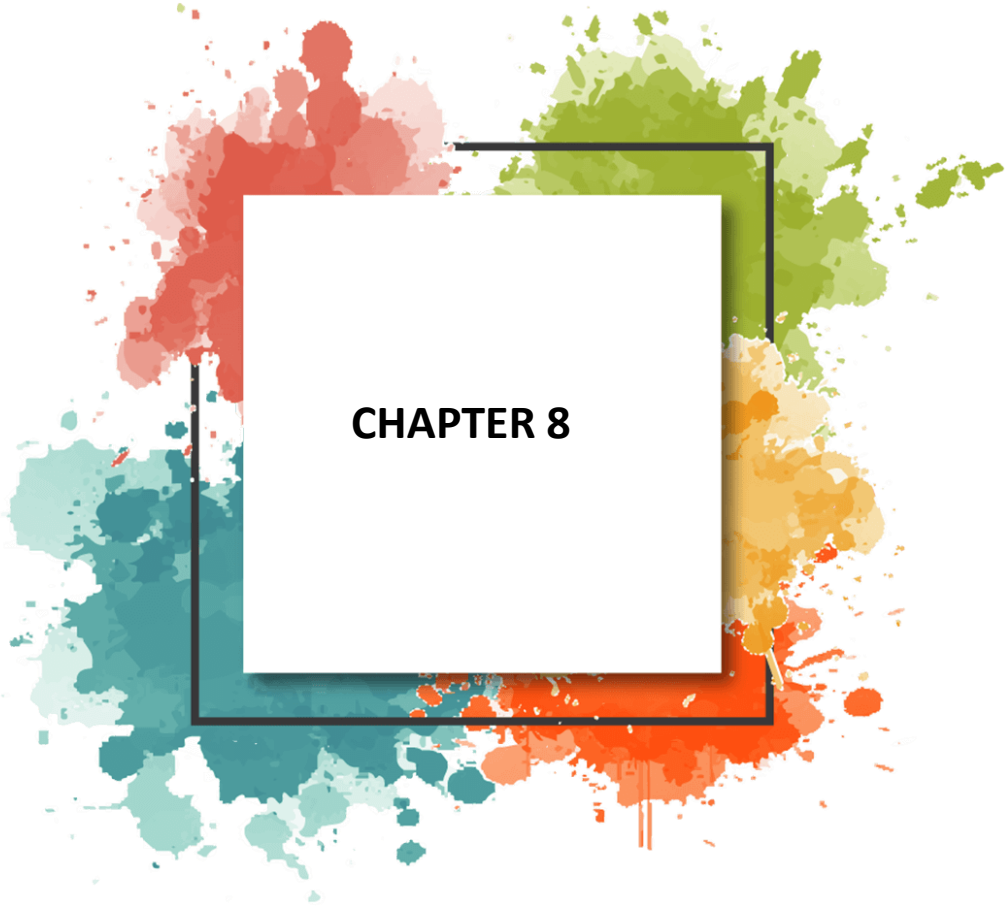
CHAPTER 8

Plant Extract-Based Nanoemulsions: Antimicrobial Activities and Applications

Mehzat Altun[1]

Introduction

According to the World Health Organization (WHO), antimicrobial resistance (AMR) has emerged as one of the most critical challenges to global health, with estimates suggesting it could be responsible for nearly 10 million deaths each year by 2050 and an economic burden approaching $100 trillion (WHO, 2021; Strathdee et al., 2020). The declining effectiveness of current antibiotics, alongside the slow development of new agents, poses a critical challenge. Limited financial returns, difficulties in discovering new compounds, inevitable resistance development, and complex regulatory requirements have collectively discouraged pharmaceutical investment in antibiotic research (Ayukekbong et al., 2017; Dutescu et al., 2021; Haroon et al., 2020).

AMR progression is accelerated by the frequent and improper use of antimicrobials in clinical, veterinary, and agricultural settings. Inadequate sanitation, limited access to clean water, and inadequate infection control measures facilitate the spread of resistant pathogens. Additionally, insufficient investment in the development of novel antimicrobials, alternative therapies, and robust surveillance systems hampers effective intervention. Collectively, these factors highlight the urgent need for a coordinated, multifaceted global response to curb the emergence and persistence of AMR (Puri et al., 2025).

In response to these urgent issues, growing research attention has been directed towards exploring the antimicrobial properties of plant-derived extracts. These natural products encompass numerous bioactive phytochemicals, such as phenolic compounds, terpenoids, alkaloids, organosulfur molecules, and antimicrobial peptides (Khameneh et al., 2019; Zouine et al., 2024). Among these phytochemicals, flavonoids and tannins show substantial antibacterial and antifungal properties through mechanisms involving membrane destabilisation, enzymatic inhibition, and suppression of virulence traits like biofilm production (Pérez-Flores et al., 2025; Vaou et al., 2021). This evidence highlights plant-derived compounds as promising candidates for mitigating the global threat of AMR.

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Although plant extracts exhibit considerable antimicrobial potential, their practical implementation as therapeutic agents is challenged by several factors. Variability in phytochemical content due to genetic, environmental, and processing influences complicates standardization and reproducibility (Pérez-Flores et al., 2025). In addition, limited bioavailability, potential toxicity at therapeutic concentrations, and regulatory restrictions hinder the translation of laboratory discoveries into clinical and commercial applications (Vaou et al., 2021).

Recently, nanoemulsion technology has attracted considerable interest in microbiology and biomedical research owing to its nanoscale droplet size, prolonged stability, and capacity to increase solubility, enhance bioavailability, and enable controlled release of bioactive agents, thus providing promising prospects for antimicrobial treatments, drug delivery systems, and diagnostic applications. Moreover, nanoemulsions can increase microbial uptake of bioactive molecules, reduce the required therapeutic dose, and improve the overall stability of plant-based constituents (Naseema et al., 2021; Nirmal et al., 2018). Therefore, nanoemulsion-based delivery systems represent a promising platform to harness the antimicrobial efficacy of herbal extracts against resistant bacteria. This chapter aims to investigate the antimicrobial properties of plant extract-based nanoemulsions, emphasising their mechanisms of action, technological advantages, and future applications in combating antimicrobial resistance.

Antibacterial Properties and Mechanisms of Medicinal Plant Extracts

Plant extracts possess notable antimicrobial properties, representing natural alternatives to synthetic agents to inhibit bacterial and fungal growth, including drug-resistant strains. These extracts comprise a diverse range of secondary metabolites, including flavonoids, terpenoids, alkaloids, and phenolic acids, many of which possess antimicrobial activities (Loukili et al., 2025).

The findings demonstrated that extracts from *Quercus coccifera*, *Ocimum gratissimum*, and *Curcuma longa* displayed the strongest antibacterial effects against *P. aeruginosa* (MIC: 4 µg/mL), *S. aureus* (MIC: 5 µg/mL), and *E. coli* (MIC: 7.58 µg/mL), respectively. These results underscore the significant antibacterial potential of medicinal plant extracts, which is frequently linked to their phytochemical profiles and the methodologies employed for extraction (Zouine et al., 2024).

The ethanolic extracts of *Loranthus acaciae* and *Cymbopogon proximus* exhibited potent inhibitory activity toward clinical pathogens such as *E. coli*, *S. aureus*, *Streptococcus* spp., and *Pseudomonas* spp., with Gram-negative bacteria displaying higher susceptibility (Jia et. al., 2025). Additionally, the aqueous

extract from mature leaves of *Asystasia variabilis* exhibited potent, dose-dependent antibacterial effects against wound-associated bacteria, such as *S. aureus, B. subtilis, P. aeruginosa,* and *E. coli*, likely due to synergistic actions of its phytochemical constituents (Wijerathna et al., 2025). Altun (2023) reported that the endemic *Sideritis trojana* ethanolic extract exhibited potent antibacterial activity against both Gram-positive and Gram-negative bacteria, with the highest efficacy observed against *B. subtilis* and *P. aeruginosa* (MIC: 15.625 µg/mL).

In a study conducted by Asfa et al. (2025), the crude extracts of three chosen medicinal plants—*Nicotiana tabacum, Psidium guajava,* and *Solanum incanum*—**were** assessed for their antibacterial efficacy against respiratory pathogens, specifically caused by *Pasteurella multocida* and *Mannheimia haemolytica***.** The methanol and chloroform extracts exhibited significant inhibitory effects comparable to commercial antibiotics, including gentamicin, oxytetracycline, and streptomycin**.**

Furthermore, crude extracts from *Syzygium aromaticum* buds, *Morinda citrifolia*, *Psidium guajava*, *Ocimum basilicum*, *Carica papaya*, and *Citrus limon* leaves, *Azadirachta indica* seed oil, and *Zingiber officinale* roots exhibited varying antibacterial efficacy against fifteen food-associated pathogens, including *Clostridium perfringens, Citrobacter youngae, Enterobacter aerogenes, Enterobacter amnigenus, Enterobacter cloacae, Escherichia coli, Hafnia alveia, Klebsiella oxytoca, Klebsiella pneumoniae, Morganella morganii, Proteus vulgaris, Proteus mirabilis, Photobacterium damselae, Raoultella ornithinolytica,* and *Vibrio alginolyticus*, with *Morinda citrifolia* showing the highest efficacy, suggesting the presence of bioactive compounds potentially useful for novel antimicrobial agent development (Singh et al., 2023). Extracts obtained from the seeds of *Urtica dioica*, *Nigella sativa*, and *Moringa oleifera* exhibited notable antibacterial activity against gastrointestinal pathogens, including *E. coli, P. vulgaris, B. subtilis,* and *S. aureus*, with MIC_{50} values ranging from 14.15 to 271.44 mg/mL (Sawalha et. al., 2025).

These antimicrobial effects are attributed to bioactive constituents that compromise microbial cell membrane integrity, prevent biofilm formation, and inhibit nucleic acid and protein synthesis (Pérez-Flores et al., 2025). Differences in extraction techniques, solvent systems, and evaluation approaches hinder cross-study comparisons, highlighting the need for standardized methodologies and comprehensive phytochemical characterization to confirm the efficacy and safety of plant-based antimicrobial agents (Vaou et. al., 2021).

Plant-derived compounds exert antimicrobial effects primarily by mechanisms involving damage to microbial cell membranes, efflux pump inhibition, interference with nucleic acid synthesis, and inhibition of biofilm development. Moreover, compounds with elevated antimicrobial potential often

share structural characteristics, including hydroxyl substituents on defined positions of phenolic rings, delocalized electron systems, and double bonds (Loukili et. al., 2025).

For example, *Ampelopsis grossedentata* (vine tea) exerts antibacterial activity against *S. aureus* by disrupting cell wall architecture, increasing membrane permeability, and inhibiting overall protein synthesis as well as the activity of specific metabolic enzymes (Liang et al., 2020). Additionally, these compounds may interact with DNA via intercalation and groove binding. Similarly, extracts derived from *Thymus vulgaris*, *Hibiscus sabdariffa*, *Syzygium aromaticum*, and *Rosmarinus officinalis* have been reported to impair cell wall integrity and alter cytoplasmic pH in *E. coli* and *S. aureus* cells (Melander et. al., 2020).

Curcuma longa L. exhibits broad-spectrum antimicrobial effects through multiple mechanisms, including compromising bacterial cell integrity, suppressing vital enzymatic activity, and modulating host immunity (Dai et. al., 2022). Curcumin enhances membrane fluidity, disrupts biofilms, and inhibits enzymes critical for bacterial replication. Additionally, its antioxidant and immune-supporting properties safeguard host cells. Together, these features and its rich phytochemical profile position *Curcuma longa L.* as a potential source for innovative antimicrobial approaches (Dai et. al., 2022; Murtadlo et. al., 2023).

Nanoemulsions: Definition, Composition, and Stability

Nanoemulsions are kinetically stable mixtures of two immiscible phases, oil and water, with droplet diameters generally between 20 and 200 nm. They are stabilised by suitable amphiphilic surfactants or co-surfactants and usually exhibit a transparent or semi-transparent appearance. The extremely small droplet sizes confer a substantial interfacial surface area, which improves the solubilisation of poorly water-soluble compounds and consequently enhances their bioavailability. Furthermore, nanoemulsions can encapsulate both hydrophilic and lipophilic molecules, offering a versatile and efficient platform for therapeutic, pharmaceutical, cosmetic, and food-related applications (Elsewedy et al., 2025; Preeti et al., 2023).

Nanoemulsions are generally categorised as oil-in-water (o/w), water-in-oil (w/o), or multiple systems (w/o/w, o/w/o), depending on the dispersed phase and droplet structure. o/w systems are primarily employed for the delivery of hydrophobic drugs, w/o systems for the encapsulation of hydrophilic compounds, and multiple emulsions for the controlled and sustained release of bioactive agents (Preeti et al., 2023).

Generally, the aqueous phase is composed of water, while the oil phase contains essential oils, triglycerides, fatty acids, waxes, and other lipophilic substances (Pavoni et al., 2020). Among these, long-chain triglycerides are

considered the most suitable due to their economic, nutritional, and functional benefits. The overall stability of nanoemulsions depends on the physicochemical properties of the oil phase, particularly interfacial tension, viscosity, polarity, and chemical stability (Jafari et al., 2013).

Nanoemulsions act as advanced delivery platforms for therapeutic agents, biologically active compounds, and nucleic acids with controlled-release challenges. They facilitate the transport of hydrophobic substances, approximately 40% of which are inherently insoluble in water. In pharmaceutical applications, nanoemulsions enhance the solubility of lipophilic drugs, thereby improving their bioavailability (Jaiswal et al., 2015).

Preparation of nanoemulsions

The formulation of nanoemulsions requires the careful selection of suitable components to ensure stability and functionality. Low-toxicity or naturally derived (GRAS) surfactants, such as Tween and Span, along with food-based biopolymers (proteins and polysaccharides), are commonly employed (Sridhar et al., 2021). Bio-based films and coatings can be classified according to their structural matrix, including lipids, proteins, polysaccharides, or composite systems. The chemical stability of nanoemulsions can be increased through the addition of antioxidants or chelating agents, adjustment of interfacial properties, and regulation of environmental factors such as temperature, pH, oxygen levels, and light exposure (Liu et al., 2019). Nanoemulsions can be formulated using either low- or high-energy techniques, depending on the manner in which energy is imparted to the system, and preparation can also be facilitated by modifying the chemical composition, temperature, or oil-to-water ratio of the formulation (Elsewedy et al., 2025; Preeti et al., 2023).

Low-Energy Methods

Low-energy (LE) approaches, developed subsequent to high-energy techniques, exploit the spontaneous formation of nanoscale oil droplets within oil–water–surfactant mixtures, triggered by alterations in formulation parameters or surrounding conditions (Gulotta et al., 2014; Komaiko & McClements, 2014). Primary LE strategies include Spontaneous Emulsification, Emulsion Phase Inversion, Phase Inversion Composition, and Phase Inversion Temperature. LE methods are cost-effective, energy-efficient, non-destructive, and straightforward to implement (Mushtaq et al., 2023). Key physicochemical parameters affecting droplet formation, which are crucial for these strategies, include temperature, solubility, and formulation composition (Salvia-Trujillo et al., 2017).

High-Energy Methods

High-energy (HE) approaches utilise mechanical forces to fragment droplets and generate kinetically stable nanoemulsions. Frequently employed HE techniques include rotor-stator emulsification, high-pressure homogenization, microfluidization, and ultrasonic homogenization. These methods exploit shear, turbulence, cavitation, and hydraulic forces to disperse oil and water phases into nanoscale droplets (Espitia et al., 2019; Wang et al., 2015).

Antimicrobial Potential of Plant Extract-Based Nanoemulsions

Plant-derived nanoemulsions act on microbial cell walls, compromising membrane integrity and preventing biofilm development. Specifically, phenolic compounds exhibit antimicrobial activity by inhibiting key enzymatic functions within microbial cells (McClements et al., 2021).

Antimicrobial nanoemulsions have been demonstrated to suppress microbial growth, maintain food quality, and prolong the shelf-life of diverse food products (Das et al., 2020; Salvia-Trujillo et al., 2015). These formulations are active against both Gram-positive and Gram-negative bacteria (Ferreira et al., 2010; Sutcliffe et al., 2008), which is primarily attributed to their capacity to compromise microbial cell wall integrity and functionality (Baker et al., 2003) or to interfere with intercellular communication, thereby preventing biofilm formation (Joe et al., 2015).

The ethanolic extract nanoemulsion of anise seeds, prepared using an ultrasound-assisted method, exhibited antimicrobial activity against foodborne pathogenic bacteria (Ghazy et al., 2021). Similarly, *Crocus sativus* (saffron) and *Achillea millefolium* (yarrow) extract-based nanoemulsions, prepared via the low-energy method, particularly *C. sativus*, demonstrated strong antimicrobial and anti-aflatoxigenic properties, highlighting their potential as natural agents for food preservation and safety enhancement (Abu Safe et al., 2023).

Plant-derived nanoemulsions from *Ocimum tenuiflorum* and *Azadirachta indica* also showed significant antimicrobial activity against *Neisseria gonorrhoeae* at higher concentrations (1000 μM) without causing haemolytic toxicity (Naicker et al., 2025). The *Woodfordia fruticosa* flower extract nanoemulsion, prepared via ultrasound-assisted technique (1–10% w/v), exhibited droplet sizes ranging from 149.25 to 244.33 nm and demonstrated higher antimicrobial activity against *P. aeruginosa*, *S. aureus*, and *C. albicans*, with the strongest effect against *C. albicans* (Najda et al., 2022). Differences in microbial susceptibility are attributed to cell wall structures, while the nanoscale size of the emulsions enhances contact with microorganisms and facilitates penetration of bioactive agents (Da et al., 2022; Gong et al., 2019).

Applications of Plant-Derived Nanoemulsions

Plant-origin nanoemulsions are receiving considerable scientific attention because of their favourable biocompatibility, structural stability, and effectiveness in incorporating lipophilic active compounds. Their utilisation extends to multiple cross-disciplinary areas:

Plant-Based Nanoemulsions in Pharmaceuticals and Drug Delivery

Nanoemulsions derived from plants have gained attention as effective delivery platforms in pharmaceutical applications owing to their capacity to enhance the solubility, stability, and bioavailability of drugs with poor water solubility. By reducing droplet size to the nanometre scale, these systems increase the surface area available for drug dissolution and facilitate absorption across biological membranes. Moreover, they serve as efficient carriers for the controlled and targeted release of therapeutic agents, thereby enhancing pharmacological efficacy while minimising adverse effects.

In addition to these pharmacokinetic benefits, nanoemulsions also provide practical formulation advantages.

They can be incorporated into a variety of dosage forms, such as gels, creams, foams, aerosols, and sprays, using cost-efficient standard methods, and can be delivered via oral, topical, intravenous, intrapulmonary, intranasal, and intraocular routes. Due to their versatility and efficiency in improving drug solubility and stability, nanoemulsions represent an advanced technology for enhancing bioavailability across multiple therapeutic contexts. This approach plays a key role in the advancement of innovative pharmaceutical formulations and holds significant potential to improve drug delivery results as investigations in the field progress (Preeti et al., 2023).

Plant-Based Nanoemulsions in the Food Industry

The application of bioactive compounds, including lipids, essential oils, vitamins, polyphenols, and carotenoids, in food products is frequently challenged by their limited aqueous solubility and instability. Nanoemulsion systems offer protection for these compounds during storage and allow for their controlled release under targeted conditions, such as in the oral cavity for flavor delivery or within the gastrointestinal tract for nutraceuticals. Additionally, incorporation into o/w nanoemulsions improves the solubility and bioavailability of hydrophobic bioactives by facilitating their integration into mixed micelles in the simulated gastrointestinal environment (Liu et al., 2019).

Plant-Based Nanoemulsions in Agriculture

The development of nano-insecticides represents a crucial area of research, offering promising avenues for creating environmentally sustainable and green alternatives to conventional pesticides while mitigating environmental contamination. In particular, plant-based nanoemulsions containing bioactive oils—such as *Portulaca oleracea*, *Raphanus sativus*, and *Rosmarinus officinalis*—have demonstrated significant potential in controlling major agricultural pests, including *Aphis gossypii*, *Spodoptera littoralis*, and *Tetranychus urticae*, with the most pronounced effects recorded against *Aphis gossypii*. These findings indicate that plant-derived oil nanoemulsions can serve as effective, environmentally friendly alternatives to traditional chemical pesticides, contributing towards sustainable farming practices through enhancing pest management strategies and reducing reliance on synthetic chemicals (Abd-Elnabi et al., 2025). Moreover, nanoemulsions loaded with *P. leptostachya* extract have also been identified as promising eco-friendly insecticidal formulations, providing high efficacy against *Acyrthosiphon pisum* while remaining safe for non-target organisms and host plants (Yang et al., 2025).

Conclusion

AMR poses a major global health challenge, driven by overuse of antimicrobials and slow development of new drugs. Plant-derived phytochemicals, including phenolics, terpenoids, and alkaloids, show potent activity against pathogens, including multidrug-resistant strains, but their efficacy is limited by variability, low bioavailability, and potential toxicity. Nanoemulsion systems enhance solubility, stability, and targeted delivery of these bioactives, disrupting microbial membranes, inhibiting key enzymes, and preventing biofilm formation. Beyond medical applications, they improve food preservation and provide sustainable alternatives to chemical pesticides. Plant-based nanoemulsions thus represent versatile platforms for combating AMR, warranting further research on standardised formulations, mechanisms, and safety.

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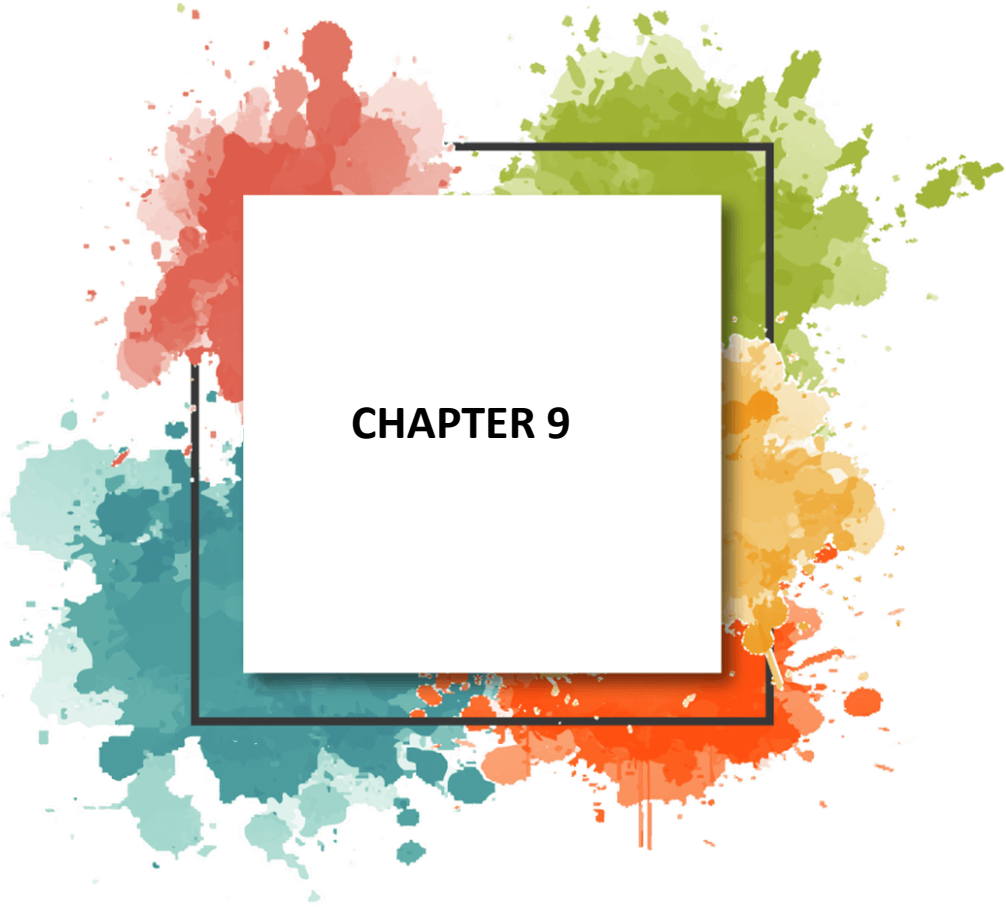
CHAPTER 9

Beliefs and Perspectives of Breastfeeding Mothers on Human Milk Banking: A Qualitative Study

Türkan Akyol Güner[1]

Introduction

Breast milk is an unparalleled source of nutrition for infants, providing essential benefits for growth, immune system development, and long-term health outcomes. It contains a balanced composition of proteins, fats, carbohydrates, vitamins, and minerals, along with antibodies, hormones, enzymes, and bioactive components, making it both nutritive and protective against infections (Victora et al., 2016). The World Health Organization (WHO) and UNICEF recommend exclusive breastfeeding for the first six months of life and continued breastfeeding alongside appropriate complementary feeding up to two years of age (World Health Organization, 2021; UNICEF, 2024). Sustaining breastfeeding practices not only reduces childhood infections, obesity, and diabetes risk but also lowers the incidence of breast and ovarian cancers in mothers (Victora et al., 2016).

From a public health perspective, breastfeeding plays a critical role in achieving the Sustainable Development Goals (SDGs), improving maternal and child health outcomes, and reducing healthcare costs worldwide. However, not all mothers are able to breastfeed adequately due to physiological (e.g., hypogalactia), psychological (e.g., anxiety, depression), or socioeconomic factors, leading to insufficient milk production. This situation poses a serious health risk, especially for preterm or low-birth-weight infants who depend on human milk for survival and immune protection (Gaya, Heine, & Gribble, 2024).

Within this context, human milk banking has emerged as an essential public health intervention that ensures equitable access to donor human milk for vulnerable infants. It involves the collection, screening, pasteurization, and safe distribution of excess breast milk donated by healthy lactating mothers (Coutsoudis & Fadnes, 2022). Globally, milk banks have been established as part of neonatal nutrition and preventive health policies across Europe, North and South America, and Asia. According to the European Milk Bank Association (EMBA, 2023), there are over 280 active milk banks in Europe, while Brazil's national network of more than 220 milk banks has been instrumental in

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significantly reducing neonatal mortality rates (Arslanoglu, Coutsoudis, & McGuire, 2021; Victora et al., 2016).

Despite these advances, the implementation of human milk banking in Türkiye remains limited and controversial, primarily due to religious and cultural sensitivities. In Islam, the concept of *milk kinship* (*ridā'a*) establishes a familial bond between children breastfed by the same woman, making marriage between them religiously prohibited (Alnakshbandi, Alkhalifa, & Abulaban, 2020; Saptarini & Suprapto, 2021). Consequently, concerns regarding milk mixing, donor anonymity, and religious permissibility have hindered public acceptance (Alnashwan, Rahman, & Abdullah, 2022).

The national milk banking initiative launched in Türkiye in 2013 was suspended following objections from religious authorities concerning the risks of milk kinship confusion (Şahin, Demirci, & Uslu, 2020). Subsequent studies have indicated that mothers' awareness of milk banking is low, while religiously motivated reservations remain strong (Şahin et al., 2020; Kılıç & Bayram, 2022). For milk banking to be effectively integrated into Türkiye's health system, it must be designed as a transparent, culturally sensitive, and ethically acceptable public health model supported by trust and religious approval.

Recent literature suggests that milk banking systems in Muslim-majority countries can achieve societal acceptance through culturally adapted frameworks, such as collaboration with religious leaders, establishment of milk kinship registries, and transparent donation processes (Gribble, Arif, & Dwi, 2025; Alnakshbandi et al., 2020). Accordingly, investigating the beliefs, knowledge, and attitudes of breastfeeding mothers in Türkiye toward human milk banking holds substantial importance for public health. Such insights can guide the development of national strategies aimed at improving infant nutrition equity, strengthening breastfeeding promotion programs, and supporting the health and survival of vulnerable newborns.

METHOD

Study Design

This study was conducted using a **phenomenological qualitative design** to explore in depth the beliefs and perceptions of breastfeeding mothers regarding human milk banking. The phenomenological approach aims to reveal individuals' lived experiences, perceptions, and the meanings they ascribe to a particular phenomenon.

Setting and Duration

The study was carried out between **February and July 2025** with breastfeeding mothers who applied to **Zonguldak Rat Family Health Center** in Türkiye. This primary healthcare institution was selected because it provided accessible contact with mothers and facilitated voluntary participation in a safe and familiar environment.

Participants and Sampling

The study group consisted of **20 volunteer mothers** who had at least one child whom they were currently breastfeeding or had breastfed previously. Participants were recruited using **purposive sampling**. Efforts were made to ensure diversity in terms of age, education level, number of children, and socioeconomic status. The sample size was expanded until **data saturation** was reached, when no new themes emerged from the interviews.

Data Collection Tools

Data were collected using a two-part form developed by the researchers:

1. **Descriptive Information Form:** This form included questions about participants' sociodemographic characteristics such as age, education level, employment status, number of children, and breastfeeding duration.
2. **Semi-Structured Interview Form:** Developed by the researcher based on a literature review, this form contained open-ended questions designed to explore mothers' knowledge, religious and cultural beliefs, attitudes toward milk donation and milk receiving, and their suggestions regarding the implementation of a human milk banking system.

Data Collection Process

Data were gathered through face-to-face interviews conducted at times convenient for the participants. All interviews were held in a quiet environment that ensured participants' privacy. Each session lasted approximately 25–35 minutes. With participants' permission, the interviews were audio-recorded, and the recordings were later transcribed verbatim by the researcher.

Data Analysis

Data were analyzed using the thematic analysis method. Each interview transcript was read several times and coded manually by the researcher to identify key concepts. Initial codes were organized into subthemes and main themes. The coding and thematic framework were reviewed and validated by two independent

experts experienced in qualitative research—one specializing in Maternity and Women's Health Nursing and the other in Pediatric Nursing. Each expert independently developed a thematic structure, and consensus was reached on the final themes (see Table 1).

Trustworthiness and Rigor

The trustworthiness of the study was ensured through member checking, expert validation, and data triangulation. Summaries of the findings were shared with several participants to confirm the accuracy of the interpretations. In addition, Lincoln and Guba's (1985) criteria for qualitative trustworthiness—credibility, transferability, dependability, and confirmability—were applied to enhance the transparency and rigor of the research process.

Ethical Considerations

Ethical approval was obtained from the Zonguldak Bülent Ecevit University Human Research Ethics Committee (Approval No: 05.12.2024/570136), followed by institutional permission. All participants were informed about the purpose of the study and confidentiality principles, and written informed consent was obtained. Participants' personal information was kept confidential, and all data were used solely for scientific purposes.

RESULTS

The participants were aged between 20 and 38 years, were married, and had completed either high school or university education. Sixty percent of the mothers had two or more children. The majority were unemployed and reported that their household income was at a moderate level.

Through thematic analysis of the interview data, six main themes and seventeen subthemes were identified:

1. Lack of Knowledge About Human Milk Banking
2. Religious Beliefs and Perceptions of Milk Kinship
3. Emotional and Cognitive Attitudes Toward Milk Banking
4. Concerns About Safety and Hygiene
5. Views on Milk Donation and Milk Receiving
6. Proposed Conditions for Acceptance and the Need for Cultural Adaptation

Table 1. Research Questions and Themes

Research Question	Main Theme	Subthemes / Codes
1. What is the level of knowledge of breastfeeding mothers about human milk banking?	Lack of Knowledge	Lack of understanding of the concept, misconceptions, hearing from media, lack of information from health institutions
2. What are the beliefs and religious approaches of breastfeeding mothers toward human milk banking?	Religious Beliefs and Perception of Milk Kinship	Fear of marriage prohibition due to milk kinship, perception of sin, concern about milk mixing, expectation of approval from the Religious Authority
3. What are mothers' emotional and cognitive attitudes toward human milk banking?	Emotional Attitudes	Perception of solidarity and spiritual reward, sense of shared motherhood, feelings of discomfort, ambivalent attitudes
4. What are mothers' opinions about safety and hygiene in human milk banking?	Concerns About Safety and Hygiene	Fear of milk mixing, fear of disease transmission, uncertainty about storage conditions, expectation of state supervision
5. What are mothers' attitudes toward donating or receiving human milk?	Attitudes Toward Milk Donation and Receiving	Willingness to donate milk, hesitation to receive milk, acceptance only in necessity, emotional dilemma
6. What are mothers' suggestions for ensuring social acceptance of human milk banking?	Conditions for Acceptance and Cultural Adaptation	Transparency, disclosure of donor and recipient information, approval from religious authorities, milk donation to same-gender infants, need for record-keeping system

1. Lack of Knowledge About Human Milk Banking

Most participants stated that they had heard of the concept of human milk banking for the first time during the study or had only encountered it superficially through the media. The lack of knowledge also led to misunderstandings about how the system functions.

"*I don't really know what a milk bank is—do they store the milk or sell it*?" (P5)
"*I've only heard about it on the news, but I don't have detailed information*." (P2)

Some mothers perceived the concept as similar to a "blood bank," while others confused it with the traditional practice of "wet-nursing."

"*I guess it's something like wet-nursing—mothers give their milk to other babies*." (P11)

A notable factor underlying this lack of awareness was the absence of any education or information provided on the topic within healthcare institutions.

2. Religious Beliefs and Perceptions of Milk Kinship

One of the most prominent themes identified in this study was the participants' hesitancy toward milk banking due to religious concerns. The majority expressed anxiety about the possibility of marriage prohibition resulting from milk kinship, which in Islam establishes a familial bond between children who are breastfed by the same woman.

"In Islam, there is milk kinship—those children become siblings, and marriage between them is forbidden. If milk gets mixed, it would be a great sin." (P9)

"*If my milk goes to another baby, that baby becomes my child's milk sibling, and later no one would know who is related to whom."* (P14)

Some participants stated that they might view milk banking more positively if **religiously appropriate conditions** were ensured, such as preventing the mixing of milk and disclosing donor and recipient identities.

"*If it's clear which milk goes to which baby and there's no mixing, maybe it could work—but I still wouldn't feel comfortable*." (P20)

3. Emotional and Cognitive Attitudes Toward Milk Banking

Although most mothers viewed human milk banking positively in terms of solidarity and helping others, they also expressed mixed emotions about the idea. Some described feeding another woman's baby as creating a spiritual bond, while others found it emotionally disturbing.

"*If another baby were fed with my milk, it would be a good deed, but I would still feel strange inside."* (P8)

"Feeding my baby with another woman's milk would feel odd to me; I wouldn't be at ease with it." (P18)

For some mothers, milk donation was perceived as **"**sharing motherhood," which evoked both compassion and a sense of jealousy at the same time.

"*On one hand, it's an act of kindness, but it also feels like I'd be giving away a part of my motherhood."* (P16)

4. Concerns About Safety and Hygiene

Participants expressed significant concerns regarding hygiene, the mixing of milk, storage conditions, and the risk of infectious diseases. They particularly emphasized the importance of knowing from whom the milk was obtained, how it was tested, and how it was stored.

"*If we don't know whose milk it is, how can we trust it?"* (P3)

There's always the fear of infection. I worry that if my milk gets mixed, it might harm the baby." (P17)

Several participants stated that these concerns could be alleviated if hygiene and safety were guaranteed under state supervision and within hospital settings.

"*If it's controlled by the government and done in hospitals, maybe then people would trust it."* (P12)

5. Views on Milk Donation and Milk Receiving

Approximately half of the mothers expressed a positive attitude toward donating milk, with "helping others" and "earning spiritual merit" being the main motivations for donation.

"*If my baby nurses well and I have extra milk, I'd like to give it—my milk could give life to another baby*." (P10)

However, the same willingness was not observed for receiving milk. More than half of the participants considered feeding their own babies with another mother's milk as religiously or emotionally inappropriate.

"*I would give my milk to others, but I wouldn't feed my own baby with someone else's milk."* (P17)

Some participants stated that they would consider receiving donor milk only in life-threatening or emergency situations.

"*If I didn't have milk and my baby would go hungry, then I would consider it."* (P19)

6. Proposed Conditions for Acceptance and the Need for Cultural Adaptation

Most participants indicated that they would support the implementation of a human milk banking system if certain conditions were met. The key requirements mentioned were:

No mixing of milk

Disclosure of donor and recipient identities

Donation to infants of the same gender

Pasteurization under hygienic conditions

Approval from religious authorities

"*If the milk isn't mixed and we know exactly whose milk goes to which baby, and everything is clean, then maybe it could work."* (P4)

"If it's religiously approved, why not? It would be beneficial for the babies." (P18)

Several participants emphasized that the milk banking system in Türkiye should be culturally tailored, taking religious sensitivities into consideration.

"Our culture is a bit different; maybe if it's approved by the Religious Authority, people would accept it." (P12)

DISCUSSION

This study qualitatively explored the beliefs, knowledge, and attitudes of breastfeeding mothers in Türkiye regarding human milk banking. The findings revealed that the concept of milk banking is not well-known among the general public, that religious beliefs and cultural values significantly influence its acceptance, and that mothers express ambivalence toward milk donation and milk receiving.

Lack of Knowledge and Limited Awareness

The majority of participants lacked adequate knowledge about human milk banking. This may be attributed to the fact that a milk banking system has not yet been implemented in Türkiye. Similarly, Alnakshbandi, Alkhalifa, and Abulaban (2020) reported that only 18% of mothers in Saudi Arabia had heard of milk banking, and many perceived it as a form of "commercial milk selling." Comparable results were found in Iran, where the lack of awareness was

identified as the most influential factor affecting mothers' decisions to donate milk (Aghajani, Ebrahimi, & Shokrpour, 2025).

Globally, studies have shown that educational programs and public awareness campaigns led by healthcare professionals increase mothers' willingness to donate milk (Arnold, 2021). These findings highlight the need to strengthen the educational and counseling roles of nurses and lactation consultants in Türkiye.

Religious Beliefs and Concerns About Milk Kinship

The most prominent concern expressed by the participants was related to milk kinship. In Islamic culture, milk kinship establishes a familial bond equivalent to biological kinship, which prohibits marriage between milk siblings (Gribble, Arif, & Dwi, 2025). Similar findings have been reported in predominantly Muslim countries such as Malaysia (Alnashwan, Rahman, & Abdullah, 2022) and Indonesia (Saptarini & Suprapto, 2021), where milk banking initiatives launched without prior consultation with religious leaders were socially rejected due to religious concerns

Likewise, the first milk bank established in Pakistan was discontinued shortly after its inception for religious reasons (Khaliq, Ahmed, & Hussain, 2024). In contrast, the Islamically Compatible Milk Banking Model implemented in Singapore demonstrated that collaboration with religious authorities and the establishment of systematic milk-kinship records can successfully address such concerns (Gribble et al., 2025).

In Türkiye, the 2013 statement of the Presidency of Religious Affairs (Diyanet İşleri Başkanlığı) indicated disapproval of milk banking because of the potential risk of milk mixing. Therefore, developing a registered, transparent system in collaboration with religious authorities and based on Islamic principles is essential for social acceptance.

Emotional Attitudes and Perceptions of Motherhood

The findings indicated that mothers perceived milk donation as an act of helping others and earning spiritual reward, but they hesitated to feed their own babies with another mother's milk. This ambivalence reflects the social and emotional meanings of motherhood. Coutsoudis and Fadnes (2022) reported that mothers described milk donation as "giving life to another baby," yet perceived receiving donor milk as culturally challenging. Similarly, Ugwu, Nwachukwu, and Eze (2024) found that Nigerian mothers expressed limited support for milk donation due to religious and cultural factors, although the notion of altruism positively influenced their attitudes.

These findings suggest that both milk donation and milk receiving behaviors are shaped by emotional and moral values, rather than purely informational or practical factors.

Concerns About Safety, Hygiene, and Health Assurance

Nearly all participants reported concerns about the **safety, hygiene, and** health reliability of donated milk. This reflects not only a lack of information but also an underlying need for institutional trust. International guidelines emphasize that donor screening, pasteurization, and traceability systems are mandatory in human milk banking (Human Milk Banking Association of North America [HMBANA], 2023).

Similarly, both the European Milk Bank Association (EMBA, 2023) and HMBANA guidelines recommend that donated milk should only be distributed under medical necessity and regulated conditions. The mothers' emphasis on "state supervision, hospital settings, and system transparency" aligns with these international standards.

A recent analysis published in Nature also warned that the rise of commercial milk markets poses ethical and safety risks, reinforcing the need for publicly regulated, non-profit milk banking systems (Arslanoglu, Coutsoudis, & McGuire, 2023).

Cultural Adaptation and Conditions for Acceptance

The participants' suggestions—preventing milk mixing, recording donor identities, matching infants by gender, and obtaining religious approval—underscore the importance of cultural adaptation. The "Conditional Identified Milk Banking System (CIMBS)" model proposed by Gribble et al. (2025) offers a potentially viable framework for milk banking in accordance with Islamic principles.

Moreover, a recent Swiss study demonstrated that the sustainability of milk donation depends on multilevel support at policy, institutional, and community levels (Witzig, Frei, & Amstad, 2025). Similarly, in Türkiye, the sustainability and public acceptance of a milk banking system would require cultural sensitivity, public engagement, and policy coordination among healthcare institutions and religious authorities.

CONCLUSION AND RECOMMENDATIONS

The findings of this study revealed that the majority of breastfeeding mothers were unfamiliar with the concept of human milk banking and often misunderstood its purpose and process. This widespread lack of awareness appears to be a consequence of the absence of a national milk banking system in

Türkiye and the limited provision of education and counseling on this topic within healthcare institutions.

From a public health perspective, these findings highlight a critical gap in maternal and child health education. Human milk banking, when implemented within ethical and culturally appropriate frameworks, has the potential to reduce neonatal morbidity and mortality, particularly among preterm and low-birth-weight infants who are most vulnerable to nutrition-related complications. Ensuring access to safe donor milk is therefore not only a nutritional intervention but also a matter of equity and public health responsibility.

The study further demonstrated that religious and cultural values strongly influence mothers' acceptance of milk banking. The concept of milk kinship, deeply rooted in Islamic tradition, continues to shape perceptions of milk sharing and often creates hesitation or rejection. Nevertheless, many mothers expressed willingness to support milk banking under specific conditions—such as transparent record-keeping, hygienic processing, and approval by religious authorities—indicating that culturally sensitive adaptation is both necessary and feasible.

Trust, hygiene, and religious compliance emerged as the key determinants of public acceptance. Addressing these factors through government-regulated systems, health professional training, and faith-based collaboration could transform milk banking into a socially accepted and ethically sustainable public health service.

In conclusion, there is an urgent need to establish a national human milk banking policy in Türkiye that aligns with religious values, cultural norms, and international safety standards. Developing such a transparent and reliable system would strengthen neonatal health outcomes, reduce health inequalities, and advance the nation's progress toward the Sustainable Development Goals related to maternal and child health.

Public health authorities, policymakers, and nursing professionals should collaborate to:

- Increase public awareness through community-based education and media campaigns.
- Integrate milk banking education into maternal and child health services.
- Engage religious and community leaders in designing culturally compatible models.

- Ensure that milk donation and distribution processes meet the highest standards of hygiene and traceability.

Ultimately, human milk banking represents an opportunity to enhance health equity, social solidarity, and the survival of vulnerable infants—key priorities within the broader mission of public health.

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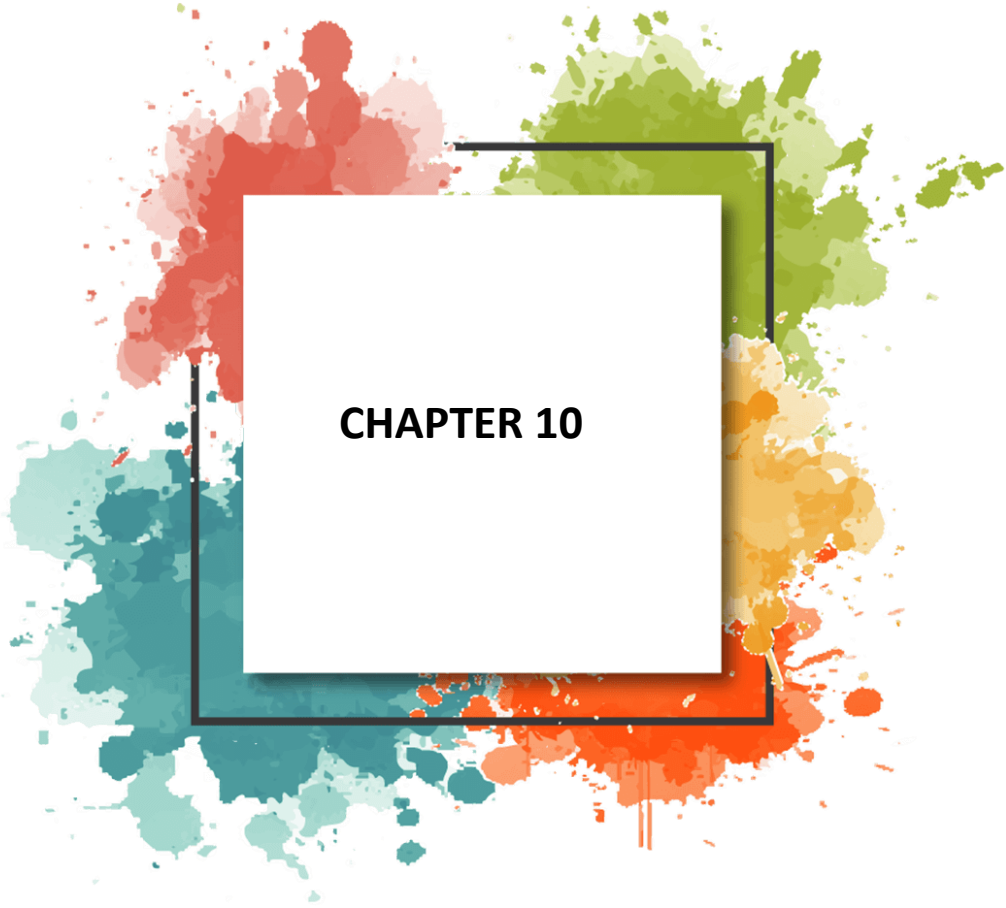
CHAPTER 10

Orthodontic Forces Effects on Periodontal Tissues and Multidisciplinary Management

Hacer Umay Uğurlu[1] & Hasan Basri Bircan[2]

Introduction

Orthodontics and periodontology are two fundamental disciplines of dentistry concerned, respectively, with correcting dental alignment and occlusion and with preserving the health of the supporting tissues of the teeth.[8,9] Although these fields pursue different primary aims, they meet on a common ground in terms of the biology and function of periodontal tissues. The main goal of periodontology is to preserve the health and integrity of the periodontium; indeed, orthodontic treatment is often required to correct pathological tooth movements or positional abnormalities that arise as a consequence of periodontal tissue loss.[1] Similarly, orthodontic problems such as malocclusion and crowding may adversely affect periodontal health. It has been shown that the density of periodontal pathogens is higher in crowded areas than in well-aligned segments.[2] Therefore, orthodontic alignment can contribute positively to periodontal tissue health by facilitating oral hygiene and eliminating traumatic occlusal forces.[3,2]

When properly indicated and accompanied by appropriate maintenance, orthodontic treatment can improve periodontal health; however, especially during fixed appliance therapy, undesirable changes may occur in periodontal tissues under poor oral hygiene. Orthodontic brackets and bands increase plaque accumulation and predispose to gingivitis.[4] In fact, individuals undergoing fixed orthodontic therapy may exhibit increased gingival inflammation and slight clinical attachment loss during treatment, with an average ~0.1 mm of alveolar bone resorption reported.[5] Consequently, orthodontic treatment has been described in the literature as a "double-edged sword," with both beneficial and adverse effects on periodontal tissues.[2] Although the long-term effects of orthodontic treatment on periodontal tissues are debated,[2] it has been reported that, under appropriate conditions, the risk of complications is quite low.[3]

In managing potential periodontal changes and risks associated with orthodontic treatment, it is crucial to determine the patient's individual periodontal risk profile. Poor oral hygiene, the presence or history of active periodontal disease, smoking, and systemic conditions such as diabetes are the main factors that increase the risk of periodontal complications during

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orthodontic therapy.[5,3] Therefore, before initiating orthodontic treatment, a comprehensive periodontal examination should be performed, active diseases should be treated, issues such as gingival recession should be addressed, and it should be ensured that the patient has an effective oral hygiene routine.[5] During orthodontic treatment, regular monitoring of periodontal status, professional cleaning with sustained patient motivation to maintain plaque control, and temporary interruption of orthodontic procedures where necessary to allow periodontal healing are critical to minimizing potential harm.[5]

The interaction between orthodontics and periodontology can also vary by patient age group. In young patients, periodontal tissues are generally healthy, and gingival changes occurring during orthodontic treatment usually remain at the level of superficial inflammation due to plaque accumulation. In contrast, adults and elderly patients may present with existing losses in periodontal support; in such cases, orthodontic treatment planning should be carried out in collaboration with a periodontist, and stabilization of periodontal health prior to treatment is critical.[3] It has been reported that the biological response of periodontal tissues to orthodontic forces slows with aging and that hyalinization foci in the periodontal ligament (PDL) can form more readily.[1] Nevertheless, chronological age alone is not an absolute contraindication to orthodontic therapy; when appropriate biomechanical approaches are employed, orthodontic tooth movement can be achieved even at advanced ages.[1]

In light of all these considerations, interdisciplinary collaboration between orthodontists and periodontists is indispensable for successful treatment outcomes. In periodontal-risk cases, planning and execution of orthodontic treatment should be performed in coordination with the periodontist. Especially in patients with periodontal problems, elimination of periodontal infections prior to orthodontic therapy, management of gingival recessions or bone defects, and regular periodontal follow-up throughout orthodontic treatment are of great importance.[5,6] The literature emphasizes that establishing a common language and communication between orthodontists and periodontists improves treatment outcomes.[2] Indeed, thanks to a multidisciplinary approach, it has been reported that periodontal health can be preserved and supported and that functional and esthetic improvement can be achieved with orthodontic treatment, including in cases with periodontitis.[6]

In conclusion, the interaction between orthodontics and periodontology is of great importance in dentistry, both in terms of biological foundations and clinical reflections. In this introductory section, the interaction between the two disciplines, their shared biological mechanisms, clinical significance, and the necessity of interdisciplinary collaboration have been outlined in general terms in light of the literature. In the following sections, the biological and clinical

changes produced by orthodontic forces on periodontal tissues, periodontal effects and risks associated with orthodontic treatment, age-specific approaches, and shared applications across orthodontics and periodontology will be addressed in detail.

2. Periodontal Tissues and Orthodontic Forces

Biological Effects of Orthodontic Forces on Periodontal Tissues

During orthodontic treatment, tooth movement results from the application of controlled forces to teeth. Removable appliances apply intermittent tipping forces, whereas fixed appliances can generate continuous multidirectional forces to produce torque, intrusion, extrusion, rotation, and bodily movement.[30,1] When teeth are moved orthodontically, the entire supporting apparatus—including osseous structures, the periodontal ligament (PDL), and soft tissue components—moves with the tooth.[33] Brown (1973) examined the effects of uprighting molars on the periodontium in four patients. Seven months after treatment commenced, a 2.5-mm greater reduction in probing depth was observed at the relevant sites of the uprighted molars compared with control teeth. Improvement in gingival architecture and less plaque accumulation on uprighted teeth were also recorded. In a follow-up study on 22 patients an average of 3.5 years later, probing depths on the mesial surfaces of uprighted mandibular molars were reported to be shallower than those of control teeth.[34]

Case reports have also indicated that decreases in probing depths can be achieved in intrabony defects following dental extrusion.[35,36] Additionally, cases of juvenile localized periodontitis have been reported in which eruption of teeth led to reduced probing depths.[37,38] In a healthy periodontium, the benefits of forced vertical extrusion for exposing tooth structure to facilitate prosthetic treatment have also been described.[39] The use of extrusive and intrusive forces in a healthy periodontium has been investigated in animals[40] with favorable outcomes when oral hygiene is maintained. In adult patients with marginal bone loss and deep overbite, intrusion of incisors has been associated with root resorption ranging from 1 to 3 mm. Optimal intrusion is recommended with low forces (5–15 g/tooth) and in the presence of periodontal health.[40]

Cellular and Tissue-Level Responses

The response of the periodontium to orthodontic forces is a coordinated process at the cellular and tissue levels. Mesenchymal cells and fibroblasts of the PDL and osteoblasts/osteoclasts in the alveolar bone secrete inflammatory mediators such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and prostaglandins upon mechanical stimulation; thus, collagen degradation and osteoclast differentiation begin within the connective tissue.[3,4] The RANK/RANKL/OPG system plays a pivotal role in this process: RANKL

released from PDL cells on the pressure side stimulates osteoclast precursors and accelerates bone resorption, whereas, on the tension side, the relative influence of OPG makes osteoblastic activity predominant.[4] Consequently, a marked increase in cytokines such as IL-1 is observed in the early stages of orthodontic tooth movement, and the ensuing inflammatory response enables remodeling of periodontal tissues through reorganization of collagen fibers and osteoclast/osteoblast activities. At the cellular level, PDL fibroblasts synthesize new collagen fibers on the tension side, whereas collagen fibers are degraded on the pressure side.[5] At the tissue level, blood vessels within the PDL constrict on the pressure side and dilate on the tension side, regulating regional blood flow and enabling shifts in the osteoclastic-osteoblastic balance.[3]

Tissue Responses According to Force Type and Magnitude

The effects of orthodontic forces on periodontal tissues vary according to the type (continuous, intermittent, or single application) and magnitude of the force. Continuous, light forces stimulate cellular activity without completely interrupting blood flow within the PDL and allow uninterrupted tooth movement. Therefore, the optimum orthodontic force should induce cell differentiation in the PDL to achieve tooth movement without fully collapsing the vasculature.[3] In intermittent force applications—e.g., with removable appliances—the PDL periodically has the opportunity to rest; compared to continuous fixed forces, this may result in less hyalinization and a more physiological tissue response (Ong & Bleakley, 2005).[1] Conversely, excessive or prolonged forces crush PDL fibers and lead to local ischemic necrosis, delaying bone resorption; tooth movement halts until the hyalinized necrotic tissue is removed.[2] Movement then resumes following elimination of hyalinization via "undermining" resorption from adjacent marrow spaces of the alveolar bone.[2] Excessive forces also increase cemental surface resorption and thus the risk of root resorption.[6] Forces that are too light may fail to provide sufficient biological stimulus and can slow tooth movement. Therefore, the force magnitude and duration should be adjusted according to the patient's age and periodontal tissue status, and optimal forces within biological limits should be used in every case.[5]

Dynamics of the Periodontal Ligament, Alveolar Bone, and Cementum

Periodontal Ligament (PDL): The PDL is a collagen-rich connective tissue that anchors the tooth to the alveolar bone. Upon application of orthodontic force, the PDL responds dynamically: the PDL space narrows on the pressure side and fibers are compressed, leading to catabolic reactions (collagen degradation, propensity for cell death); the PDL space widens on the tension side, fibroblasts synthesize new collagen, and extracellular matrix deposition increases.[5] The vasculature adapts similarly: vessels collapse on the pressure side and dilate on the tension side, increasing blood flow to relevant regions.[3] Thus, the PDL acts

like a sensory organ that perceives the applied force and initiates bone resorption-formation processes.

Alveolar Bone: The bone surrounding a tooth subjected to force responds as follows[30,31]: resorption occurs on the pressure side and new bone forms on the tension side. When pressure is applied to a tooth, compression of the PDL produces an initial movement phase of six to eight days. Compression of the PDL compromises blood flow in a region of the PDL and creates an avascular, acellular area called "hyalinization." When hyalinization occurs, tooth movement stops. This delay in movement can vary from short periods under light forces to longer periods under heavier forces. The hyalinized area is removed via PDL regeneration achieved by remodeling from marrow spaces (undermining resorption) and from areas adjacent to the unaffected PDL and alveolar bone. When the hyalinized area is eliminated, tooth movement can resume. If inflammation is present in periodontal tissues, PDL regeneration does not occur.[32] Therefore, inflammation must be controlled with periodontal therapy. As the tooth moves within bone, the bone remodels to follow the tooth to its new position. Since the periodontal attachment apparatus moves as a unit with the tooth, marginal bone level is largely maintained during orthodontic tooth movement under healthy conditions.[2] In other words, surrounding alveolar bone can maintain its health at the tooth's new position under appropriate force and hygiene, adapting with new bone apposition on both pressure and tension sides.
Cementum: Cementum, the mineralized tissue covering the root, can be affected particularly when excessive orthodontic forces are applied. Severe and uncontrolled forces may cause partial resorption of the cementum at the root surface, resulting in root resorption.[1] Microscopic cemental losses that occur under normal orthodontic force levels are generally termed "surface root resorption" and can be repaired after treatment via cementoblastic activity.[42] However, if cementum and dentin loss is advanced enough to shorten the root length, the change is irreversible and may adversely affect the long-term health of the affected tooth.[43]

Examples of Reversible and Irreversible Changes

Some changes in periodontal tissues under orthodontic forces are reversible, while others are irreversible. The literature indicates that, when ideal conditions are provided, orthodontic treatment does not create permanent damage in periodontal tissues; however, under poor oral hygiene or in the presence of active periodontal disease, treatment may lead to significant periodontal deterioration.[7,6] Many changes induced by orthodontic forces within physiological limits regress after treatment. For instance, slight mobility of teeth and widening of the PDL space during treatment are expected findings; after an adequate retention period, tooth mobility returns to normal and periodontal fibers reorganize.[5]

By contrast, certain adverse changes are irreversible and are recorded as permanent complications of orthodontic treatment. Root resorption exceeding a certain threshold is irreversible. Marked shortening of root length (e.g., resorption of more than one-third of the original length) is a permanent change that may negatively affect the tooth's prognosis.[6,4] Similarly, clinical attachment loss and reduction in alveolar bone height due to poor oral hygiene during treatment are also permanent; once bone loss occurs, it does not spontaneously return to its original state after treatment.[5] Gingival recession occurring when the tooth is moved excessively beyond the alveolar housing is likewise generally irreversible unless additional surgical intervention is undertaken.[2] Therefore, to minimize the risk of irreversible damage while supporting reversible adaptation processes, orthodontic treatment should be planned to protect periodontal health, employ optimal force levels, and ensure strict oral hygiene control.[1,5]

3. Orthodontic Treatment and Periodontal Health

Effects of fixed orthodontic appliances on periodontal tissues: Fixed appliances such as brackets and archwires can lead to direct and indirect effects on periodontal tissues by creating niches that favor plaque retention on tooth surfaces. Their presence can cause mechanical irritation at the gingival margin and increased microbial adherence, thereby intensifying gingival inflammation.[43] In vivo studies have shown that, even in patients with good oral hygiene, mild transient gingivitis and pseudo-pocket formation may occur during fixed orthodontic treatment, generally resolving shortly after appliance removal.[44] Conversely, when oral care is inadequate, fixed appliances may predispose to significant adverse periodontal changes. With increased microbial retention, the composition of the subgingival microbiota changes, contributing to exacerbation of periodontal inflammation.[45] In the presence of fixed appliances, slight clinical attachment loss and gingival enlargement have been reported, particularly due to plaque accumulation around bands and brackets. However, these changes largely depend on the patient's hygiene level and the treatment protocol.[4] The overall consensus is that, when good oral hygiene is maintained, the risk of permanent periodontal damage is minimal.[45]

Oral hygiene and microbial plaque accumulation: Effective oral hygiene during orthodontic treatment is critical for maintaining periodontal health.[46] Microbial dental plaque is the fundamental etiologic factor for gingivitis and periodontitis, and because plaque accumulation is facilitated in patients wearing fixed appliances, the risk of gingivitis increases.[2] Research shows that, in the absence of plaque control, orthodontic forces—particularly movements such as tipping or intrusion—can lead to angular bony defects and connective tissue attachment loss.[2] In contrast, when plaque accumulation is effectively controlled, gingival health can be preserved even under orthodontic treatment and no

permanent damage occurs in the supporting periodontal tissues.[2,5] Histologic findings regarding the impact of banded appliances on gingival health indicate that achieving excellent oral hygiene during and after band placement is essential to prevent permanent periodontal destruction. Therefore, a rigorous plaque-control program supported by regular professional debridement, fluoride applications, and patient motivation should be implemented throughout orthodontic therapy. With good oral hygiene, undesirable effects of orthodontic treatment on periodontal tissues can be minimized.[47]

Relationship between treatment duration and periodontal health: As the duration of orthodontic treatment increases, the time teeth remain covered with brackets and wires also increases, potentially raising periodontal risks. Over long treatments, maintaining patient motivation for oral hygiene may become more difficult, and plaque accumulation may pose greater problems over time. Short-term changes most commonly seen after appliance placement include mild gingivitis and gingival hyperplasia; these usually regress rapidly after treatment ends.[2] Long-term follow-up studies in adult patients suggest that, even years after orthodontic treatment, there are no significant differences in periodontal support compared with controls.[2] This finding indicates that—regardless of treatment duration—orthodontic therapy may not cause permanent harm to the periodontium when appropriate maintenance and follow-ups are provided. Nonetheless, some researchers have noted that unnecessarily prolonging treatment can impair maintenance of oral hygiene and negatively affect gingival health. Therefore, it is important to complete treatment in the shortest clinically reasonable time and perform regular periodontal evaluations throughout therapy. In summary, the effect of treatment duration on periodontal outcomes depends largely on hygiene management and individual risk factors; when conditions are appropriate, periodontal health can be preserved even in prolonged treatments.[4]

Age-related differences in periodontal response: Age is another factor influencing the periodontal response to orthodontic forces. In younger individuals, cellular activity in the PDL and alveolar bone is higher, and adaptation and remodeling occur more rapidly in response to applied forces. With advancing age, regenerative capacity decreases and collagen content increases; therefore, during orthodontic tooth movement in older patients, the tissue response may be slower and more limited. For example, in adults, hyalinization foci on the compression side of roots can form more readily under orthodontic force, causing transient pauses in tooth movement as part of a histologic adaptation.[1] Nevertheless, advanced age alone is not a contraindication to treatment. The literature reports that, provided appropriate periodontal preparation is undertaken, orthodontic therapy can be safely applied even in middle-aged and older adults, with successful adaptation of periodontal tissues.[4,2]

However, because older patients may present with additional factors—existing restorations, gingival recession, or prior periodontal loss—these must be considered in treatment planning, with closer periodontal monitoring than in younger patients. In adolescents, it should be remembered that gingival inflammation may be more likely due to hormonal changes, and hygiene instruction should emphasize this risk.

Clinical approaches in high-risk cases: Patients considered periodontal-risk typically include those with active periodontal disease or reduced periodontal support. In these cases, periodontal health must be stabilized before initiating orthodontic treatment.[6] After initial periodontal therapy—such as scaling and root planing—and plaque control are completed and inflammation is resolved, orthodontic treatment can be planned. Periodontal care should continue throughout orthodontic therapy, with regular periodontal assessments and professional cleaning to monitor gingival health.[5] Treatment planning should consider the patient's periodontal condition, and lighter, more controlled orthodontic forces are recommended, since excessive force can increase the risk of unwanted attachment loss and resorption in compromised periodontal tissues. In the presence of recession or a thin biotype, mucogingival procedures such as free gingival grafts may be considered before tooth movement to prevent potential defects related to movement. A multidisciplinary approach is essential in periodontally compromised cases: orthodontists and periodontists should work in coordination from pretreatment planning through treatment and maintenance. Where indicated, biologically supportive interventions—such as selective alveolar decortication and regenerative bone grafting—may be employed to facilitate tooth movement and improve periodontal tissue healing. Recent reviews emphasize that, in advanced periodontitis cases, orthodontic treatment performed after active periodontal therapy can facilitate plaque control by correcting dental alignment, reduce traumatic occlusion, and even stimulate new bone formation in defect areas, promoting stabilization of bone levels.[4,3] Nevertheless, a strict periodontal follow-up protocol should be implemented to detect adverse gingival changes early during orthodontic treatment. In summary, in periodontal-risk patients, orthodontic treatment—when properly indicated and meticulously executed—can yield gratifying outcomes for both occlusion and periodontal health.

4. Gingival Changes and Orthodontic Treatment

The relationship between orthodontic treatment and periodontal tissues is critical for successful treatment outcomes and gingival health. The literature emphasizes that, in patients undergoing orthodontic therapy, failure to achieve good oral hygiene markedly increases the risk of gingivitis and other periodontal complications.[4,5] In a systematic review by Bollen et al.,[10] orthodontic treatment,

compared with no treatment, was associated with 0.13 mm alveolar bone loss and a 0.23 mm increase in periodontal probing depth. Therefore, clinicians should perform a comprehensive periodontal evaluation before initiating orthodontic therapy. Any active periodontal disease should be treated first, and a robust preventive program should be established.[11,12] Etiology-oriented treatment principles should be applied.[13,14] Especially fixed orthodontic appliances can facilitate plaque accumulation and lead to undesirable gingival changes such as gingivitis, gingival hyperplasia, and recession.[2,3] Conversely, with appropriate periodontal preparation and maintenance, orthodontic treatment can in some instances support periodontal health.[3] The main gingival changes accompanying orthodontic therapy and their management are discussed below.

4.1 Gingival Hyperplasia

Another important change observed during orthodontic treatment is gingival hyperplasia (gingival enlargement) or pseudo-pocket formation. Plaque accumulation around brackets and bands leads to chronic inflammation and hyperplastic transformation of gingival tissues (Kwon et al., 2024).[5] Clinically, this presents as increased volume of papillary and marginal gingiva, a swollen appearance, and increases in probing depths. The literature indicates that gingival enlargement is among the short-term effects of orthodontic treatment and is particularly pronounced in patients with poor oral hygiene (Dannan, 2010).[2] Nevertheless, even in individuals with good oral hygiene, localized gingival enlargements may be observed due to mechanical irritation from bands or brackets.[2] Gingival hyperplasia may acquire a fibrotic character and may not fully regress spontaneously. Therefore, management should first aim to eliminate the causative plaque. Patient education for effective home care and professional scaling should be instituted to control inflammation.[15] If these conservative measures do not achieve sufficient regression, surgical gingivectomy may be required for persistent fibrotic tissues. To prevent recurrence, maintaining optimal plaque control throughout orthodontic treatment is crucial.[5] Clinicians should emphasize to patients the importance of removing dental plaque—the primary etiologic factor—to prevent unnecessary surgical procedures. After appliance removal, with appropriate maintenance, gingival enlargements have been reported to regress substantially in most cases and gingival health can be re-established.[3]

4.2 Mucogingival Problems

During orthodontic therapy, patients may have an increased risk of developing mucogingival deformities.[16] This is particularly common when planned orthodontic movements involve buccal displacement of teeth.[17,18] Therefore, careful mucogingival assessment should be part of the comprehensive periodontal evaluation before initiating orthodontic treatment.[5]

Mucogingival assessment should include determining the width of keratinized tissue, measuring any existing gingival recession, and evaluating the presence of high frenulum attachment.[5] Where necessary, appropriate mucogingival interventions (e.g., gingival grafting) should be performed before starting orthodontic therapy. During active orthodontic treatment, mucogingival conditions should be monitored regularly for potential deterioration or progression. If adverse changes are detected, mucogingival treatment may be required simultaneously with orthodontic therapy.[5]

Gingival recession is a condition characterized by apical displacement of the gingival margin that can occur during or after orthodontic treatment. Especially in patients with a thin biotype, buccal movement of teeth increases the risk of dehiscence and attachment loss, thereby predisposing to gingival recession.[2,5] Clinical studies have shown that 5–12% of patients may develop new or increased gingival recessions at the end of orthodontic therapy, with the mandibular anterior segment particularly affected.[6] Thin attached gingiva and insufficient bone thickness predispose to mucogingival problems related to orthodontic treatment.[6]

If the initial biotype is very thin or a recession is already present, periodontal procedures such as soft tissue grafting to increase the width of attached gingiva should be considered before or during orthodontic treatment.[5] Patients should be closely monitored for recession development during treatment. If progression is noted, protective grafting can be applied to the affected area. On the other hand, when properly indicated, orthodontic treatment can also help correct certain mucogingival problems. For example, when roots are orthodontically moved toward the center of the alveolar housing, existing mucogingival deformities may improve. In a cohort of twelve adults with mandibular incisors exhibiting gingival recession and roots positioned outside the alveolar envelope, moving the teeth toward the center of the alveolar arch reduced recession depth by 23% and width by 38%.[19]

Therefore, orthodontic planning should anticipate potential effects on gingival health in each case, and orthodontic treatment should be conducted within an integrated approach combined with periodontal therapy.

4.3 High Frenulum Attachment

During orthodontic treatment, a high frenulum attachment may hinder effective plaque removal in the affected area.[20] The presence of orthodontic attachments and a high frenulum can make manipulation of the toothbrush around the marginal gingiva difficult, leading to pronounced residual plaque accumulation and initiating localized periodontal inflammation and disease.[5] Although debated, a high frenulum attachment may further increase the risk of mucogingival deformity due to a traction effect.[16,21] From an orthodontic

perspective, a high frenulum attachment can make closure of a maxillary midline diastema more difficult.[22,23] Therefore, clinicians should carefully examine patients when planning orthodontic treatment and decide whether frenal detachment or frenectomy would benefit the patient.

4.4 Root Resorption

During orthodontic treatment, external root resorption may occur, leading to shortening of roots.[17,24] The prevalence ranges from 98.1% for mild resorption to 2.9% for severe resorption.[17,25] As a result, teeth may exhibit excessive mobility during active orthodontic treatment and their long-term periodontal prognosis may be adversely affected. Radiographic assessment during active orthodontic therapy should therefore include evaluation of the roots, especially in patients with risk factors for root resorption. These risk factors include family history of root resorption, prolonged active orthodontic therapy, tooth extraction, and a history of trauma.[17,26] If root resorption is detected, the clinician should inform the patient, discuss potential risks, and suspend active orthodontic movement for at least 2–3 months and up to 6 months to allow healing.[17,27] After completion of orthodontic therapy, teeth with root resorption should be strictly monitored to prevent periodontal disease development. As long as a healthy periodontium is maintained, morbidity risk is not higher for teeth with shortened roots.[26] Therefore, clinicians should establish a robust preventive program that includes regular monitoring, home care, supportive periodontal therapy, and occlusal management (e.g., intraoral or extracoronal splinting, night guard therapy, or occlusal adjustment to eliminate fremitus or premature contacts).[5]

4.5 Post-Orthodontic Gingival Healing and Relapse

Upon completion of orthodontic treatment, a marked improvement in gingival condition is usually observed. Following removal of fixed appliances, a decrease in plaque accumulation and provision of professional cleaning lead to substantial regression of gingival inflammation that developed during treatment.[48] Especially gingivitis and mild hyperplasia arising during orthodontic therapy can completely resolve in the post-treatment period with effective home care. However, to maintain periodontal health, regular follow-ups and supportive periodontal care must continue after treatment.[49] Otherwise, with deterioration in oral hygiene, gingival inflammation can readily recur and the achieved improvement may not be permanent.

Another issue at the end of orthodontic treatment is dental relapse and its relationship with periodontal tissues. During stabilization of teeth in their new positions, the PDL and supracrestal gingival fibers have a memory of their previous position, and there is a tendency for especially rotated teeth to return to their former position. A well-planned retention program is therefore essential to

prevent post-treatment relapse. In addition to retainers, circumferential supracrestal fiberotomy, which involves severing the transseptal and gingival fibers around the teeth, can be applied where indicated.[1] By preventing tension generated by collagen fibers in teeth corrected by rotation, fiberotomy helps maintain the position achieved through orthodontic therapy. In this way, both positional stability and prevention of gingival relapse (e.g., recurrent inflammation or recession) are supported after treatment. In conclusion, regular monitoring of both tooth positions and periodontal status at scheduled intervals after orthodontic therapy, along with supportive periodontal treatments as needed, is indispensable for successful long-term outcomes.[49]

6. Clinical Applications and Special Topics

In the intersection of orthodontics and periodontology, many clinical applications and special situations can yield successful esthetic and functional outcomes when managed with a multidisciplinary approach. This section evaluates, in light of the literature, periodontal surgeries adjunctive to orthodontic therapy, orthodontic approaches in patients with periodontal loss, the orthodontic–implant relationship, and esthetic/functional special topics.

Periodontal surgical procedures adjunctive to orthodontic treatment may be preferred to shorten treatment time, prevent relapse, or enhance esthetic outcomes. In particular, circumferential supracrestal fiberotomy (CSF) is recommended to reduce relapse risk after rotational corrections.[1] This procedure reduces the tendency of teeth to return to their former positions by severing the supracrestal collagen fibers around teeth. Similarly, alveolar decortication and procedures such as accelerated osteogenic orthodontics can be used to facilitate orthodontic tooth movement and support periodontal regeneration.6 However, such surgeries should be applied only under correct indications and in close collaboration with periodontology.

Orthodontics in patients with periodontal loss has attracted growing interest in recent years. In patients with attachment loss due to advanced periodontitis, orthodontic treatment can achieve both functional and esthetic improvements. Controlled intrusion and extrusion, in particular, can favorably modify the morphology of bone defects and contribute to periodontal therapy.[3] Nevertheless, in such cases, orthodontic forces must be kept light, applied in a stable environment after active periodontal therapy, and monitored in coordination with a periodontist throughout the process.[4,2] In individuals with periodontal loss, orthodontic treatment not only provides an esthetic smile, but by correcting tooth position, it facilitates plaque control and reduces occlusal trauma, contributing to long-term periodontal stability.[6]

The orthodontic–implant relationship is an important special topic clinically. Since implants cannot move like natural teeth, they can be used as anchorage sources in orthodontic treatments.5 Mini-implants placed especially in posterior regions make tooth movements more controlled and prevent excessive forces on periodontal tissues. In cases with missing teeth, orthodontic treatment can be planned to provide the ideal space and bone support for implants.[3] However, aligning the teeth orthodontically before implant placement is critical to obtaining the most favorable esthetic and functional outcomes.

During active orthodontic treatment, bone grafting may be performed for future implant therapy.[5] When guided bone regeneration is carried out simultaneously with orthodontic treatment, healing has been shown to accelerate significantly in line with the principles of the regional acceleratory phenomenon.[18,28] Biologically, osteoblasts and osteoclasts already present in the region during orthodontic treatment can be harnessed to facilitate resorption of graft particles and their replacement with autogenous bone. This may be applied to orthodontic patients who need hard tissue augmentation for future implants. For example, an adolescent orthodontic patient with a congenitally missing incisor often presents with an atrophic alveolar ridge. During orthodontic treatment, this atrophic ridge can be successfully augmented using the regional acceleratory phenomenon. Near the completion of orthodontic therapy, hard tissue healing will also have been achieved, and the patient can proceed to implant treatment without delay. The same approach can be applied to adults requiring implants to rehabilitate an atrophic edentulous area alongside orthodontic treatment.5 Where necessary, a definitive implant fixture can be used to enhance the orthodontic outcome. For example, appropriate biomechanics for uprighting an adjacent tooth can be achieved by using an osseointegrated implant complex as orthodontic anchorage.[29]

Esthetic and functional special situations require joint management by orthodontics and periodontology. For example, “black triangles” that arise during space closure can cause both esthetic and phonetic problems. In such cases, in addition to orthodontic treatment, papilla regeneration techniques or composite restorative procedures can be planned. Likewise, gingival asymmetries that arise during or after orthodontic treatment can be corrected with esthetic gingivoplasty procedures. Crown lengthening performed alongside orthodontics to enhance smile esthetics has also been described in the literature.[4]

In conclusion, clinical applications and special situations accompanying orthodontic treatment can be managed successfully when carried out in close collaboration with periodontology. Orthodontics can enhance the effectiveness of periodontal therapies, while periodontal surgeries can make orthodontic outcomes more stable in the long term. Therefore, a multidisciplinary approach

and patient-specific planning are fundamental requirements for achieving satisfactory esthetic and functional results in clinical practice.

7. Conclusion and Future Perspectives

The relationship between orthodontics and periodontology is a critical factor determining treatment success and long-term prognosis in dentistry. Current literature shows that orthodontic treatment can have both beneficial and adverse effects on periodontal tissues. Especially the presence of fixed appliances increases plaque accumulation and elevates the risk of gingivitis and gingival hyperplasia,4–5 whereas in individuals with appropriate plaque control, these changes are mostly transient and reversible. Conversely, in individuals with impaired periodontal health, incorrect or uncontrolled application of orthodontic forces can lead to permanent attachment loss.

Orthodontic treatment not only provides esthetic and functional improvement but, when properly planned, also supports periodontal health. By correcting malocclusions, plaque control is facilitated, traumatic occlusion is eliminated, and periodontal stability is reinforced.[3] Nevertheless, it is clear that treatment planning in periodontal-risk patients must be carried out with a multidisciplinary approach. Collaboration between periodontists and orthodontists at all stages of the treatment process (before, during, and after) ensures long-term stability of dental alignment and the health of the periodontium.

From a future perspective, molecular-level research is needed to better understand the biological processes during orthodontic treatment. Studies focusing on cytokines, growth factors, and genetic markers can elucidate in more detail the response of periodontal tissues to orthodontic forces. In addition, prospective clinical studies evaluating outcomes of orthodontic approaches combined with regenerative periodontal surgery will contribute to advances in this field. With the development of digital orthodontics and biocompatible materials, methods that inflict less harm on periodontal tissues and shorten treatment time are anticipated to be used more frequently in the near future.

In conclusion, collaboration between orthodontics and periodontology is a fundamental necessity for patient-centered and successful long-term outcomes in contemporary dentistry. An interdisciplinary approach not only integrates existing periodontal problems into orthodontic treatment but also provides the most reliable strategy for meeting esthetic and functional requirements. Therefore, more in-depth examination of the intersections between these two fields in both clinical practice and research will guide future generations of dental practice.

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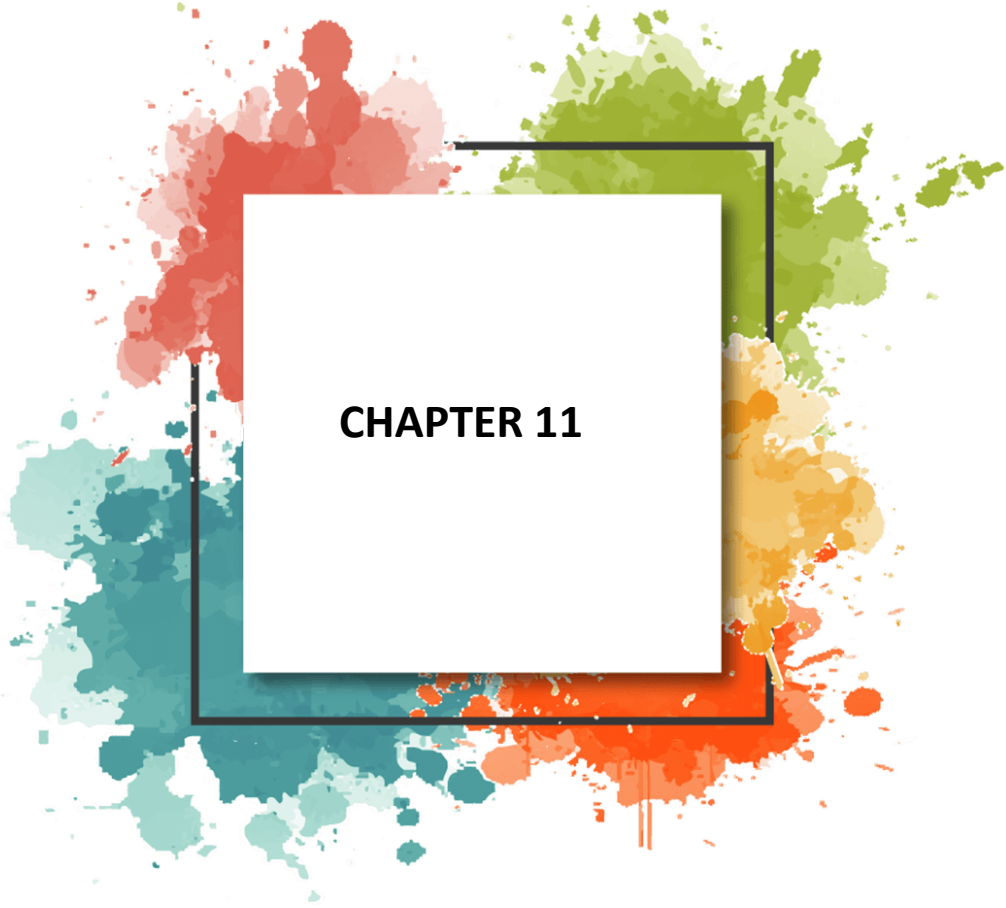
CHAPTER 11

New Horizons in Cancer Immunotherapy

Elif Erturk[1]

1. Tumor Immunology

The immune system is the body's system capable of recognizing foreign antigens and destroying them more rapidly when they are detected again. This mechanism protects against cancer cells that develop throughout life. The organs that make up the immune system are the central lymphoid organs (bone marrow and thymus), and the peripheral lymphoid organs (lymph nodes, spleen, and mucosa-associated lymphoid tissue). Impairments in the immune system lead to the inability to destroy cancer cells, their uncontrolled proliferation, spread, and death (1). Based on this research, immunotherapy has become one of the leading drugs in cancer treatment today.

The immune system can be examined under two main headings. The first is "innate immunity," which is innate, and the other is "acquired immunity," which is acquired later. Acquired immunity is further divided into cellular and hormonal immunity. In innate immunity, a very rapid response develops against previously unrecognized foreign antigens. The main cells involved in this response are neutrophils, leukocytes, macrophages, phagocytes, natural killer (NK) cells, and dendritic cells. The target itself or molecules expressed on its surface are recognized, and the phagocytosed target is destroyed by the complement or lectin system. Acquired (acquired) immunity, on the other hand, is antigen-specific. The response rate is slower, but subsequent encounters with the stored antigen elicit a more rapid response. The primary cells involved are B and T lymphocytes. Acquired immunity is examined under two headings. In cellular immunity, antigen-specific T lymphocytes are involved, and these are the primary cells in cancer immunotherapy. The other is humoral immunity, characterized by the antigen-specific antibody synthesis of B lymphocytes (2).

2. Immune System Cells

Lymphoid precursor cells give rise to T, B, and NK lymphocytes, while bone marrow myeloid precursor cells give rise to neutrophil leukocytes, dendritic cells, and macrophages. These cells, called T lymphocytes because they originate in the thymus, are responsible for cellular immunity. They recognize and activate antigens by interacting with the Major Histocompatibility Complex on the surface of antigen-presenting cells (APCs) via the T-cell receptor (TCR). T cells are able

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to recognize cancer cells thanks to specific tumor antigens secreted by cancer cells. MHC class 1 molecules, found on all nucleated cells, can be recognized by CD8+ T lymphocytes, while MHC class 2 antigens on the surface of APCs are recognized by CD4+ T cells. T cells are activated in two ways. The first is the formation of a TRC-MHC complex. The second is costimulation. The interaction between CD28 on the T cell surface and CD80 (B7-1) and CD86 (B7-2) on APCs is called the priming phase and occurs in the lymph nodes. Negative regulatory cell surface receptors such as cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed cell death 1 (PD1) are expressed on the surface of activated T lymphocytes. Binding of PD1 with its ligand (PDL-1), present in the tumor and tumor microenvironment, inhibits phase 2, while binding of CD80 and CD86 with CTLA4 inhibits phase 1. Immune checkpoint inhibitors used in immunotherapy exert their effects at these stages (3).

CD4+ T lymphocytes are divided into two types: T helper 1 (Th1), which secretes interleukin 2 (IL2) and interferon (IF), and T helper 2 (Th2), which secretes IL4, IL5, and IL13. They are important in regulating immune system function. CD8+ T cells, also called cytotoxic T lymphocytes, distinguish cancer cells from normal cells and destroy them with granules that initiate apoptosis (4).

B lymphocytes originate from the bone marrow and function in the immune system by producing specific or nonspecific antibodies. NK cells are cells capable of killing through antibody-dependent cytotoxicity without the need for antigen presentation or cell surface IgG. If MHC class 1 activation in cancer cells decreases, NK cells induce lysis via IFN and TNF. NK cells are considered the first cells encountered by metastasizing cancer cells, and it is known that cancer cells metastasize by inhibiting NK cells. Macrophages are cells found primarily in tissues that destroy damaged, dead, and mutant cells in the body. After destroying cancer cells with toxic oxygen radicals and TNF, they present their antigens to T cells. Cancer cells can avoid this pathway through antiphagocytic "don't eat me" signals. Phagocytosis is inhibited after the interaction of SIRPα on the surface of macrophages with CD47 on the tumor cell surface. Dendritic cells are considered the most advanced antigen-presenting cells (APCs). They activate T cells by carrying MHC class 2 (5).

3. Immunotolerance and Immunosurveillance

Immunotolerance is the state of unresponsiveness to the body's own antigens in a healthy immune system. This can occur naturally or through external immune system induction.

This characteristic develops in immune system cells in two ways. First, it occurs centrally, during the developmental stages of cells in the bone marrow and thymus, and second, it develops in the peripheral tissues and lymph nodes where

mature T and B lymphocytes reside. If this state is disrupted, the individual's own immune system attacks its own cells, leading to autoimmune diseases, allergies, and organ transplant rejection.

Immune surveillance is the immune system's ability to recognize and destroy cancer or precancerous cells through specific antigens or molecules with increased synthesis. Two other ways the immune system prevents cancer formation are to prevent the development of tumors that involve viruses in their etiology by eliminating viruses and to shorten the inflammatory process that accelerates tumor formation by eliminating pathogens (6).

4. Tumor Immunoregulatory (Immunediting)

After the immune system's effectiveness in preventing cancer development was demonstrated, and the immune system's contribution to cancer development was also established, the term tumor immunoregulation was chosen to broadly describe the role the immune system plays in cancer development. This is a three-phase process. It is referred to as the 3 E's of tumor immunology. The phases that comprise this process are similar to the concept of elimination and immune surveillance. *Equilibrium* is the quiescent period of tumor cells that cannot be destroyed by the immune system. *Escape* is the period during which tumor cells, which can be stopped during the equilibrium period, evade the immune system and undergo uncontrolled proliferation and growth (7).

5. Immunotherapy in Cancer Treatment

Historically, the first attempts at immunotherapy began in the 18th century. In 1866, Wilhelm Busch observed that tumors shrank when streptococcal skin infections developed on the skin overlying tumors. He then attempted to treat tumors by inducing infections in cancer patients (5). In 1891, Coley treated soft tissue tumors by intratumorally administering a vaccine he had obtained from inactivated Streptococcus pyogenes and Serratia Marcescens bacteria (8).

6. Immunotherapy Drugs Used in Clinical Practice

6.1. Monoclonal Antibodies

Monoclonal antibodies are the most approved and widely used drugs in cancer treatment. Monoclonal antibodies initiate immune activity by binding to cell surface receptors. They have been developed to target cancer-promoting growth factors such as vascular endothelial growth factor (VEGF), epithelial growth factor (EGFR), and human epidermal receptor (HER2), or antigens specifically produced on cancer cells, such as CD52 and CD20. In recent years, drugs developed based on immune checkpoints (PD1, PDL-1, and CTLA4) have ushered in a new era in immunotherapy for cancer patients.

6.2. Immune Checkpoint Inhibitors

This group of drugs has become a dominant force in current cancer treatment over the last 10 years. PD1 (programmed death receptor), PDL-1 (PD1 binding ligand), and CTLA4 (cytotoxic T-lymphocyte-associated antigen 4) have been selected as targets for therapeutic drugs in this area. These three molecules act as immune checkpoints, and their activation inhibits T-cell-mediated antitumor activity. Studies conducted with the idea that blocking this process could lead to an enhanced immune response against tumor cells have yielded positive results (9,10).

6.3. Anti-PD-1 Monoclonal Antibodies

6.3.1. Pembroluzumab: As an anti-PD-1 monoclonal antibody, significant gains in survival rates have been achieved in many cancer types (11). It is currently the first-line drug of choice for suitable patients in lung cancer, the most common cancer and the leading cause of cancer-related death (12). In patients with malignant melanoma, a skin tumor, it has significantly increased the time to recurrence and survival times as an adjuvant in advanced-stage and surgical cases (13). It is effective in lymphoma, esophageal, stomach, colon, bladder, kidney, cervix, and endometrial cancers. The presence and degree of PD-1 expression as a biomarker has not yet been clearly accepted. Its use in tumors with high microsatellite instability (MSIH), regardless of organ or site (tumor agnostic), has been a first in cancer treatment (14).

6.3.2. Nivolumab: This is the second anti-PD-1 monoclonal antibody used. It has been used successfully in many cancer types, including non-small cell lung cancer, melanoma, renal cell cancer, head and neck cancers, and lymphoma. It is also effective in the treatment of MSI-H tumors, regardless of anatomic location (15-17).

6.4. Anti-PDL-1 Monoclonal Antibodies

6.4.1. Atezolumumab: A significant advantage has been achieved in triple-negative breast cancer, especially when used in conjunction with chemotherapy, despite its poor prognosis (18). It is used in advanced-stage small cell lung cancer and bladder cancer (19,20).

6.4.2. Avelumumab: It is used in metastatic Merkel cell carcinoma and bladder cancer (21,22).

6.4.3. Durvalumumab: This is an approved drug with demonstrated efficacy in advanced-stage small cell lung cancer and bladder cancer (23).

6.5. Anti-CTLA-4 Monoclonal Antibodies

6.5.1. Iplimumab: This is the first CTLA-4-targeted drug to be discovered. It has been found to be effective in the treatment of melanoma and has begun to be used, but due to the high number of side effects and the greater effectiveness of anti-PD-1-targeted drugs, it is not used alone. Currently, it is used in combination with other immunotherapy drugs (24,25).

6.5.2. Tremlimumab: While used in mesothelioma and hepatocellular carcinoma, it is currently under investigation in other cancer types (26).

Studies targeting other immune checkpoints are ongoing, and among these, T-cell immunoglobulin ITIM domain (TIGIT), lymphocyte activation gene 3 (LAG-3), V region immunoglobulin suppressor T-cell activation (VISTA), T-cell immunoglobulin (TIM), and OX40 stand out as targets with positive results (27).

7. Cytokines

Cytokines, which play an important role in cell signaling, are proteins that enable the development, organization, and communication between distant sites of immune system cells. Tumor cells also contain high levels of cytokines (1). The most commonly used are interleukin-2 and interferons. IL-2 has been used in renal cell cancer, and interferons in melanoma and renal cell cancer. However, due to the development of more effective treatments, they are no longer included in current treatment plans (28).

8. Adoptive Immunotherapy

Those T cells (tumor-infiltrating T cells/TILs, CAR-T cells, BITE/bispecific T cell enganger), which have been immunologically activated and expanded in the laboratory, are administered back to the patient to elicit a strong, tumor-specific immune response. This is a highly toxic and challenging method (29). It can be used in the treatment of prostate cancer, but is not preferred due to its cost and difficulty of application (30). Good results have been obtained in treatments with CAR-T and BITE, especially in hematological malignancies, and they have entered clinical use (31,32).

9. Oncolytic Virus Treatment

Oncolytic viruses are natural or engineered viruses that can survive and replicate in tumor cells. The powerful signals sent to dendritic cells by these viruses enhance the antitumor immune response and accelerate tumor cell lysis. However, when administered intravenously, they are destroyed by the humoral system and, when administered intratumorally or in the peritumoral area, they lack sufficient viral replication. Oncolytic herpes simplex virus 1 (talimogen laherparepvec/T-Vec) expressing GM-GCSF is used in metastatic malignant melanoma (33).

10. Cancer Vaccines

The formation of an immune response is a process that begins with antigen presentation. Cancer vaccines are used to activate adaptive immunity or strengthen existing responses. Cancer vaccines can be antigen-based (tumor-specific or tumor-associated antigens). Tumor-specific antigens are ideal targets for antitumor therapy. They are protein products of mutated normal cell genes and are produced only by cancer cells. They are foreign to the immune system and therefore elicit high-affinity antitumor T-lymphocyte responses. Antigen vaccines are antigens with protein-peptide and ganglioside structures. These antigens are injected into the patient's cancerous area, and the immune system stimulates the production of antibodies or cytotoxic T cells (34). Tumor cell-based vaccines aim to enhance the immunogenicity of tumor cells with low immunogenicity and enhance the antitumor response (35).

Their disadvantages include increased autoimmunity and T-cell allergy. Dendritic cells (DCs) are the most important and potent T cell stimulators among antigen-presenting cells (APCs). DC-based cancer vaccine studies have historically been conducted primarily on melanoma patients. However, the only approved use currently is sipuleucel-T in prostate cancer. The limited efficacy of DC vaccines depends on the source and strength of the DCs used in the application and their ability to migrate to the lymph nodes (36). Genetically based vaccines include DNA and RNA-based vaccines. Following cell transfection, bacterial plasmids express their encoded proteins, and these antigenic proteins initiate or accelerate the adaptive and innate immune response process. DNA-based vaccines have not yet yielded satisfactory results (37). RNA-based vaccines, on the other hand, are promising because they are rapidly degraded in the body, cause fewer autoimmune reactions, and enhance immune responses (38). Anti-idiotypic cancer vaccines consist of antibodies with three-dimensional immunogenic idiotopes that can bind to cell receptors. The variable region of B-lymphocyte membrane immunoglobulin is used as the antigen in idiotypic vaccines. It is still in the experimental stage and is not used clinically (39).

11. Conclusion

Chemotherapeutic agents are intended to target malignant cells, exerting cytotoxic effects that will lead to tumor regression and increased survival. One of the main problems limiting the effectiveness of chemotherapies is the development of drug resistance. Resistance to chemotherapy leads to inadequate drug uptake, poor prognosis, and disease relapse. The search for alternative cancer treatments to prevent the development of resistance has led to the development of immunological therapies. Various clinical trials are ongoing in combination with immunotherapeutic agents, cytotoxic chemotherapy, and/or radiation, all of which strive to provide longer-term disease control. These

treatments, compared to traditional treatments, aim to enhance immune responses by activating or enhancing the immune system against the tumor. The success of cancer immunotherapy depends largely on the identification of tumor antigens and the development of biomarkers. Immune Checkpoint Inhibitors (ICIs) can halt tumor progression in some patients with advanced malignancies. For example, when pembrolizumab treatment was reported to help more than 15 percent of people with advanced small cell lung cancer live at least five years, the average five-year survival rate for this type of cancer was reported to be only 5.5 percent when it began in 2012 (40). Although studies on cancer immunotherapy are ongoing, its clinical use remains limited, and its efficacy and safety are debated. Regarding efficacy, only some patient groups have been reported to respond to immunotherapies (41). Furthermore, more studies have been conducted on hematological cancers (42). In recent years, although the success of immunotherapies in solid tumor treatment studies appears promising, few drugs have yet been approved by the FDA for the treatment of solid tumors (43). This is partly due to the high costs of solid tumor research and the difficulty of selecting biomarkers. Despite this, the intensification of clinical trials is promising. Identifying biomarkers is crucial for patient selection and predicting patient responses. Therefore, strategies to develop patient-specific immunotherapies based on biomarkers expressed on cancer cells and to evaluate combination therapy options to improve response rates should be further studied.

Regarding the safe use of immunotherapy, immunotherapy can cause autoimmune side effects in some patients, leading to attacks on healthy tissues. Many immunotherapies can cause severe hypotension, fever, renal dysfunction, and other potentially fatal side effects. Although CAR-T cells have shown tremendous promise in the treatment of hematological tumors, due to the immunosuppressive TME, CAR-T cells are ineffective at infiltrating solid tumor tissue. CAR T cells can recognize and interact with normal cells expressing target antigens, even at low levels. When activated in normal cells such as the heart, liver, or lung, they can potentially lead to death (44). Additionally, cytokine release syndrome and tumor lysis syndrome may occur after high-dose CAR T-cell infusion (45). Therefore, numerous clinical trials are underway to improve the safety and high efficacy of CAR T-cell research. Furthermore, PD-1 and PD-L1 blockade is one of the most active areas of clinical research in ICI therapies. A recent phase II randomized trial in patients with advanced BRAF V600 E/K mutant melanoma demonstrated increased progression-free survival and duration of response in patients treated with dabrafenib plus trametinib and pembrolizumab compared to those treated with dabrafenib plus trametinib (46). One of the most recent studies with preliminary results is the COMBI-I study, which investigated trametinib and placebo-dabrafenib, trametinib, and the anti-PD-1 agent PDR001 in 178 patients. This clinical trial yielded preliminary results

reporting a 94% disease control rate and a 33% complete response rate. Despite the benefits of such combinations in enhancing antitumor efficacy, immunotherapy combinations carry a significant risk of toxicity. In melanoma, combinations of dabrafenib, trametinib, and anti-PD-1 resulted in higher rates of grade 3/4 adverse events than expected compared to monotherapies (47). Furthermore, the failure of most patients to develop durable antitumor responses after ICI treatment due to developing resistance limits the success of immunotherapy.

Despite the beneficial results of immunotherapies, it should be noted that long-term use can lead to autoimmune reactions and the development of resistance to the immunotherapeutic agents used. While the agents used in traditional treatment methods target cancer cells, they also attack normal cells in the body. Severe side effects and resistance to chemotherapy limit the success of these treatments. To overcome these limitations in immunotherapies, intensive research is underway on biomarkers, and immunotherapy has achieved significant success in treating cancer since 2011. In the future, immunotherapy may be an approach that can address the limitations of personalized treatments by identifying biomarkers that will result in fewer immune-related adverse events compared to traditional treatment methods.

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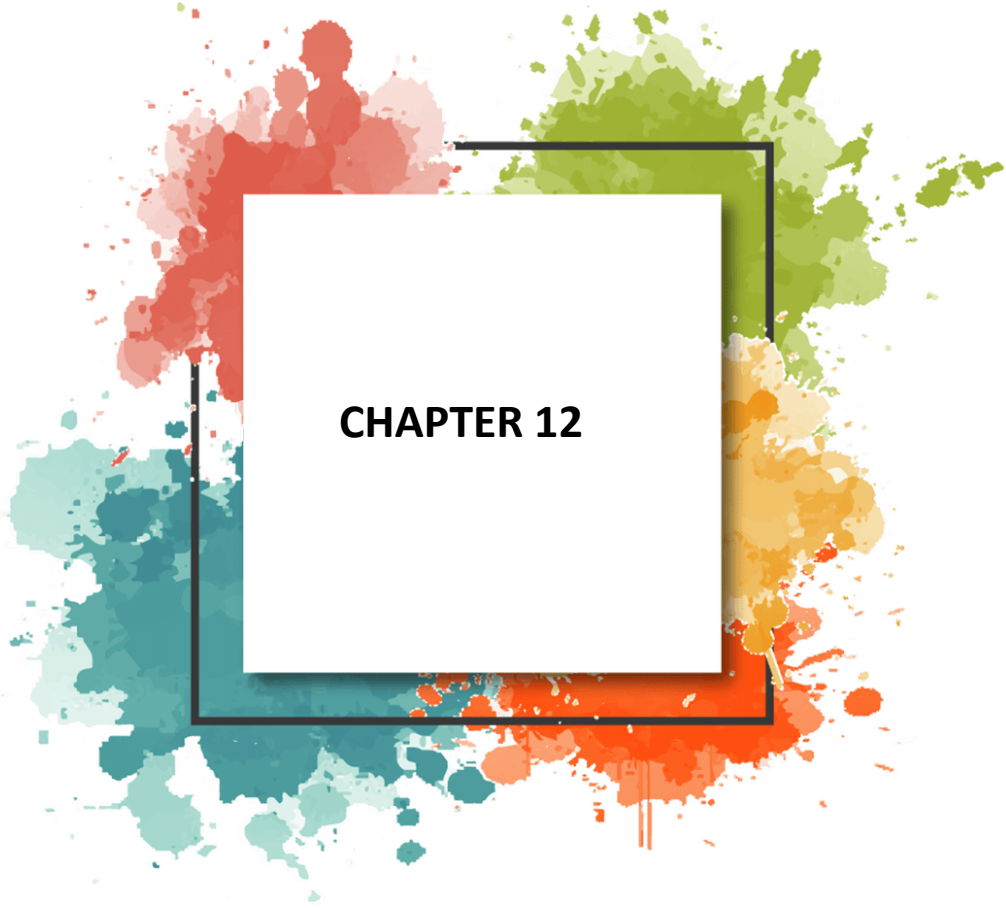
CHAPTER 12

Keratinized Primary Lesions Superinfected with *Candida*

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Among the microorganisms inhabiting the oral cavity, *Candida* species are the most frequently encountered opportunistic pathogens (Lalla, Patton, & Dongari-Bagtzoglou, 2013). Although they normally exist as commensals in the oral flora of healthy individuals, disruptions in systemic or local immune balance can promote their transition to pathogenic form, leading to infection. Systemic factors, including immunosuppressive therapy, immune disorders, diabetes, and malnutrition, as well as local factors such as reduced salivary flow rate, poor oral hygiene, smoking, atrophic oral mucosa, radiotherapy, ill-fitting dentures, and mucosal diseases, play a role in the development of oral candidiasis (Patil, Rao, Majumdar, & Anil, 2015).

Since *Candida* carriage varies with age, lifestyle, and genetic susceptibility, it is difficult to provide a prevalence rate for the general population. However, rates ranging from 17% to 75% have been reported (Meurman, Siikala, Richardson, & Rautemaa, 2007). Epidemiological studies have shown *Candida* carriage in 45 to 65% of healthy children and 30 to 45% of healthy adults, with the prevalence increasing with age and in the presence of predisposing conditions (Axéll, Henriksen, Nilner, & Heimdahl, 1990). In Türkiye, *Candida* carriage has been reported in 26.3% of healthy children aged 0–12 years, with *Candida (C.) albicans* accounting for 22.3% of isolates (Kadir, Uygun, & Akyüz, 2005). In another study conducted in Türkiye, an overall *Candida* carriage rate of 37.2%, with *C. albicans* accounting for the highest proportion (75.8%) among the isolated species, was reported (Ak, Erturan, Ünür, & Yeğenoğlu, 1998).

Although more than 150 *Candida* species have been identified to date, only a limited number are capable of colonizing human hosts. Approximately 65% of these species cannot grow at human body temperature (37°C) (Silva *et al*., 2012). *C. albicans* is the most frequently isolated species in oral candidiasis; other species such as *C. kefyr*, *C. krusei*, *C. dubliniensis*, *C. guilliermondii*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis* also play a role (Casu, Pinna, Denotti, Murgia, & Orru, 2023). *C. albicans* is responsible for approximately 95% of fungal

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infections in the oral cavity (Vila, Sultan, Montelongo-Jauregui, & Jabra-Rizk, 2020). However, in recent years, an increased incidence of species such as *C. glabrata* and *C. krusei*, displaying specific resistance to antifungal treatment, has been reported (Meurman *et al*., 2007; Byadarahally Raju & Rajappa, 2011).

Oral candidiasis is classified as primary and secondary. Primary oral candidiasis is subdivided into four types, which are acute forms, chronic forms, *Candida*-associated lesions, and primary keratinized lesions superinfected with *Candida* (Singh, Verma, Murari, & Agrawal, 2014). Secondary oral candidiasis includes disseminated and persistent infections that are generally seen in association with systemic immunodeficiency syndromes. This form encompasses candidal infections of the oral mucosa, particularly in congenital and acquired immunodeficiency syndromes such as thymic aplasia (DiGeorge syndrome) and candidiasis-endocrinopathy syndrome (Gümrü, 2025; Singh *et al*., 2014).

1. PATHOGENESIS OF CANDIDIASIS

C. albicans exhibits several adaptive mechanisms to maintain its viability and pathogenicity in the host, including thermotolerance, adhesion and invasion capability, nutrient acquisition, immune evasion, and resistance to antifungal agents (Lopes & Lionakis, 2022). These traits, known as virulence factors, contribute significantly to the pathophysiological processes of colonisation, onset of infection, and its progression (Talapko *et al*., 2021). Specifically, the primary virulence factors responsible for the pathogenicity of *C. albicans* are adhesion and invasion, germ-tube formation, dimorphism, phenotypic switching, production of toxic substances, and hydrolytic enzyme secretion (Gümrü, 2025).

Infection of oral epithelial cells by *C. albicans* occurs via two primary mechanisms: induced endocytosis and active penetration. Induced endocytosis leads to invasion of epithelial and endothelial cells by hyphal components, and host factors are important at this stage. Active penetration, on the other hand, leads to direct cell damage and depends on fungal activity (Bartie, Williams, Wilson, Potts, & Lewis, 2004; Talapko *et al*., 2021).

1.1. Dimorphism and Germ-Tube Formation

C. albicans is a budding pathogen that can exist in a variety of cellular forms, ranging from yeast to pseudohyphae, or true hyphae (Gow, 1997). All three morphological forms can coexist within a colony, and their proportions can determine the appearance and pathogenic potential of the colony. The yeast form of *C. albicans* is considered avirulent, and its ability to transform from yeast to hyphal form is critical for pathogenicity (Sudbery, 2001). This morphological change not only increases its ability to adhere to epithelial cells but also improves tissue invasion capacity (Moyes *et al.*, 2016; Lopes & Lionakis, 2022). Adhesion is the first stage of *Candida* invasion, and germ-tube formation is thought to

contribute to this stage (Ellepola & Samaranayake, 1998). Germ-tubes have been shown to adhere more easily to oral epithelium and surfaces such as dental materials (Gümrü, 2005).

1.2. Phenotypic Switching

Among the virulence factors of *C. albicans* is the phenotypic switching between "white" and "opaque" cells. Phenotypic diversity enables rapid responses to environmental changes (Talapko *et al*., 2021). The white phenotype produces smooth-surfaced, white, round budding cells, while the opaque phenotype produces broad-surfaced, flat, rough, grey colonies. These cells differ in hyphal formation ability, germ-tube formation duration, and sensitivity to temperature changes (Gümrü, 2005). Morphological changes and phenotypic transitions are transcriptionally stabilized and remain stable across multiple generations (Talapko *et al*., 2021).

1.3. Adhesion and Invasion

The process of *Candida* adhesion to the epithelium is quite complex and multifactorial. Since both the epithelial and fungal cell surfaces are negatively charged, long-range repulsive forces must be overcome for binding to occur. This binding occurs through the interaction of adhesins on the candidal surface with specific ligands on the host cell surface (Williams et al., 2013).

Among the primary proteins involved in the adhesion process are products of the agglutinin-like sequence (Als) gene family. The Als3 and Ssa1 proteins belonging to this family facilitate adhesion by interacting with E-cadherin on epithelial cells and N-cadherin on endothelial cells (Sun *et al.*, 2010; Liu & Filler, 2011; Williams *et al.*, 2013). Furthermore, Als3 interacts with the epidermal growth factor receptor (EGFR) and Her2, facilitating the entry of hyphal forms into cells via endocytosis. The same protein also plays a role in iron supply by binding to host cell ferritin (Liu & Filler, 2011; Lopes & Lionakis, 2022).

Another important adhesion molecule, hyphal wall protein 1 (Hwp1), enhances epithelial attachment by forming covalent bonds with host proteins through transglutaminase activity and plays a critical role in oral colonization (Williams *et al.*, 2013).

The β-1,3-glucan structure present in the cell wall of *C. albicans* has been shown to interact with the Dectin-1 receptor on the surface of phagocytic host cells, including dendritic cells in the oral epithelium. This interaction limits inflammatory pathology by triggering IL-10 production, helps to ensure fungal persistence in the host, and promotes long-term immunity. Dectin-1, a receptor that triggers cellular activation, may also participate in the development of inflammatory diseases (Tsoni & Brown, 2008).

A more recently discovered virulence factor, candidalysin, is a hyphal form-associated peptide toxin and one of the main virulence factors of *C. albicans*. It directly damages epithelial cell membranes, triggering intracellular calcium influx and activating the epithelial immune response. The release of candidalysin leads to membrane instability, increased intracellular stress, and cell death, facilitating fungal translocation (Richardson *et al.*, 2022). It has been demonstrated that *C. albicans* strains lacking candidalysin expression are unable to activate or damage epithelial cells and cannot establish mucosal infection (Moyes *et al.*, 2016). Interestingly, candidalysins from *C. dubliniensis* and *C. tropicalis* have been reported to exhibit higher membrane binding and permeability capacity compared to *C. albicans*, but lower epithelial damage (Richardson *et al.*, 2022).

1.4. Hydrolytic Enzyme Secretion

During active penetration into epithelial tissue, *C. albicans* secretes hydrolytic enzymes to facilitate nutrient uptake and damages host tissues (Lopes & Lionakis, 2022). These enzymes are divided into three main groups: secretory aspartic proteases (SAPs), phospholipases, and lipases.

The SAP family consists of ten members, some of which are secreted on the cell surface and others extracellularly. SAPs trigger neutrophil infiltration and induce the release of proinflammatory cytokines such as IL-1β and TNF-α, causing epithelial damage (Peter, Kumar, Kalyan, Thomas, & Saraswathi, 2018). SAP activity is considered one of the most important virulence determinants, particularly for *C. albicans* and *C. tropicalis*, and its expression has been shown to be significantly higher in individuals with oral diseases (Schaller, Borelli, Korting, & Hube, 2005).

While phospholipases target the host cell membrane, lipases also support the virulence process (Peter *et al.*, 2018). Phospholipases, which damage the host cell membrane by hydrolysing the ester bonds in glycerophospholipids - essential components of the cell membrane - are divided into subtypes as phospholipase A, B, C, and D based on the structure of the target phospholipids and their mode of action (Gümrü, 2005; Schaller *et al.*, 2005). Phospholipase A activity is associated with germ-tube formation, whereas phospholipase B is the predominant isoform secreted in pathogenic *C. albicans* strains. Phospholipase C breaks down phosphatidylcholine and, through a receptor-bound signalling pathway, leads to cellular activation such as secretion and growth via the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG). It has been demonstrated to be secreted in greater amounts during the hyphal phase. In contrast, phospholipase D is a membrane-bound enzyme, and its activity is induced during the dimorphic transition (Niewerth & Korting, 2001).

Phospholipase activity appears to be specific to *C. albicans*. Screening of clinical isolates revealed that 79% of *C. albicans* strains possessed phospholipase activity, whereas none of the *C. tropicalis*, *C. glabrata*, or *C. parapsilosis* isolates exhibited such enzymatic production (Schaller *et al.*, 2005).

2. *CANDIDA* AND ORAL CARCINOGENESIS

Alterations in the oral mucosa may further facilitate candidal invasion. Epithelial changes such as atrophy, hyperplasia, and dysplasia disrupt barrier integrity, predisposing tissues to infection. In this context, *Candida* species play a role in the etiopathogenesis of oral premalignant lesions because they may create a microenvironment that supports cell proliferation and the growth of genetically modified epithelial cells (Peter *et al.*, 2018).

Moreover, studies have shown that metabolic processes of *Candida* can produce potentially carcinogenic compounds such as nitrosamines, N-nitrosobenzylmethylamine, and acetaldehyde (Gall *et al.*, 2013; Sankari, Gayathri, Balachander, & Malathi, 2015). Strains isolated from lesions with higher degrees of dysplasia have been shown to exhibit higher nitrosation potential (Sankari *et al.*, 2015). Individuals with chronic oral candidiasis since childhood have been reported to have a significantly increased risk of developing oral carcinoma at a younger age (Gall *et al.*, 2013).

In healthy individuals, secretory IgA produced in the gastrointestinal mucosa suppresses the filamentous form of *C. albicans*, maintaining it in its yeast form. Furthermore, *C. albicans* residing in the intestinal flora contributes to the release of systemic antifungal IgG from B lymphocytes, helping to prevent candidemia (Lopes & Lionakis, 2022).

Meanwhile, *C. albicans* also contributes to maintaining a stable microbiota and suppressing local inflammatory responses. It has been reported that it may reduce immune exhaustion following acute inflammation by inhibiting the production of proinflammatory cytokines such as IL-1β, TNF-α, and INF-γ through its secreted N-glycan structures (Lopes & Lionakis, 2022).

3. KERATINIZED PRIMARY LESIONS PREDISPOSED TO *CANDIDA* SUPERINFECTION

3.1. Oral Leukoplakia

Leukoplakia is defined as a white lesion of the oral mucosa with the potential for malignant transformation in cases where other white lesions at risk of malignancy have been clinically and histopathologically excluded. While it most commonly affects the buccal mucosa, alveolar mucosa, and lower lip, lesions located on the floor of the mouth, lateral borders of the tongue, and lower lip are suggested to have a higher risk of dysplasia and malignant transformation

(Gürbüz, 2012). Leukoplakia is the most common premalignant lesion of the oral mucosa (Öztürk *et al.*, 2009).

It is more common in men, with a reported global prevalence rate ranging from 1.7 to 2.7%. Factors such as smoking, alcohol consumption, tobacco chewing, and poor oral hygiene play an important role in its aetiology (Petti, 2003). A strong association has been established between smoking and leukoplakia (Casu *et al*., 2023).

Oral leukoplakia is classified into two main clinical forms: the homogeneous form, which presents as a flat, uniform white lesion, and the non-homogeneous form, which includes nodular, verrucous, and erythroleukoplakia subtypes. The nodular type presents as small, polypoid, and predominantly white nodules. The verrucous type is characterized by a raised, proliferative, or corrugated surface, and typically exhibits aggressive behaviour, resistance to treatment, a high recurrence rate, and increased risk of malignant transformation. Erythroleukoplakia presents as predominantly white lesions interspersed with red areas (Parlatescu, Gheorghe, Coculescu, & Tovaru, 2014).

The average annual malignant transformation rate of leukoplakia varies considerably among populations and geographic regions, ranging from 1% to 43% (Parlatescu *et al.*, 2014). Overall, the average transformation rate is approximately 9.5% (Casu *et al*., 2023). Factors associated with an increased risk of malignancy include female sex, long-standing lesions, specific intraoral locations, and occurrence in non-smokers. Furthermore, non-homogeneous morphology and the degree of epithelial dysplasia are critical for assessing the risk of malignant transformation (Ribeiro, Salles, da Silva, & Mesquita, 2010).

3.1.1. Treatment approach in oral leukoplakia

Assessing the potential for malignant transformation pf the lesion is fundamental to treatment planning. Firstly, risk factors such as smoking and alcohol consumption should be eliminated. Lesions showing low-grade dysplasia histopathologically can be completely excised or monitored clinically, depending on their size, location, and the patient's cooperation. Surgical treatment is recommended in cases of moderate or severe dysplasia. Surgical treatment options include conventional surgical excision, cryotherapy, electrocauterization, and laser ablation. The postoperative recurrence rate is over 10% (Ribeiro *et al*., 2010; Peter *et al.*, 2018; Casu *et al*., 2023).

In cases where surgical treatment is not appropriate, medical treatment options can be considered. Pharmaceutical agents such as vitamin A and retinoids, vitamin C, systemic beta-carotene, lycopene, and ketorolac can be used. Alternatively, a "watch and wait" approach can be adopted and the patient can be

monitored under regular clinical and histopathological follow-up (Ribeiro *et al*., 2010; Parlatescu *et al.*, 2014).

3.1.2. Oral leukoplakia and *Candida*

The close relationship between oral leukoplakia and candidal infection was first recognized in 1965. Lehner described chronic candidal infection with a leukoplakia-like appearance as "candidal leukoplakia." Until the mid-1980s, the terms chronic hyperplastic candidiasis (CHC) and candidal leukoplakia were used interchangeably. However, when the term CHC was later used for chronic mucocutaneous candidiasis (CMC), a systemic disorder affecting multiple mucocutaneous sites, CHC was reclassified into two groups to avoid confusion. Group 1 CHC refers exclusively to oral candidal lesions, while Group 2 CHC lesions occur on other mucocutaneous surfaces in addition to the oral mucosa (Sitheeque & Samaranayake, 2003).

As previously mentioned, oral leukoplakia is most commonly observed on the buccal mucosa, particularly in the commissural region. Oral leukoplakia lesions, especially those located in the buccal mucosa, are thought to be associated with angular cheilitis (Sitheeque & Samaranayake, 2003).

In oral leukoplakia, varying degrees of chronic inflammatory cell infiltration are observed in the lamina propria, along with ortho- or parakeratization and irregular cell stratification in the surface epithelium. Candidal hyphae have been observed to invade the epithelial surface at right angles, and candidal invasion does not extend beyond the junction between the parakeratotic layer and the stratum spinosum. Another characteristic histopathological feature of candidal leukoplakia is clusters of neutrophils (PMNL) forming "microabscesses" associated with candidal hyphae (Sitheeque & Samaranayake, 2003).

Candidal invasion induces a hyperplastic response in the epithelium. An increase in mean epithelial thickness of up to 66% has been observed in individuals with CHC. The tighter intercellular junctions in hyperplastic epithelium and its responsiveness to antifungal therapy suggest that hyperplasia is a consequence of host defense against fungal invasion (Sitheeque & Samaranayake, 2003).

The prevalence of fungi isolated from patients with leukoplakia has been reported to range from 15.9% to 82% (Krogh, Holmstrup, Thorn, Vedtofte, & Pindborg, 1987; Novo *et al.*, 2024). Studies have reported a higher frequency of dysplastic changes in *Candida*-infected oral leukoplakia lesions (55.9%) compared with uninfected lesions (33.5%). This supports the hypothesis that *Candida* infection increases the risk of malignant transformation (Shukla *et al.*, 2019). However, the role of non-*albicans Candida* species in the neoplastic transformation of oral leukoplakia remains unclear (Peter *et al.*, 2018).

Both local and systemic factors may predispose leukoplakia lesions to candidal superinfection. Traumatic irritation from natural and artificial teeth may induce epithelial hyperkeratinization. Although not definitely proven, hyperkeratinization is suggested to contribute to the development of oral leukoplakia. Dentures may also constitute significant fungal reservoirs. Other contributing factors include reduced salivary flow rate, epithelial changes, and smoking (Sitheeque & Samaranayake, 2003).

The greatest risk factor for *Candida* superinfection in oral leukoplakia lesions is advanced age (Novo *et al.*, 2024). Tobacco use further increases this risk. In a study by Lipperheide *et al*. (1996), *Candida* species were isolated from 77.1% of patients with oral leukoplakia and from 100% of smokers.

Endogenous nitrosamines and acetaldehydes produced during *Candida* metabolism, along with overexpression of p53, Ki-67, and COX-2, are thought to contribute to malignant changes in the oral epithelium. Studies have suggested that nitrosamines such as N-nitrosobenzylmethylamine (NBMA) can induce oral and oesophageal cancer in rats (Krogh, 1990; Shukla *et al.*, 2019). Interestingly, while fewer *C. albicans* strains were isolated from non-homogeneous leukoplakias exhibiting more advanced dysplastic changes, the isolated strains showed higher nitrosation potential (Krogh, Hald, & Holmstrup, 1987; Krogh, 1990). At sufficient concentrations, acetaldehyde increases the risk of cancer by causing mutagenic effects on DNA. P53 and Ki-67 are key proteins and markers involved in the regulation of cellular proliferation, and their overexpression increases the risk of malignancy. Overexpression of COX-2 has also been found to be associated with colorectal cancer (Shukla *et al.*, 2019).

Candida, as an opportunistic pathogen, may colonize the damaged epithelial surfaces within oral leukoplakia lesions and increase the risk of malignant transformation; therefore, controlling *Candida* infection is considered critical in reducing cancer risk (Gall *et al.*, 2013; Casu *et al*., 2023).

3.2. Oral Lichen Planus

Lichen Planus (LP) is a chronic, idiopathic, T-cell-mediated autoimmune disease that can affect the skin and mucosa (Manchanda, Rathi, Joshi, & Das, 2023). LP affects approximately 0.22 to 5% of the general population, while the prevalence of oral lichen planus (OLP) has been reported to range from 0.5 to 2% (Baek & Choi, 2018). While OLP is more common in women, cutaneous LP affects both sexes at similar rates. OLP has been reported to develop approximately 10 years after the onset of cutaneous lesions (Jainkittivong, Kuvatanasuchati, Pipattanagovit, & Sinheng, 2007).

Although OLP most commonly affects the buccal mucosa, it may also involve the tongue, gingiva, palate, and labial mucosa (Baek & Choi, 2018). According

to Andreasen's clinical classification, OLP is categorized into six subtypes: reticular, erosive, atrophic, papular, plaque-like, and bullous (Andreasen, 1968). The most common form is the reticular form, while the bullous form is the least frequent. The erosive and atrophic forms are usually symptomatic and are noted as erythematous ulcerative areas surrounded by white striae (Elenbaas, Enciso, & Al-Eryani, 2022).

OLP may cause symptoms such as dysphagia, pain during swallowing, taste disturbances, and sensitivity to spicy foods. Mucosal lesions are generally more persistent and resistant to treatment than cutaneous lesions (Manchanda *et al.*, 2023).

3.2.1. Treatment approach in oral lichen planus

OLP has been associated with various systemic diseases, including thyroid disorders, hypertension, diabetes mellitus, anaemia, metabolic syndrome, and dyslipidaemia. It has been suggested that psychological stress may also trigger the onset or exacerbate the disease. The primary goals of treatment are to alleviate symptoms, prolong remission, and reduce the risk of potential malignant transformation. There is no universal treatment protocol for OLP; treatment planning is tailored to the patient's clinical presentation and response to treatment (Manchanda *et al.*, 2023).

Corticosteroids are the first-line treatment for OLP. Topical corticosteroids are preferred over systemic corticosteroids because they have a lower side effect profile, but systemic corticosteroids may also be used in severe cases (Elenbaas *et al.*, 2022; Manchanda *et al.*, 2023). In cases where topical and systemic corticosteroid treatments are ineffective, intralesional corticosteroid injections can be considered as an alternative treatment option (Elenbaas *et al.*, 2022). However, before prescribing corticosteroids, patients should be carefully evaluated for contraindications such as gastric ulcer, diabetes, tuberculosis, hypertension, and viral infections. It should also be considered that long-term use of glucocorticoids may increase the risk of osteoporosis (Elenbaas *et al.*, 2022).

While surgical excision may be considered for refractory cases that do not respond to conventional therapy, it is not recommended for atrophic or erosive forms. Cryosurgery has been tried in the treatment of erosive OLP, but a high recurrence rate has been reported. Maintaining optimal oral hygiene plays an important role in reducing both the incidence and severity of OLP (Elenbaas *et al.*, 2022). In cases that are refractory to treatment or in which conventional therapy is contraindicated, eliminating predisposing factors may be a more prudent treatment strategy.

3.2.2. Oral lichen planus and *Candida*

Candidal superinfections are known to exacerbate symptoms, particularly in the erosive type of OLP. *Candida* metabolism can produce potentially carcinogenic substances such as nitrosamines and acetaldehyde. Candidal superinfection is of greater importance in individuals exposed to risk factors for malignant transformation, such as smoking and alcohol consumption, which are implicated in the etiopathogenesis of OLP, and these patients require close monitoring for malignant transformation (Rodriguez-Archilla & Fernandez-Torralbo, 2022).

In a meta-analysis by Rodriguez-Archilla and Fernandez-Torralbo (2022) including 24 studies from 13 countries, *C. albicans* was reported to be isolated from 37% of 1303 OLP patients. Furthermore, the incidence of *Candida* was found to be significantly higher (2.48 times) in patients with OLP compared to healthy individuals. The likelihood of developing *Candida* superinfection was also determined to be 2.53 times higher in patients with erosive OLP. The incidence of non-*albicans Candida* species was found to be 2.33 times higher in OLP lesions superinfected with *Candida* (Rodriguez-Archilla & Fernandez-Torralbo, 2022).

Although the relationship between OLP and *Candida* colonization is not clear, it is thought that topical corticosteroids, which are widely used in the treatment of OLP, may facilitate *Candida* colonization by disrupting the oral microbiota.

Topical corticosteroids used in the treatment of OLP are effective because they suppress inflammation and the immune response. However, one of the most common side effects of this treatment is oral candidiasis. It has been suggested that the suppression of inflammation and the immune response accelerate the transition of *Candida* from the commensal to the pathological form (Yaltkaya, Aykut, Yalçınkaya, & Gümrü, 2025).

Secondary candidiasis is more common in patients treated with corticosteroids, with an incidence ranging from 11.4% to 76.7% (Yaltkaya *et al.*, 2025). Candidiasis is reported to occur mostly within approximately 60 days after the onset of the treatment (Saepoo, Kerdpon, & Pangsomboon, 2023).

It has been reported that clinical symptoms may worsen in OLP patients with candidal superinfection, that symptoms regress with antifungal treatment, and that erosive OLP lesions may transform into reticular form (Jainkittivong *et al.*, 2007; Mehdipour *et al.*, 2010).

3.3. Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that affects multiple organs, including the skin, joints, hematopoietic system, kidneys,

and central nervous system. It is most common in women aged 15-45, and the mortality rate is 2-3 times higher than the general population, with infections and cardiovascular diseases being the primary causes of death (Barber *et al*., 2021; Ameer *et al*., 2022).

Although its aetiology has not been fully elucidated, genetic predisposition, environmental factors, and autoimmune mechanisms are suggested to play a role. It has been reported to be associated with silica, smoking, oral contraceptives, UV-B rays, certain medications, and Epstein-Barr virus (EBV) infections (Lahita, 2010; Firestein *et al.*, 2024).

Diagnosis is based on laboratory tests combined with clinical findings; at least four of the 1997 American College of Rheumatology (ACR) criteria must be met (Lahita, 2010).

Clinically, weight loss, fever, fatigue, malar rash, arthritis, lupus nephritis, glomerulonephritis, alopecia, and mucocutaneous lesions are common (Lahita, 2010; Lazar & Kahlenberg, 2023). Oral manifestations are also frequent, with only 25% of patients showing no oral involvement (Hammoudeh, Al-Momani, Sarakbi, Chandra, & Hammoudeh, 2018). Mucocutaneous manifestations are more prevalent in juvenile SLE cases (Schmidt, 2022).

Oral lesions include painless ulcers, erythematous areas, honeycomb-like plaques, petechiae, and cheilitis. The lips are frequently affected, and discoid lesions usually begin on the lips as pink-red plaques (Schmidt, 2022). The risk of dental caries increases due to xerostomia, and oral ulcerations may occur in 7-41% of patients. Additionally, the prevalence of periodontal disease is high (Rutter-Locher, Smith, Giles, & Sofat, 2017). *Candida* infections are also common findings following corticosteroid treatment (Hammoudeh *et al*., 2018).

Lesions often present as erythematous areas with central white papules in the palatal region. Differential diagnosis with OLP may be necessary. Rarely, squamous cell carcinoma may develop; therefore, careful monitoring for malignant transformation is recommended (Hammoudeh *et al*., 2018).

3.3.1. Treatment approach in lupus erythematosus

Treatment of SLE is challenging due to its multisystem involvement and clinical variability and requires a multidisciplinary approach. The primary goals of treatment are to prevent flares, maintain remission, and minimize medication-related side effects. Disease activity is assessed using various indices, and treatment is tailored accordingly (Ameer *et al*., 2022; Ermurat & Tezcan, 2022).

Hydroxychloroquine, an antimalarial agent, is commonly used as first-line treatment. While highly effective, its long-term use carries a risk of retinal toxicity. Glucocorticoids, administered to control acute symptoms during flare-

ups, can later be replaced by hydroxychloroquine or immunosuppressive agents. In cases where frequent relapses occur despite hydroxychloroquine therapy, immunosuppressive drugs such as methotrexate or azathioprine may be added to treatment regimen (Ameer *et al*., 2022; Lazar & Kahlenberg, 2023).

3.2.3. Lupus erythematosus and *Candida*

In a large cohort study conducted by Fangtham *et al*. (2014), oral candidiasis was diagnosed in 325 of 2,258 SLE patients (14%). Oral candidiasis was associated with African-American ethnicity, elevated white blood cell count, a history of bacterial infection, and the use of prednisone and immunosuppressive agents. The same study also reported a higher prevalence of proteinuria, while the use of hydroxychloroquine was found to reduce the risk of oral candidiasis (Fangtham, Magder, & Petri, 2014).

In a study conducted in Taiwan by Su *et al*. (2021), the incidence and mortality risk factors of invasive fungal infections in patients with SLE were investigated. The mortality rate was found to be 26.7%, and *Candida* species were identified as the most common fungal pathogens, accounting for 52.8% of cases. Intravenous steroid therapy, in particular, was identified as the most significant risk factor for invasive *Candida* infections. Furthermore, several immunosuppressive agents have been reported to increase the pathogenic potential of fungal organisms (Su *et al*., 2021).

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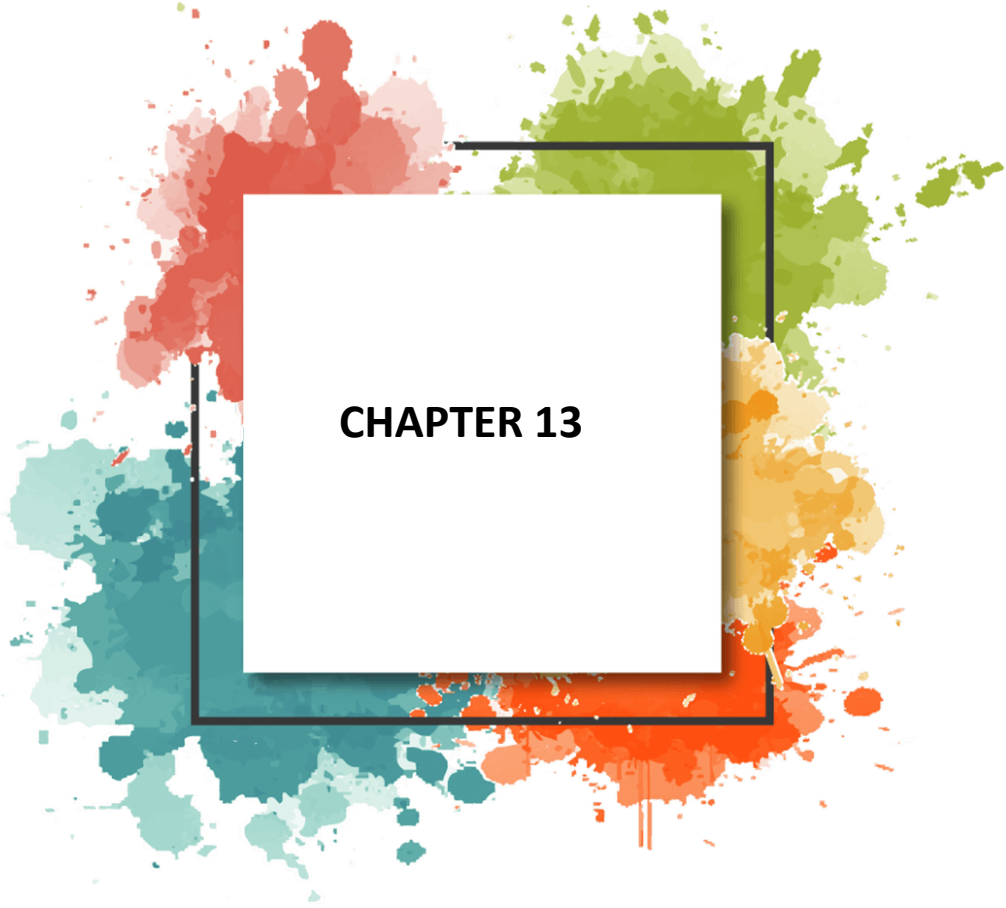
CHAPTER 13

Prematurity (Preterm Birth)

Can Bilginer[1]

Preterm birth (PTB)—delivery before 37 completed weeks of gestation—is a leading cause of neonatal morbidity and mortality worldwide. An estimated 13.4 million babies (95% credible interval: 12.3–15.2 million) were born preterm in 2020, representing nearly 10% of all live births (Ohuma et al., 2023). Despite advances in obstetric care, complications of preterm birth remain the top cause of under-five mortality (Ohuma et al., 2023). This chapter reviews the epidemiology, risk factors, pathophysiology, prediction, prevention, and management of preterm birth. It synthesizes current evidence from major professional organizations (ACOG, SMFM, ISUOG, WHO) and contemporary literature and offers practical guidance for clinicians and public health professionals. Figures and tables summarize classification, risk factors and the multifactorial pathways leading to preterm parturition. The chapter emphasizes evidence-based interventions—including lifestyle modification, progesterone supplementation, cervical cerclage, tocolytic therapy, antenatal corticosteroids, magnesium sulphate for neuroprotection, antibiotics for preterm rupture of membranes and strategies for neonatal care—and highlights public health strategies to reduce the global burden of PTB.

1. Introduction

Preterm birth (PTB) is defined as any delivery before 37 completed weeks (259 days) of gestation. Global epidemiological analyses estimate that 1 in 10 babies are born preterm (Ohuma et al., 2023), and PTB accounts for approximately three-quarters of perinatal mortality and more than one-half of long-term neonatal morbidity. In high-income countries, survival rates for extremely preterm infants (<28 weeks) exceed 90%, whereas fewer than 10% of such infants survive in low-income settings (Ohuma et al., 2023), underscoring the profound disparity in outcomes.

1.1 Definition and Classification

Preterm births are stratified by gestational age because neonatal outcomes vary dramatically with each week of prematurity. The World Health Organization and major obstetric societies classify PTB as follows:

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Table 1. Classification of preterm birth by gestational age

Category	Gestational age range	Clinical notes
Extremely preterm	<28 weeks	High mortality; survivors have severe respiratory and neurologic morbidities. Survival: 10–70% depending on resources. Common complications: respiratory distress syndrome, bronchopulmonary dysplasia, intraventricular hemorrhage, periventricular leukomalacia, necrotizing enterocolitis, retinopathy of prematurity, sepsis, patent ductus arteriosus. Long- term: cerebral palsy, cognitive impairment, visual and hearing deficits, chronic lung disease.
Very preterm	28 to <32 weeks	Require intensive neonatal care; risk of chronic lung disease and neurodevelopmental impairment. Survival: 80–90% in high-income settings. Complications: RDS, IVH (grades I–III), sepsis, hyperbilirubinemia, feeding difficulties. Neurodevelopmental impairment is less frequent but still above term baseline.
Moderately preterm	32 to <34 weeks	Generally good survival; feeding and thermoregulation problems common. Survival: >90%. Complications: transient tachypnea of the newborn, jaundice, hypoglycemia, temperature instability. Many require short NICU stay; long-term outcomes approach those of term infants.
Late preterm	34 to <37 weeks	Often appear "term" but have increased risk of respiratory distress, jaundice and feeding difficulties. Survival: ≈99%. Complications: feeding difficulties, jaundice, respiratory distress, thermoregulation issues. Often considered "near term" but still at higher risk than term infants; early discharge should be avoided.

Term deliveries are further classified into **early term** (37–38⁶⁄₇ weeks), **full term** (39–40⁶⁄₇ weeks) and **late term** (41 weeks). Late-preterm infants account for the majority of preterm births in many countries (Liang,

2024). The distinction between late preterm and early term is clinically important because even a few weeks' difference in gestational age can alter neonatal risk profiles.

Global Burden of Preterm Birth

The recent "Born Too Soon" report and associated analyses highlight that in 2020 an estimated 13.4 million infants (9.9% of live births) were born preterm worldwide(Ohuma et al., 2023). Preterm birth is a global problem with marked geographic disparities. The majority of preterm births occur in southern Asia and sub-Saharan Africa (≈13%), and survival differs dramatically based on where a child is born; more than 90% of extremely preterm infants die within the first days of life in low-income countries compared with <10% in high-income settings (Ohuma et al., 2023). This reflects disparities in antenatal care, availability of neonatal intensive care and socio- economic determinants such as nutrition and maternal education.

Preterm birth complications were responsible for roughly 1 million neonatal deaths in 2022 (Ohuma), making PTB the leading cause of under-five mortality. Temporal trends show no substantial decline in global PTB rates during the past decade (Ohuma et al., 2023).

Regional Disparities

Low- and middle-income countries (LMICs) carry a disproportionate burden of preterm morbidity and mortality. Contributing factors include limited access to prenatal care, high prevalence of infections, malnutrition, short inter-pregnancy intervals, lack of trained birth attendants, and inequitable distribution of resources.

Economic and Social Impact

The economic cost of preterm birth encompasses immediate expenditures for neonatal care as well as long-term societal costs due to neurodevelopmental disabilities, chronic health problems and educational support. Families experience psychological stress and financial hardship, and survivors may suffer from lifelong sequelae such as cerebral palsy, cognitive impairment, sensory deficits and cardiometabolic disorders. Reducing PTB rates is therefore a public health priority.

2. Epidemiology and Global Trends

2.1. Preterm Birth Rates by Region

Despite steady improvements in obstetric and neonatal care, preterm birth rates have changed little in the past decade. Table 2 summarizes the approximate preterm birth rates for selected regions and countries based on recent literature.

In the United States the preterm birth rate rose to 10.49% of live births in 2021—the highest since 2007— driven largely by an increase in late-preterm deliveries. In the Middle East, a population-based registry from Qatar reported a

late-preterm birth rate of 6.4% and early-term births accounting for 33.7%. China reported that 5.17% of live births were late preterm, representing 75% of preterm deliveries and reflecting an increase of 8.8% between 2012 and 2018. Brazil's late-preterm rate is estimated at 7.3%, whereas limited data from southern Asia suggest that late-preterm births may constitute up to 12.7% of all live births. In high-income European countries such as Iceland and the United Kingdom, late-preterm rates are lower (3–6%) but have been increasing in parallel with provider-initiated deliveries (Liang, 2024)(Liang et al., 2024).

Table 2. Selected estimates of preterm or late-preterm birth rates

Region/country	Estimated preterm/late-preterm birth rate	Notes
Southern Asia	≈12.7% of live births	Limited data; estimated late-preterm births
United States (2021)	10.49% preterm; 7.67% late-preterm	Highest US rate since 2007
Brazil	7.3% late-preterm	Early-term ≈35% of live births
Qatar	6.4% late-preterm; 33.7% early-term	Based on population registry
China	5.17% late-preterm	75% of preterm deliveries
High-income Europe (e.g., Iceland)	3–6% late-preterm	Increasing trend with provider-initiated deliveries

2.2. Visualizing Regional Differences

Public-health programmes should be tailored to local epidemiology: in countries with high rates of medically induced late-preterm birth (e.g., the United States), quality improvement initiatives may reduce non-indicated early deliveries, whereas low-income regions need broader interventions addressing nutrition, infection, and access to antenatal care.

2.3. Trends Over Time

Long-term epidemiological analyses reveal that global incidence of preterm birth declined modestly between 1990 and 2016 but has plateaued or increased slightly thereafter. A 2024 global burden of disease (GBD) analysis reported that the age-standardized incidence rate (ASIR) decreased from 358.94 per 10,000 population in 1990 to 348.41 per 10,000 in 2021. However, after a period of decline, the ASIR began to rise again after 2016. This upturn highlights emerging drivers such as increasing maternal age, obesity, assisted reproductive technology

(ART), caesarean delivery and environmental stressors. The analysis also demonstrated stark regional differences: low socio-demographic index regions experienced a 43% increase in annual incidence from 1990 to 2021, whereas high-income regions saw a 9.6% decline (Arham & Wróblewska-Seniuk, 2025). These disparities underscore the need for global actions that address upstream determinants of preterm birth.

3. Etiology and Pathophysiology

3.1. Underlying Biological Pathways

Preterm birth is a syndrome rather than a single disease; multiple pathways converge on activation of parturition before term. Preterm labour and birth result from multiple overlapping pathways that converge on activation of the maternal-fetal decidua and myometrium leading to contractions and cervical change. Four principal biological mechanisms have been identified:

1. **Infection and inflammation** — Ascending genital tract infections incite decidual and fetal membrane inflammation, leading to increased production of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and chemokines. These mediators upregulate cyclooxygenase-2 (COX-2), increase prostaglandin synthesis and matrix metalloproteinases, reduce progesterone responsiveness and trigger uterine contractions and membrane rupture. Microbial invasion of the amniotic cavity triggers production of pro-inflammatory cytokines, prostaglandins and matrix metalloproteinases that weaken fetal membranes and induce uterine contractions. Subclinical intra-amniotic infection is thought to underlie many spontaneous preterm births, particularly at earlier gestations (Goldenberg et al., 2008.).
2. **Decidual hemorrhage or vascular disease** — Abruption, bleeding and thrombin generation cause release of proteases and inflammatory mediators that stimulate uterine contractions. Maternal vascular disease (hypertensive disorders) leads to uteroplacental hypoperfusion and oxidative stress. Placental abruption and decidual bleeding release thrombin and tissue factor, leading to uterine contraction and rupture of membranes. Vaginal bleeding in the second and third trimester is thus a warning sign (Goldenberg et al., 2008.).
3. **Uterine overdistention** — Mechanical stretch from multifetal gestation, polyhydramnios or fetal macrosomia activates myometrial oxytocin receptors and inflammatory pathways, increasing prostaglandins and promoting contractility. Multiple gestation, polyhydramnios or macrosomia cause mechanical stretching of the myometrium that stimulates oxytocin receptors and gap junction

formation. Uterine over- distension also induces inflammatory pathways (Goldenberg et al., 2008.).

4. **Maternal-fetal stress** — Activation of the hypothalamic-pituitary-adrenal (HPA) axis elevates CRH, cortisol and fetal adrenal steroids. CRH stimulates prostaglandin production; elevated cortisol accelerates fetal maturation and myometrial responsiveness. Activation of the hypothalamic-pituitary-adrenal axis with increased corticotropin-releasing hormone (CRH) and cortisol results in production of prostaglandins and matrix metalloproteinases. Maternal stress may be psychological (e.g., anxiety, intimate partner violence) or physiological (e.g., chronic disease, malnutrition) (Goldenberg et al., 2008.).

These pathways are not mutually exclusive; rather they often act synergistically. For example, infection may be more common in the setting of cervical insufficiency or uterine over-distension, and inflammation may be exacerbated by maternal stress or environmental exposures. The fetus and placenta also influence parturition through production of surfactant proteins, fetal fibronectin and inflammatory mediators.

The interplay between these pathways culminates in cervical ripening, membrane weakening, myometrial activation and fetal endocrine maturation. Genetic susceptibility plays a role as well—family history of preterm birth increases risk, and genome-wide association studies have identified loci related to inflammation and uterine contractility. Epigenetic modifications induced by environmental exposures (e.g., smoking, air pollution, endocrine disruptors) may alter gene expression in the placenta and uterus, contributing to transgenerational risk.

3.2. Spontaneous vs. Provider-Initiated Preterm Birth

Preterm births can be broadly classified as **spontaneous** (resulting from preterm labour with intact membranes or pre-labour rupture of membranes) or **medically indicated** (provider-initiated). Spontaneous preterm labour accounts for roughly 40–45% of cases and often follows the biological pathways described above (Goldenberg et al., 2008.). In contrast, provider-initiated preterm birth results from obstetric or medical indications such as severe preeclampsia, fetal growth restriction, placental abruption, placenta previa, vasa previa, severe congenital anomalies or maternal illness (e.g., uncontrolled diabetes, cardiomyopathy). Although medically indicate preterm births may reduce maternal-fetal morbidity, they contribute significantly to the rising proportion of late- preterm and early-term deliveries in high-income countries (Liang et al., 2024).

4. Risk Factors and Social Determinants

4.1. Established Risk Factors

Risk factors can be broadly categorized into maternal, fetal/placental and socio-demographic domains. Preterm birth is a multifactorial syndrome influenced by genetic, physiological, obstetric, socio-economic and environmental factors. Table 3 summarizes key risk factors identified in large cohort and case-control studies. Many of these factors are potentially modifiable and therefore targets for primary prevention.

Table 3. Risk factors for spontaneous preterm birth

Domain	Factors	Key notes
Obstetric factors	Prior spontaneous PTB; multifetal gestation; short cervical length; preterm prelabor rupture of membranes (PPROM); placenta previa; placental abruption; uterine anomalies; polyhydramnios or oligohydramnios; fetal anomalies	A prior spontaneous PTB is the strongest predictor of recurrence. Women with ≥1 prior second-trimester loss or preterm birth due to painless cervical dilation have particularly high risk. The gestational age and circumstance of the prior PTB should be documented. Multifetal pregnancies increase uterine stretch, promoting preterm labour. Short cervix (<25 mm at 20– 24 weeks) is associated with higher PTB risk (Iams, 1996). Multiple gestation accounts for ~20% of late-preterm births (Liang et al., 2024).
Medical and infectious conditions	Maternal hypertension, pre- eclampsia, diabetes, renal disease, autoimmune disorders; genital tract infections (bacterial vaginosis, sexually transmitted infections), urinary tract infection; systemic inflammatory conditions (periodontitis, pyelonephritis); vaginal bleeding; intra-amniotic infection or chorioamnionitis	Infections trigger release of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and prostaglandins, leading to uterine contractions and cervical ripening. Pre-existing diabetes mellitus and hypertensive disorders strongly associated with medically indicated preterm birth and with spontaneous late preterm and early term deliveries (Liang et al., 2024).

Lifestyle and demographic factors	Maternal age <17 or >35; low body mass index (BMI); poor nutrition; smoking, alcohol or illicit drug use; high stress levels; lack of prenatal care; low socioeconomic status; Black race; short inter-pregnancy interval	Both teenage mothers and women over 35 have higher preterm birth risk, partly due to psychosocial stress, chronic disease and use of assisted reproduction. Smoking and substance use are modifiable and significantly associated with PTB. Dose- dependent increase in spontaneous preterm birth risk (Goldenberg et al., 2008.). Racial disparities reflect interactions between structural inequities, discrimination and epigenetic factors. In the United States, women of African descent have higher rates of spontaneous preterm birth even after adjusting for socio-economic status (Goldenberg et al., 2008.). Low educational attainment associated with limited prenatal care, poor nutrition and increased stress (Liang, 2024). Short inter-pregnancy interval (<12 months) - insufficient recovery between pregnancies increases risk (Arham & Wróblewska-Seniuk, 2025).
Uterine and cervical factors	Cervical insufficiency; congenital uterine anomalies (bicornuate or septate uterus); uterine fibroids; history of cervical surgery (cone biopsy, loop electrosurgical excision procedure)	Cervical trauma and structural anomalies weaken the cervix, increasing susceptibility to dilation under the weight of the pregnancy. Short cervix (<25 mm before 24 weeks) - cervical shortening is a key predictor of spontaneous preterm birth; risk is highest when combined with prior preterm birth (Iams et al., 1996).

Domain	Factors	Key notes **Structural abnormalities may lead to cervical incompetence or poor placental implantation.**
Placental and fetal factors	Placental abruption, placenta previa, vasa previa; fetal growth restriction or major anomalies	Cause bleeding and stress leading to medically indicated preterm birth (Goldenberg et al., 2008.). Often necessitate early delivery to prevent stillbirth.
Environmental and lifestyle factors	Cigarette smoking and substance use (alcohol, cocaine, opiates); exposure to air pollutants and high ambient temperatures; maternal stress, depression and intimate partner violence; poor nutrition and anemia	Dose-dependent increase in spontaneous preterm birth risk (Goldenberg et al., 2008.). Linked to increased late-preterm and early-term deliveries (Liang et al., 2024). Activate the hypothalamic- pituitary-adrenal axis and inflammatory pathways; trait anxiety correlates with late-preterm delivery (Liang et al., 2024). Associated with low BMI, infection susceptibility and placental dysfunction.
Psychosocial stress and environmental exposures	Intimate partner violence, psychosocial stressors, low social support, shift work, environmental toxins, air pollution and exposure to heavy metals	Chronic stress activates the maternal hypothalamic-pituitary- adrenal axis, elevating corticotropin-releasing hormone (CRH) and cortisol, which stimulate myometrial activity and prostaglandin production.
Assisted Reproductive Technology	ART pregnancies	Associated with multiple gestation and placenta-mediated complications; ART pregnancies have higher preterm birth rates even after adjusting for plurality (Liang et al., 2024).

The interaction of these factors determines the individual risk; however, many cases of PTB occur without identifiable risk factors, highlighting the multifactorial nature of the syndrome.

3.3. Emerging and Controversial Factors

Microbiome and infection — The vaginal and placental microbiomes have been implicated in preterm birth pathogenesis. Dysbiosis, especially overgrowth of *Ureaplasma* or *Mycoplasma* species, may trigger inflammatory cascades. Randomized trials of probiotics and antibiotics have yielded mixed results; routine antibiotic use is not recommended because it has not reduced preterm birth and may increase neonatal necrotizing enterocolitis.

Genetic and epigenetic factors — Genome-wide association studies have identified variants in genes related to interleukin signaling, collagen metabolism and progesterone receptors. Epigenetic modifications such as DNA methylation and histone acetylation—often influenced by environmental exposures like smoking—may regulate gene expression relevant to uterine contractility.

Environmental toxins — Exposure to phthalates, heavy metals and endocrine disrupting chemicals has been associated with spontaneous preterm birth in observational studies. Climate change may also influence preterm birth via heat stress, air quality changes and food insecurity.

5. Prediction and Screening

Effective prediction allows targeted prophylaxis. Screening modalities include clinical history, biochemical markers and sonographic assessment. Early identification of women at risk of preterm birth allows clinicians to initiate preventive measures and arrange timely referral to tertiary care. Prediction strategies combine risk scoring, biophysical tests and biomarkers. While no single test can predict all preterm births, combining modalities improves accuracy.

5.1. History and Obstetric History

A detailed obstetric history remains the cornerstone for risk stratification. The simplest prediction tool is a careful maternal history. Women with a prior spontaneous preterm birth have a recurrence risk of approximately 30%. Previous spontaneous preterm birth confers the greatest risk of recurrence; risk increases with the number and gestational age of prior events. The gestational age and circumstance of the prior PTB should be documented (e.g., PPROM, spontaneous preterm labour, medically indicated). Additional history of cervical surgery, uterine anomalies, multifetal gestation or exposure to diethylstilbestrol is pertinent. Other elements include maternal age, body mass index, smoking status, socioeconomic factors and chronic diseases. Several risk scoring systems (e.g., the Creasy scale) assign points for these variables, but their predictive value is limited. Clinicians should not rely solely on risk scores but use them to inform counselling.

5.2. Cervical Length Measurement

Transvaginal ultrasound measurement of cervical length (CL) at 18–24 weeks is the most validated screening tool. Transvaginal ultrasonography is the gold-standard method for measuring cervical length. A CL ≤25 mm identifies women at increased risk for PTB. A short cervix (<25 mm) before 24 weeks is highly predictive of spontaneous preterm birth (Iams et al., 1996). Serial measurements can refine risk stratification and guide prophylactic interventions such as progesterone therapy or cerclage. In women with a prior spontaneous PTB, universal CL screening is recommended by ACOG and SMFM. Screening is recommended for women with a history of preterm birth and is often performed at the mid-trimester anatomy scan. In low-risk populations, universal cervical length screening remains controversial because of costs and false positives, but some guidelines advocate routine screening between 18 and 24 weeks. Cervical length is dynamic; serial measurements may be useful in high-risk women. Women with a singleton pregnancy, prior spontaneous preterm birth and a short cervix (<25 mm) before 24 weeks should be offered progesterone therapy or cerclage depending on their obstetric history.

5.3. Biochemical Markers

Several biomarkers have been investigated for predicting spontaneous preterm labour, including fetal fibronectin (fFN), phosphorylated IGFBP-1, cervicovaginal interleukins, placental alpha-microglobulin-1 (PAMG-1) and metabolites of CRH. Several biochemical markers have been investigated for preterm birth prediction:

- **Fetal fibronectin (fFN)** — A glycoprotein produced by fetal membranes. A qualitative vaginal fFN test performed between 24 and 34 weeks has a high negative predictive value (NPV >99%); a negative result

indicates <1% risk of delivery within 7 days. Positive fFN testing between 22–35 weeks is associated with increased PTB risk; however, the negative predictive value of fFN is more clinically useful—women with a negative test have a <1% risk of delivery within 7 days. However, the positive predictive value is low (~13%), limiting its usefulness. False positives occur after digital examination, vaginal bleeding or intercourse. Combining fFN testing with cervical length measurement improves predictive accuracy.

Combined CL measurement and fFN testing improve risk prediction but have not demonstrated clear benefit in low-risk populations.

- **Placental alpha microglobulin-1 (PAMG-1)** — A protein detected by the PartoSure test, which may have higher positive predictive value than fFN. Data are limited, and routine use is not yet recommended.

- **Insulin-like growth factor binding protein-1 (IGFBP-1)** — The Actim Partus test detects phosphorylated IGFBP-1 in cervicovaginal secretions; like fFN it has high NPV but low PPV.

- **Serum biomarkers** — Low levels of pregnancy-associated plasma protein A (PAPP-A) and placental growth factor (PlGF) in the first trimester are associated with preeclampsia and may also predict preterm birth. Elevated inflammatory markers (C-reactive protein, interleukins) correlate with early delivery but are nonspecific.

Emerging omics technologies may yield more precise predictive signatures in the future.

5.4. Uterine Electrical and Contraction Monitoring

Home uterine contraction monitoring and electrohysterography have been studied but have not consistently shown clinical benefit in predicting PTB. Excessive false positives lead to unnecessary interventions. Therefore, current guidelines do not recommend routine use outside research protocols. While increased uterine activity may precede preterm labour, these methods have poor specificity and are not routinely used. Research into wireless uterine monitoring and machine-learning algorithms may improve predictive performance in the future.

5.5. Risk Stratification Table

The following table summarizes common prediction tools, their indications and limitations.

Table 4. *Selected* methods for predicting preterm birth

Test/method	Target population	Advantages	Limitations
Obstetric history and risk scoring	All pregnant women	Inexpensive; identifies women with prior preterm birth and other risk factors	Low sensitivity; cannot identify first-time mothers at risk
Transvaginal cervical length measurement	Women with history of preterm birth or at high risk	High negative predictive value; guides progesterone or cerclage therapy	Requires skilled ultrasound; dynamic changes over time; screening in low-risk women debated
Fetal fibronectin test	Symptomatic women with threatened preterm labour between 24 and 34 weeks	Negative test has high NPV (>99%) for delivery within 7 days	Low positive predictive value; false positives after intercourse or examination
PAMG-1 and IGFBP-1 tests	Symptomatic women	May provide additional predictive value compared with fFN	Limited evidence; cost; unclear guidelines
Serum biomarkers (PAPP-A, PlGF, CRP)	High-risk women	Can be integrated with aneuploidy screening; research tool	Poor specificity; not recommended for routine clinical use
Home uterine activity monitoring	Selected high-risk women	Theoretically detects early contractions	Limited accuracy; not proven to reduce preterm birth

6. Prevention Strategies

The heterogeneous etiology of PTB means no single intervention can eliminate risk. Prevention strategies target modifiable risk factors, enhance uterine quiescence and correct structural or hormonal deficiencies. Prevention of preterm birth encompasses **primary prevention** (reducing risk factors before conception or early in pregnancy), **secondary prevention** (identifying high-risk women and initiating prophylactic interventions) and **tertiary prevention** (managing symptoms to prolong pregnancy). The following sections review key interventions.

6.1. Lifestyle Modification and General Measures

Preconception and early antenatal care: Encouraging women to seek preconception counselling and early antenatal care facilitates identification and modification of risk factors. Adequate intake of folic acid, iron and micronutrients, management of chronic illnesses and optimization of maternal weight are recommended.

Lifestyle and socio-economic interventions:

- **Smoking cessation and substance abuse treatment** — Tobacco, alcohol and illicit drugs have dose- dependent associations with preterm birth (Goldenberg et al., 2008.). Smoking cessation programmes, nicotine replacement therapy and behavioural interventions should be offered to all pregnant women. Brief

  interventions for alcohol and drug use reduce consumption but require integration with mental health services.

- **Optimizing maternal nutrition** — Ensuring adequate caloric intake, balanced macronutrients and micronutrients (iron, folate, vitamin D, calcium, omega-3 fatty acids) supports placental function. Diets rich in vegetables, fruits, nuts and whole grains are associated with a 12% reduction in PTB risk. Omega-3 fatty acid supplementation may exert anti-inflammatory and uterine relaxant effects, decreasing early PTB. Adequate vitamin D and zinc reduce infection-related PTB. Supplementation with docosahexaenoic acid (DHA) has modest evidence for reducing early preterm birth but not late preterm birth. Nonetheless, randomized trials on nutritional interventions have yielded mixed results; therefore, supplementation should be individualized and emphasize balanced diet. In low-resource settings, food fortification and supplementation programmes may reduce preterm birth and low birth weight.

- **Reducing physical workload and stress** — Occupational modifications, stress reduction programmes and treatment of depression or anxiety may decrease risk. Although data are inconsistent, psychosocial support is important for general well-being.

- **Inter-pregnancy interval and family planning** — Short inter-pregnancy intervals (<18 months) are associated with higher PTB risk. Educating couples about spacing pregnancies at least 12 months apart (ideally 18 months) reduces recurrence risk (Liang et al., 2024). Access to contraception and family planning services is essential. Counselling on optimal spacing reduce recurrence.

- **Periodontal care and infection treatment:** Screening and treatment of asymptomatic bacteriuria, bacterial vaginosis and sexually transmitted infections can reduce infection-mediated PTB. Good oral hygiene and treatment of periodontal disease mitigate inflammatory triggers.

- **Vaccination and infection control** — Influenza and COVID-19 vaccination during pregnancy reduce maternal illness and may prevent inflammatory triggers of preterm labour. Screening and treatment for asymptomatic bacteriuria and sexually transmitted infections are recommended.

6.2. Progesterone Supplementation

Progesterone maintains uterine quiescence by inhibiting prostaglandin production and down-regulating oxytocin receptors, and cervical competence. Two formulations are used clinically, have been used for preterm birth prevention: **vaginal progesterone** and **intramuscular 17-hydroxyprogesterone caproate (17-OHPC)**. The evidence base and guidelines have evolved in recent years.

1. **17-α hydroxyprogesterone caproate (17-OHPC):** Administered as a weekly intramuscular injection (250 mg) from 16–20 weeks until 36 weeks in women with a singleton pregnancy and a history of spontaneous PTB. Initial randomized trials demonstrated a reduction in recurrent PTB; however, subsequent data were mixed, and the US FDA withdrew approval of Makena (17-OHPC) in 2023 due to lack of confirmed efficacy ("Practice Bulletin No. 130: Prediction and Prevention of Preterm Birth," 2012). The latest ACOG practice advisory states that 17-OHPC is not recommended for routine prevention of recurrent PTB ("Practice Bulletin No. 130: Prediction and Prevention of Preterm Birth," 2012). Historically, weekly intramuscular injections of 250 mg 17-OHPC were used for recurrent preterm birth prevention. However, subsequent trials failed to replicate the benefit, and concerns about thromboembolic and hepatic adverse effects emerged. In 2023 the
2. US Food and Drug Administration withdrew approval for Makena, the only marketed 17-OHPC product. ACOG and SMFM no longer recommend its use ("Practice Bulletin No. 130: Prediction and Prevention of Preterm Birth," 2012). The SMFM suggests that intramuscular 17-OHPC may still be considered when other options are unavailable.
3. **Vaginal progesterone:** Micronized progesterone (200 mg pessary or 90 mg gel) is administered daily from 16–24 weeks until 34 weeks in women with a short cervix (≤25 mm) but no history of PTB. A meta-analysis of 974 singleton pregnancies with a short cervix showed a 44% reduction in spontaneous PTB and a 41% reduction in neonatal morbidity/mortality. Vaginal progesterone is therefore recommended

for women with a short cervix. Randomized trials show that vaginal progesterone (200 mg pessary or 90 mg gel daily) given from 16 to 36 weeks to women with a history of spontaneous preterm birth and a short cervix (<25 mm) reduces the risk of preterm birth <34 weeks by approximately 30% (Berghella et al., 2017; "Practice Bulletin No. 130: Prediction and Prevention of Preterm Birth," 2012). ACOG's 2025 practice advisory recommends offering **vaginal progesterone** to women with prior spontaneous preterm birth and a short cervix, and suggests its use may be considered in women without prior preterm birth who are found to have a short cervix on ultrasound(Berghella et al., 2017; "Practice Bulletin No. 130: Prediction and Prevention of Preterm Birth," 2012; Romero et al., 2021). The therapy is generally safe; reported side effects include vaginal discharge and pruritus. In pregnancies with both a short cervix and a prior PTB, progesterone may be used in combination with cerclage or pessary.

Natural progesterone vs. synthetic progestogens — Oral dydrogesterone and micronized progesterone have been studied but evidence is insufficient to recommend routine use. Oral formulations have lower bioavailability and more systemic side effects.

Importantly, neither intramuscular nor vaginal progesterone is effective for preventing PTB in multifetal gestation. Progesterone is not indicated in multiple gestation.

Table 5. Guideline recommendations for progesterone supplementation (ACOG 2025)

Risk profile	Progesterone regimen	Evidence/Recommendation
Singleton pregnancy with prior spontaneous preterm birth and short cervix (<25 mm) before 24 weeks	Vaginal progesterone 200 mg nightly until 36 weeks	May reduce recurrent preterm birth; offered after discussion (“Practice Bulletin No. 130: Prediction and Prevention of Preterm Birth,” 2012)
Singleton pregnancy with prior spontaneous preterm birth but cervix ≥25 mm	No progesterone	Vaginal progesterone not effective without cervical shortening(“Practice Bulletin No. 130: Prediction and Prevention of Preterm Birth,” 2012)
17-OHPC (intramuscular)	Not recommended	FDA withdrew approval; evidence does not demonstrate benefit (“Practice Bulletin No. 130: Prediction and Prevention of Preterm Birth,” 2012)
Multiple gestation	Not recommended	Progesterone has not been shown to reduce preterm birth in twins or higher multiples

6.3. Cervical Cerclage and Pessary

Cerclage is a surgical procedure in which a suture is placed around the cervix to reinforce the cervical canal, to

reinforce mechanical strength. According to SMFM guidelines, there are three indications(O’Brien et al., 2007):

1. **History-indicated cerclage:** Offered at 12–14 weeks for women with ≥2 previous second-trimester losses or spontaneous PTBs related to painless cervical dilation. Offered at 12–14 weeks gestation to women with ≥1 prior second-trimester pregnancy losses or spontaneous preterm births attributable to painless cervical dilation (O’Brien et al., 2007). The McDonald or Shirodkar techniques are commonly used. Randomized trials show a significant reduction in recurrent PTB and perinatal mortality. The McDonald or Shirodkar technique is performed at 12–14 weeks.

2. **Ultrasound-indicated cerclage:** Placed between 16–23 weeks when transvaginal ultrasound demonstrates cervical shortening (<25 mm) in women with a history of PTB or cervical surgery. Recommended for women with a **singleton pregnancy**, a **history of preterm birth** and a **current cervical length <25 mm before 24 weeks** (O'Brien et al., 2007). Evidence does not support cerclage for short cervix in women without prior preterm birth. Cerclage may be combined with vaginal progesterone when the cervix is very short (<20 mm) or funneled. Placement is generally recommended between 14 and 24 weeks.
3. **Physical exam-indicated (rescue) cerclage:** Used before 24 weeks in pregnant women with dilated cervix and exposed fetal membranes in the absence of infection or labour. Considered when a dilated cervix is noted on physical examination (<24 weeks) without contractions or infection (O'Brien et al., 2007). A systematic review of 96 studies involving 3239 women demonstrated that rescue cerclage significantly prolongs gestation and reduces delivery before 28 weeks. Success rates vary; emergency cerclage should be accompanied by antibiotics and tocolytics.

Cerclage is not recommended in multifetal pregnancies and may increase PTB risk. Cerclage is not recommended for multiple gestations without prior preterm birth and may increase risk of infection and rupture of membranes. Contraindications include active labour, chorioamnionitis, significant vaginal bleeding or fetal anomalies incompatible with life.

Cervical pessary: The Arabin® silicone pessary is a non-invasive device that encircles and tilts the cervix, thereby changing the cervical angle and redistributing intra-uterine pressure away from the internal os. Cervical pessaries are silicone devices placed around the cervix to redistribute uterine weight. Evidence for its efficacy is mixed: some randomized trials show reduced PTB in women with a short cervix, whereas others show no benefit. Trials have yielded mixed results; a meta-analysis found no significant benefit for singleton pregnancies with a short cervix, but some benefit for women with twin gestations and short cervix. Current guidelines suggest that a cervical pessary may be considered for women with a singleton pregnancy, short cervix (≤25 mm) and no prior PTB when progesterone and cerclage are unavailable or not acceptable. In twin pregnancies, a pessary does not clearly reduce PTB risk. Use is therefore individualized.

6.2. Tocolytic Therapy

The primary objective of tocolysis is to delay delivery for at least 48 hours to allow administration of antenatal corticosteroids and magnesium sulphate and

to facilitate maternal transfer to a tertiary centre. The main classes of tocolytics include beta-agonists, calcium channel blockers, magnesium sulphate, prostaglandin inhibitors and oxytocin receptor antagonists:

- **Beta-agonists (β2-adrenergic agonists):** Terbutaline stimulates β2 receptors, increasing cyclic AMP and reducing intracellular calcium. A meta-analysis of 95 randomized trials found that β2-agonists prolong pregnancy for 48 hours but do not improve neonatal outcomes; maternal side effects include tachycardia, hypotension and pulmonary edema. Due to serious maternal effects, β-agonists are used sparingly.
- **Calcium channel blockers (CCBs):** Nifedipine inhibits calcium influx into myocytes. Common regimen: 20–30 mg oral loading dose repeated every 15–20 minutes, then 10–20 mg every 4–8 hours. CCBs are effective in delaying birth beyond seven days and have fewer side effects than β-agonists.
- **Magnesium sulphate:** Competes with calcium at voltage-gated channels, decreasing myometrial contractility and providing fetal neuroprotection. A typical regimen is a 4–6 g intravenous loading dose followed by 2 g/h infusion. The BEAM trial demonstrated that magnesium sulphate administered before anticipated early preterm birth significantly reduces the risk of cerebral palsy without increasing mortality (RR 0.55, 95% CI 0.32–0.95). ACOG and SMFM recommend magnesium sulphate for women at risk of delivery before 32 weeks. Maternal adverse effects include flushing, hypotension and respiratory depression; calcium gluconate is the antidote for toxicity.
- **Prostaglandin inhibitors (NSAIDs):** Indomethacin reduces prostaglandin synthesis, decreasing contractions and cervical ripening. The typical loading dose is 50 mg orally, followed by 25–50 mg every 6 hours for up to 48 hours. Indomethacin is effective in delaying delivery and is often first-line before 32 weeks. Potential fetal risks include ductus arteriosus constriction, oligohydramnios and NEC; thus treatment is limited to short courses.
- • **Oxytocin receptor antagonists:** Atosiban (available in Europe) antagonizes oxytocin-mediated contractions and has fewer maternal side effects but limited availability and cost restrict its use.

Maintenance tocolysis beyond 48 hours does not improve outcomes and is generally not recommended. The choice of tocolytic depends on gestational age, maternal comorbidities and contraindications.

6.3. Antenatal Corticosteroids

Antenatal corticosteroids (ACS) accelerate fetal lung maturation and reduce neonatal morbidity and mortality. Betamethasone or dexamethasone cross the placenta and induce synthesis of surfactant proteins. A Cochrane meta-analysis of 21 randomized trials demonstrated that ACS decrease the risk of respiratory distress syndrome, intraventricular hemorrhage and necrotizing enterocolitis with no significant difference between agents. The recommended regimen is two doses of 12 mg betamethasone 24 hours apart or four doses of 6 mg dexamethasone 12 hours apart (total dose 24 mg). A single course is indicated for women at 24–34 weeks who are at high risk of delivery within 7 days. Repeat courses may modestly prolong gestation but are associated with smaller birthweight and potential infection and are generally reserved for women with ongoing risk after 14 days.

6.4. Magnesium Sulphate for Neuroprotection

In addition to its tocolytic effect, magnesium sulphate administered to women at risk of delivery <32 weeks reduces the risk of cerebral palsy and severe motor dysfunction in surviving infants. The regimen mirrors that used for tocolysis but the goal is neuroprotection rather than prolonging pregnancy. Administration should begin when delivery is imminent; infusion is discontinued after 12 hours if birth does not occur. Maternal monitoring includes reflexes, respiratory status and urine output.

6.5. Antibiotics

Antibiotics prolong pregnancy when PTB is related to PPROM. Earlier randomized trials suggested benefit of erythromycin or ampicillin for women with PTL and intact membranes, but subsequent studies (e.g., ORACLE II) showed no improvement in neonatal outcomes and increased risk of maternal infection. Consequently, WHO and NICE guidelines recommend antibiotics only when PPROM is present or when group B streptococcal prophylaxis is indicated. Broad-spectrum antibiotics should not be used routinely for spontaneous PTL without infection.

6.6. Aspirin and Anti-Inflammatory Interventions

The ASPIRIN trial showed that low-dose aspirin (81 mg daily) starting before 14 weeks in nulliparous women without prior medical conditions reduced PTB rates. Aspirin may exert anti-inflammatory and anti-platelet effects on placental microvasculature. Low-dose aspirin (81–150 mg daily) starting between 12 and 16 weeks reduces the risk of preeclampsia and may indirectly decrease provider-initiated preterm birth. Current guidelines recommend low-dose aspirin for prevention of preeclampsia, which may secondarily reduce iatrogenic PTB.

Randomized trials of metformin in obese women showed reduction in gestational weight gain but not preterm birth. Statins, nitric oxide donors and anti-inflammatory agents are under investigation. Ongoing research examines other anti-inflammatory agents, antioxidants and novel tocolytics (e.g., 2-aminoethoxydiphenyl borate, glycyl-H-1152).

6.7. Preeclampsia and Hypertensive Disorders

Timely diagnosis and management of preeclampsia and hypertensive disorders are essential because severe disease often necessitates medically indicated PTB. Antenatal surveillance, control of blood pressure, magnesium sulphate for seizure prophylaxis, and determination of optimal timing of delivery are critical to balancing maternal and neonatal risks.

Summary of Prevention Interventions

Table 6. Preventive interventions for preterm birth

Intervention	Target population	Evidence of benefit	Guideline recommendations
Smoking cessation	All pregnant women	Reduces risk of preterm birth and low birth weight	Recommended by all major societies
Nutritional supplementation	Women with malnutrition or low intake of specific nutrients	Iron, folic acid and DHA supplementation modestly reduce risk of preterm birth; evidence stronger for early than late preterm birth	WHO recommends balanced energy- protein supplementation in undernourished populations
Vaginal progesterone	Women with prior spontaneous preterm birth and short cervix (<25 mm)	Reduces recurrent preterm birth by ~30% (Berghella et al., 2017; "Practice Bulletin No. 130: Prediction and Prevention of Preterm Birth," 2012)	ACOG/SMFM recommend use; WHO supports use where resources allow
17-OHPC (Makena)	Women with prior spontaneous preterm birth	No benefit in recent trials; potential harms	Withdrawn from US market; not recommended ("Practice Bulletin No. 130: Prediction and Prevention of Preterm Birth," 2012)

Cerclage	Women with prior second-trimester loss and short cervix; emergency use	Reduces risk of preterm birth <34 weeks in high-risk women (O'Brien et al., 2007)	SMFM/ISUOG recommend history- and ultrasound-indicated cerclage; not recommended in unselected populations
Cervical pessary	Women with short cervix, especially twin pregnancies	Mixed evidence; possible benefit in twins	Consider in research settings or individualized care
Low-dose aspirin	Women at risk of preeclampsia	Reduces preeclampsia and associated provider-initiated preterm birth	Recommended by ACOG/SMFM/WHO for high-risk women

7. Management of Threatened Preterm Labour

When a woman presents with uterine contractions, cervical change or rupture of membranes before 37 weeks, clinicians must balance prolonging pregnancy to maximize fetal maturity against avoiding maternal or fetal harm. Management depends on gestational age, severity of symptoms and presence of complications (e.g., PPROM, infection, fetal distress). The goals are to prolong pregnancy long enough to administer ACS and neuroprotective agents, treat underlying causes, and ensure that delivery occurs in a facility equipped for preterm neonatal care. Management involves **confirming diagnosis**, **assessing maternal-fetal status**, **administering therapies** to improve neonatal outcomes, and deciding whether to transfer or deliver.

7.1. Initial Assessment

When a patient presents with symptoms of preterm labour (uterine contractions, pelvic pressure, vaginal bleeding or discharge), Women presenting with uterine contractions before 37 weeks should undergo a

comprehensive evaluation: clinicians should confirm gestational age, perform speculum and digital cervical examinations, assess uterine contraction pattern and evaluate for PPROM. confirmation of gestational age, history of vaginal bleeding or fluid leakage, assessment of risk factors, vital signs, fetal heart rate monitoring and sterile speculum examination to look for cervical dilation and ruptured membranes. Fetal monitoring for heart rate patterns and ultrasound assessment for presentation, amniotic fluid volume and cervical length are performed.

Laboratory tests may include fFN, complete blood count, urinalysis, cervical cultures and biomarkers of infection. If PPROM is suspected, sterile speculum examination with nitrazine, fern testing and ultrasound evaluation is performed. Uterine contractions alone do not mandate tocolytic therapy; the diagnosis of **preterm labour** requires regular contractions accompanied by cervical change.

7.2. Tocolytic Therapy

Rationale — Tocolytic medications are administered **not to achieve term gestation**, but to prolong pregnancy long enough (48–168 h) to allow administration of antenatal corticosteroids, magnesium sulfate for neuroprotection and, when necessary, maternal transfer to a tertiary care centre. Tocolytics are medications used to suppress uterine contractions and delay delivery for 48–72 hours. This delay permits administration of antenatal corticosteroids, magnesium sulfate for neuroprotection and in utero transfer to a tertiary center.

According to the StatPearls point-of-care review, tocolysis aims to **delay delivery for two to seven days** by creating a quiescent uterine environment (Romero et al., 2014). This window also allows determination of maternal Group B Streptococcus (GBS) status and prophylaxis (Romero et al., 2014). Importantly, tocolytics have not been shown to reduce neonatal mortality; their benefit is primarily to buy time (Romero, 2014). No tocolytic has been shown to improve long-term neonatal outcomes, and there are **no FDA-approved tocolytic drugs**; all agents are used off-label (Romero et al., 2014). There are no FDA-approved tocolytics; all are used off-label and have contraindications.

Table 7. Common tocolytic agents

Drug class	Example/route	Mechanism of action	Typical dosing	Contraindications and adverse effects
Calcium channel blockers	Nifedipine (oral)	Inhibits calcium influx in smooth muscle, reducing uterine contractility	Initial 20–30 mg, then 10–20 mg every 6–8 h for 48 h	Contraindicated in maternal hypotension or cardiac disease; side effects: flushing, headache, dizziness. First-line agent in many guidelines; avoid concomitant magnesium sulfate due to synergistic hypotension

Cyclo-oxygenase inhibitors	Indomethacin (oral or rectal)	Reduces prostaglandin synthesis	50–100 mg loading dose then 25–50 mg every 6 h for 48 h; limit to <32 weeks gestation	Risk of premature closure of ductus arteriosus and oligohydramnios; avoid after 32 weeks. Preferred for gestations <32 weeks; monitor amniotic fluid and fetal ductus. Maternal nausea, gastritis
Beta-adrenergic agonists	Terbutaline (subcutaneous)	Stimulates β_2-receptors causing myometrial relaxation	0.25 mg every 4 h (maximum 48 h)	Maternal tachycardia, pulmonary edema; terbutaline withdrawn for chronic use due to maternal deaths (Romero, 2014). Historically used; FDA warns against prolonged use due to maternal deaths; not recommended for maintenance (Romero, 2014). Tachycardia, palpitations, hyperglycemia; risk of pulmonary edema; contraindicated in cardiac disease
Oxytocin receptor antagonists	Atosiban (intravenous)	Blocks oxytocin receptor; reduces myometrial contractions	Loading 6.75 mg over 1 min; infusion 18 mg/h for 3 h then 6 mg/h up to 45 h	Widely used in Europe; limited availability in US; minimal side effects. Efficacy comparable to nifedipine

Magnesium sulfate	$MgSO_4$ (intravenous)	Competes with calcium at the cell membrane and NMDA receptors; reduces contractility and provides fetal neuroprotection	Loading 4–6 g IV over 20–30 min then 2 g/h for 24 h	Flushing, nausea, respiratory depression at high levels; used primarily for neuroprotection (see Section 7.4). Not a potent tocolytic but provides neuroprotection (Jafarabady et al., 2024). Caution in renal insufficiency
Nitric oxide donors	Nitroglycerin (transdermal/IV)	Relax smooth muscle by releasing nitric oxide	10 mg topical patch or IV	Limited evidence; side effects include headache and hypotension. Used as second-line therapy
Drug class	**Example/route**	**Mechanism of action**	**Typical dosing**	**Contraindications and adverse effects**
			infusion titrated to effect	

If gestational age is between 24 and 34 weeks and there are no contraindications (e.g., chorioamnionitis, severe fetal anomalies, intrauterine demise, maternal instability), acute tocolysis is initiated using a single agent (commonly nifedipine or indomethacin). Choice of tocolytic depends on gestational age, contraindications and clinician experience. Tocolytics are generally **contraindicated** when delivery is clearly in the interest of maternal or fetal health—for example, in the presence of severe preeclampsia, significant vaginal bleeding suggesting abruption, chorioamnionitis, lethal fetal anomaly, fetal demise or maternal contraindications to the drugs. Contraindications to tocolysis include intra-uterine infection, fetal demise, lethal fetal anomaly, severe preeclampsia/eclampsia, significant antepartum hemorrhage or maternal contraindication to the drugs. Use of multiple tocolytics simultaneously is not recommended; clinicians should select one agent tailored to the gestational age and maternal history. Maintenance tocolysis beyond 48–72 h has not been shown to confer benefit and is discouraged.

7.3. Antenatal Corticosteroids

A single course of betamethasone (12 mg intramuscularly, two doses 24 hours apart) or dexamethasone (6 mg intramuscularly, four doses 12 hours apart) accelerates fetal lung maturity, reducing respiratory distress syndrome, intraventricular hemorrhage and neonatal death. Administration of antenatal corticosteroids accelerates fetal lung maturation and reduces the incidence of respiratory distress syndrome, intraventricular hemorrhage and neonatal death. WHO guidelines recommend a single course of corticosteroids (betamethasone 12 mg intramuscularly every 24 h for two doses, or dexamethasone 6 mg intramuscularly every 12 h for four doses) for women at 24–34 weeks gestation who have a high likelihood of preterm birth within 7 days, provided that accurate gestational age is known, there is no clinical evidence of maternal infection, and adequate maternal and neonatal care is available (Romero et al., 2014). WHO and national guidelines recommend antenatal corticosteroids for women at risk of preterm birth from 24 to 34 weeks when accurate gestational age, imminent delivery and adequate care are assured (Romero et al., 2014). Betamethasone or dexamethasone should be given promptly for fetal lung maturation. Repeated or rescue courses are generally not recommended because benefits decrease and potential harms (e.g., fetal growth restriction, neurodevelopmental issues) may accrue. For women at **34–36⁄₇ weeks**, evidence for benefit is less robust; decisions should be individualized, particularly for those at risk of late-preterm delivery due to medical indications. Administration outside this window or repeated courses have uncertain benefit and may impair fetal growth. Monitoring maternal blood glucose is important in women with diabetes. If preterm labour continues beyond 7 days after an initial course of ACS, a repeat (rescue) course may be considered in women <34 weeks with persistent risk of preterm delivery. The benefits of rescue dosing must be weighed against potential adverse effects such as growth restriction and altered neurodevelopment.

7.4. Magnesium Sulphate for Neuroprotection

Administration of magnesium sulfate to women at risk of delivery before 32 to 34 weeks reduces cerebral palsy risk without affecting mortality (Jafarabady et al., 2024). In addition to its tocolytic effect, magnesium sulphate administered to women at risk of delivery <32 weeks reduces the risk of cerebral palsy and severe motor dysfunction in surviving infants. Randomized trials and meta-analyses indicate that administration of **intravenous magnesium sulfate** to women at imminent risk of preterm delivery **reduces the risk of cerebral palsy (CP)** without significantly affecting neonatal mortality (Jafarabady et al., 2024). A recent meta-analysis including 8,171 participants reported that the **prevalence of cerebral palsy decreased from 5.6% in untreated infants to 3.9% in those exposed to $MgSO_4$** (Jafarabady et al., 2024). The optimal gestational age and

dosage remain debated; However, the optimal gestational age and dosing remain controversial. most Guidelines generally recommend $MgSO_4$ for neuroprotection when delivery is expected before **32–34 weeks**; regimens typically include a 4 g IV loading dose followed by a 1–2 g/hr infusion for up to 24 hours or until birth. The regimen mirrors that used for tocolysis but the goal is neuroprotection rather than prolonging pregnancy. Administration should begin when delivery is imminent; infusion is discontinued after 12 hours if birth does not occur.

Magnesium sulphate is administered concurrently for neuroprotection if the gestational age is <32 weeks. Treatment is stopped if delivery does not occur within 24 hours. Maternal vital signs and reflexes should be monitored; calcium gluconate must be available to reverse toxicity. Careful monitoring for maternal side effects (e.g., respiratory depression, hyporeflexia) is essential. The benefits of $MgSO_4$ appear to outweigh risks, but further research is needed to define ideal candidates (Jafarabady et al., 2024). Maternal monitoring includes reflexes, respiratory status and urine output.

7.5. Antibiotics and Group B Streptococcus Prophylaxis

For **preterm pre-labour rupture of membranes (PPROM)**, broad-spectrum antibiotics (e.g., ampicillin 2 g IV q6h plus erythromycin 250 mg q6h for 48 hours followed by oral amoxicillin 875 mg twice daily and erythromycin 333 mg four times daily for 5 days) prolong latency and reduce neonatal infection. In cases of **preterm premature rupture of membranes**, broad-spectrum antibiotics prolong the latency period and reduce maternal and neonatal infection. Recommended regimens include intravenous ampicillin 2 g every 6 h and erythromycin 250 mg every 6 h for 48 h, followed by oral amoxicillin 875 mg twice daily and erythromycin 333 mg four times daily for 5 days. The combination of amoxicillin and erythromycin is chosen to provide activity against genital Mycoplasma species; co-administration of ampicillin with azithromycin is an alternative for penicillin-allergic patients. However, prophylactic antibiotics are not indicated for intact membranes because they do not prevent preterm birth and may increase necrotizing enterocolitis. Universal vaginal-rectal screening for **Group B Streptococcus** at 36–37 weeks and intrapartum penicillin prophylaxis remain cornerstones of perinatal infection prevention. Group B streptococcal prophylaxis and broad-spectrum antibiotics are administered if PPROM is present. The patient should be monitored for labour progression, maternal side effects and fetal well-being.

7.6. Management of PPROM

Preterm prelabour rupture of membranes occurs in 2–3% of pregnancies and accounts for approximately one- third of preterm births. Management depends on gestational age:

- **Before 34 weeks:** Expectant management is preferred if there is no evidence of infection or placental abruption. The patient is hospitalized for monitoring, receives a course of ACS, magnesium sulphate for
- neuroprotection, and prophylactic antibiotics (e.g., a combination of ampicillin and erythromycin for 7 days). Tocolysis is controversial; if contractions develop, a short course may be used to complete ACS. Delivery is indicated if chorioamnionitis, placental abruption, non-reassuring fetal status or advanced labour occurs.
- **34–36 weeks:** Management is individualized; many guidelines recommend induction or delivery after 34 weeks to reduce the risk of infection because neonatal outcomes improve after this gestational age.
- • **≥37 weeks:** Delivery is recommended as term infants have favourable outcomes.

7.7. Corticotropin-Releasing Hormone Antagonists and Investigational Therapies

Drugs targeting corticotropin-releasing hormone, interleukin-6 and other inflammatory mediators are being investigated. Owing to the complexity of parturition pathways, it is unlikely that a single agent will prevent preterm birth in all women. Trials of prophylactic antibiotics or probiotics for bacterial vaginosis have not consistently reduced spontaneous preterm birth and may cause harm; thus, routine use is discouraged.

7.8. Fetal Monitoring and Decision for Delivery

Continuous electronic fetal monitoring is used to detect heart rate abnormalities. Ultrasound for fetal biometry and Doppler velocimetry can assess fetal growth and placental function. Decision for delivery balances maternal condition, gestational age, fetal status and the ability to administer ACS and magnesium sulphate. A multidisciplinary team—including obstetricians, neonatologists, anesthesiologists and nurses—should be involved.

8. Neonatal Outcomes and Long-Term Consequences

Prematurity is a leading cause of childhood morbidity and mortality. Outcomes after preterm birth correlate with gestational age and birth weight. Short-term neonatal complications include respiratory distress syndrome, bronchopulmonary dysplasia, necrotizing enterocolitis, sepsis, hypothermia, jaundice, intraventricular hemorrhage and retinopathy of prematurity. Long-term

sequelae encompass cerebral palsy, hearing and visual impairment, cognitive and behavioral disorders, asthma, and metabolic syndrome. Early intervention programs and neurodevelopmental follow-up are essential.

8.1. Immediate Neonatal Outcomes

Preterm infants are susceptible to respiratory distress syndrome, due to surfactant deficiency; intraventricular hemorrhage (particularly before 32 weeks); necrotizing enterocolitis; sepsis; and metabolic problems such as hypoglycemia and hypothermia. The risk and severity of these complications increase with decreasing gestational age. Late-preterm infants, while less severely affected, still have higher rates of jaundice, feeding difficulties and hospital readmission compared with term infants.

8.2. Long-Term Neurodevelopmental Outcomes

Survivors of preterm birth are at higher risk of cerebral palsy, cognitive impairment, attention deficit hyperactivity disorder and autism spectrum disorder. A meta-analysis evaluating magnesium sulfate for

neuroprotection found a significant reduction in cerebral palsy but no difference in overall mortality (Jafarabady et al., 2024). Preterm children may also experience vision and hearing deficits, behavioral problems and difficulties in school performance (Jafarabady et al., 2024). Early neurodevelopmental follow-up and intervention programmes improve outcomes.

8.3. Cardiometabolic and Reproductive Outcomes

Preterm birth is associated with long-term cardiovascular consequences, including hypertension, ischemic heart disease and insulin resistance in adulthood. Adults born prematurely also have reduced renal mass and are at higher risk of chronic kidney disease. Women born preterm may themselves face an increased risk of preterm birth in their pregnancies, suggesting an intergenerational transmission of risk. Longitudinal studies show that individuals born preterm may have lower educational achievement, reduced income, higher rates of psychiatric disorders and increased cardiometabolic risk in adulthood. Preterm birth has intergenerational effects—women born preterm have higher risk of delivering preterm infants, suggesting epigenetic or socio- economic transmission. Providing developmental follow-up and early interventions, such as kangaroo mother care, breastfeeding support, physiotherapy, and special education, can mitigate some adverse outcomes.

Table 8. Selected complications of prematurity by gestational age

Gestational age category	Common short-term complications	Long-term consequences
Extremely preterm (<28 weeks)	High mortality; respiratory distress syndrome, patent ductus arteriosus, severe intraventricular hemorrhage, necrotizing enterocolitis, sepsis	Cerebral palsy, cognitive impairment, blindness, hearing loss, chronic lung disease
Very preterm (28–<32 weeks)	Respiratory distress, apnea of prematurity, feeding difficulties, hypothermia, jaundice	Neurodevelopmental delay, learning disabilities, behavioral problems
Moderately preterm (32–<34 weeks)	Transient tachypnea of the newborn, hypoglycemia, thermoregulatory instability	Mild neurodevelopmental impairment, attention deficit disorders
Late preterm (34–<37 weeks)	Respiratory distress, jaundice, feeding difficulties, hypoglycemia	Increased risk of hospital readmission, learning difficulties, metabolic syndrome

9. Guidelines from Professional Societies

Major obstetric societies have published evidence-based guidelines for preterm birth prevention and management. Table 9 synthesizes key recommendations from ACOG, SMFM, ISUOG and WHO. Clinicians should refer to the full guidelines for detailed criteria.

9.1. American College of Obstetricians & Gynecologists (ACOG)

The American College of Obstetricians and Gynecologists (ACOG) Practice Bulletin No. 234 and its 2023

practice advisory emphasize risk assessment and evidence-based interventions for PTB. Key recommendations include:

- **Screening** — ACOG recommends **transvaginal cervical length measurement** at 18–24 weeks in women with prior spontaneous preterm birth. Universal CL screening at 18–24 weeks for women with a prior PTB and consideration of screening in all pregnant women.

Universal screening may be considered based on local resources. **Fetal fibronectin** testing may help triage symptomatic patients.

- **Progesterone** — Vaginal progesterone for women with a singleton gestation and short cervix (≤25 mm) without prior PTB. Vaginal progesterone is offered to women with **singleton pregnancy, prior preterm birth and short cervix**; 17-OHPC is **not recommended** (Committee, 2012). Serial ultrasound monitoring and discussion of prophylactic options (progesterone, cerclage, pessary) in women with a prior PTB based on CL measurement and obstetric history. No routine use of 17-OHPC for recurrent PTB due to lack of proven benefit and withdrawal of FDA approval. Progesterone is not indicated in multiple gestation.

- **Cerclage** — History-indicated cerclage at 12–14 weeks for women with ≥2 second-trimester losses; ultrasound-indicated cerclage for short cervix in women with prior preterm birth (O'Brien et al., 2007). Consider cerclage for history-indicated or ultrasound-indicated cases; recommend aspirin for preeclampsia prevention.

- **Tocolytics** — Nifedipine and indomethacin are first-line agents; terbutaline is not used for maintenance tocolysis due to maternal risks (Romero et al., 2014). Tocolysis to delay delivery for 48 hours to facilitate ACS; nifedipine and indomethacin preferred due to favorable side-effect profiles.

- **Antenatal corticosteroids** — Administration of ACS between 24 and 34 weeks in women at risk of delivery within 7 days; consideration of rescue dosing for persistent risk. Betamethasone or dexamethasone for women at risk of preterm birth between 24–34 weeks; consider 34–36⁶⁄₇ weeks on case-by-case basis (Romero et al., 2014).

- **$MgSO_4$**— Magnesium sulphate for neuroprotection when delivery is imminent before 32 weeks. Administer for neuroprotection when delivery is anticipated before 32–34 weeks (Jafarabady et al., 2024).

- **Antibiotics** — Avoidance of maintenance tocolysis and routine antibiotic use without PPROM.

9.2. Society for Maternal-Fetal Medicine (SMFM)

SMFM emphasizes individualized counselling based on risk factors. It supports ACOG's recommendations on progesterone, cerclage and corticosteroids. SMFM advises against universal screening with fFN in asymptomatic women and underscores that tocolytics should not delay indicated

delivery for maternal or fetal indications. Similar to ACOG; supports vaginal progesterone and cerclage; discourages 17-OHPC; recommends individualized pessary use. Suggests nifedipine or indomethacin as first-line tocolytics; advises against maintenance tocolysis; recommends antenatal steroids and magnesium sulfate as per gestational age.

9.3. International Society of Ultrasound in Obstetrics & Gynecology (ISUOG)

The International Society of Ultrasound in Obstetrics & Gynecology (ISUOG) guidelines recommend CLscreening for women at risk, support the use of progesterone or cerclage for a short cervix, and emphasize accurate gestational age assessment using first-trimester ultrasound. ISUOG guidelines focus on accurate dating, early anomaly detection and cervical length screening. They encourage first-trimester ultrasound for gestational age confirmation, which is essential for timing interventions. ISUOG notes that screening for short cervix and administration of progesterone reduce spontaneous preterm birth in high-risk women. ISUOG also highlights the importance of compassionate counselling and shared decision-making when considering interventions.

Emphasizes cervical length screening and vaginal progesterone; supports cerclage for short cervix <25 mm with prior preterm birth; calls for more research on pessary. Endorses antenatal corticosteroids, tocolytics for short- term use and magnesium sulfate for neuroprotection; discourages routine bed rest.

9.4. World Health Organization (WHO)

The World Health Organization recommends routine antenatal corticosteroids for women at risk of preterm birth between 24 and 34 weeks when gestational age is accurately assessed, delivery is expected within 7 days, there is no evidence of maternal infection and adequate maternal and neonatal care is available. WHO emphasizes **respectful, woman-centred care** and equitable access to evidence-based interventions. The 2022 WHO guideline on antenatal corticosteroids recommends a **single course** for women at 24–34 weeks at risk of preterm birth when accurate gestational age assessment and adequate maternal-neonatal care are available (Romero, 2014). WHO guidance also advocates for eight antenatal contacts, dietary counselling, infection screening and early ultrasound to prevent PTB. WHO's tocolytic recommendation (2022) suggests using tocolytic therapy to delay birth when there are clear benefits (e.g., time to administer corticosteroids) but warns against routine use in all preterm labour because of potential harm and lack of long-term benefit. FIGO's 2021 guidelines on corticosteroid therapy endorse a total dose of 24 mg betamethasone/dexamethasone administered as two doses 24 hours apart. WHO also advocates **kangaroo mother care**, early initiation of breastfeeding,

continuous positive airway pressure (CPAP) and caffeine therapy for preterm infants (Romero, 2014). Recommends population-wide interventions (smoking cessation, nutritional support); advocates for antenatal corticosteroids for women at risk of delivery 24–34 weeks in facilities with adequate care; promotes kangaroo mother care and breastfeeding; suggests low-dose aspirin for preeclampsia prevention. Recommends uterine evacuation only for severe maternal/fetal indications; supports use of tocolytics to delay delivery for steroid administration; emphasizes equitable access to neonatal intensive care.

Table 9. Selected guideline recommendations

Organization	Prevention recommendations	Management recommendations
ACOG (Practice Advisory 2025)	Offer vaginal progesterone to women with prior spontaneous preterm birth and short cervix; do not use 17- OHPC; consider cerclage for history-indicated or ultrasound-indicated cases; recommend aspirin for preeclampsia prevention	**Administer antenatal corticosteroids between 24–34 weeks; consider rescue cerclage with tocolysis; magnesium sulfate for fetal neuroprotection up to 32–33⅙ weeks; avoid routine tocolysis beyond 72 hours**
SMFM	Similar to ACOG; supports vaginal progesterone and cerclage; discourages 17-OHPC; recommends individualized pessary use	**Suggests nifedipine or indomethacin as first-line tocolytics; advises against maintenance tocolysis; recommends antenatal steroids and magnesium sulfate as per gestational age**
ISUOG	Emphasizes cervical length screening and vaginal progesterone; supports cerclage for short cervix <25 mm with prior preterm birth; calls for more research on pessary	**Endorses antenatal corticosteroids, tocolytics for short-term use and magnesium sulfate for neuroprotection; discourages routine bed rest**

WHO	Recommends population-wide interventions (smoking cessation, nutritional support); advocates for antenatal corticosteroids for women at risk of delivery 24–34 weeks in facilities with adequate care; promotes kangaroo mother care and breastfeeding; suggests low-dose aspirin for preeclampsia prevention (Romero, 2014)	**Recommends uterine evacuation only for severe maternal/fetal indications; supports use of tocolytics to delay delivery for steroid administration; emphasizes equitable access to neonatal intensive care**

10. Public Health Strategies and Future Directions

Reducing the global burden of prematurity requires **public-health initiatives** that address upstream determinants and improve access to high-quality care. Key strategies include:

1. **Health-system strengthening** — Expand access to antenatal care, skilled birth attendants and neonatal intensive care, particularly in low-income regions. Train healthcare workers in early identification and management of preterm labour, and develop regional networks for timely transfer to tertiary centers. Investment in primary healthcare, midwifery, emergency obstetric care and neonatal intensive care units is needed. Implementation of evidence-based packages such as the WHO "Every Newborn Action Plan" and "Born Too Soon" initiatives can accelerate progress (Ohuma et al., 2023).
2. **Policy and legislation** — Implement smoke-free policies, maternity leave, workplace protections and environmental regulations to reduce exposure to toxins. Support universal health coverage and reduce financial barriers to prenatal services. Policies supporting paid maternity leave, safe housing and clean water are integral.
3. **Education and empowerment** — Provide preconception counselling, health education and community outreach to promote healthy behaviors and family planning. Engage fathers and families to improve adherence to interventions. Community education on danger signs, birth preparedness, nutrition, smoking cessation and contraception empowers women and families. Male partner involvement and gender equality initiatives reduce intimate partner violence and psychosocial stress.

4. **Data collection and research** — Invest in registries and surveillance systems to track preterm birth rates and outcomes. Conduct context-specific research to evaluate interventions in diverse populations and to explore gene-environment interactions. Improving vital registration systems and harmonizing definitions of PTB enable accurate surveillance and evaluation of interventions. Research priorities include understanding genetic and epigenetic determinants, discovering novel biomarkers and developing targeted therapies.
5. **Equity and advocacy** — Address socio-economic and racial disparities that contribute to preterm birth. Addressing poverty, racism, environmental exposures, pollution and workplace hazards is crucial for reducing disparities. International partnerships like the *Born Too Soon* initiative and the *Every Newborn Action Plan* aim to mobilize resources, share best practices and hold governments accountable.

10.1. Future Research Directions

Advances in genomics, microbiome science and artificial intelligence are opening new avenues to understand and prevent preterm birth. Future research priorities include:

1) Elucidating genetic and epigenetic determinants of preterm birth across diverse populations;
2) Developing accurate prediction models that integrate clinical, biochemical, imaging and genetic data;
3) Testing novel therapeutics such as anti-inflammatory agents, targeted microbiome interventions and gene therapies;
4) Addressing social determinants of health, including systemic racism, poverty and environmental exposures; and
5) Evaluating policies that reduce provider-initiated preterm births through improved obstetric practices and shared decision-making.

10.2. Clinical Practice Guidelines

Summary of Key Clinical Recommendations

Universal health coverage should ensure access to quality antenatal care, including early pregnancy dating (preferably by ultrasound), risk assessment, screening for infections and chronic diseases, nutritional support and mental health services.

11. Conclusion

Preterm birth remains a complex, multifactorial syndrome with profound implications for neonatal and long- term health. Prematurity remains a global challenge with complex and multifactorial roots. Interventions

targeting infection, inflammation, vascular pathology, uterine mechanics and maternal-fetal stress can mitigate risk, but no single strategy will eliminate PTB. Although survival has improved dramatically in high-income countries, global inequities persist. Comprehensive antenatal care—including screening for cervical shortening, judicious use of progesterone, timely cerclage, tocolytic therapy, antenatal corticosteroids and magnesium sulphate—improves outcomes. Clinicians must maintain vigilance for risk factors, utilize validated screening tools and apply evidence-based interventions to prevent or manage preterm labour. Tailored counselling and shared decision-making are paramount, particularly regarding progesterone therapy, cerclage and tocolytic use. Public health initiatives addressing socioeconomic inequities, nutrition, infection control, family planning and health system strengthening are pivotal. Ongoing research into biomarkers, genetic and epigenetic determinants and novel therapeutics offers hope for more precise prevention. Multidisciplinary collaboration between obstetricians, midwives, neonatologists, policymakers and communities is essential to reduce preterm birth and its lifelong consequences. Continued research and equitable policy initiatives are essential to reduce the global burden of prematurity.

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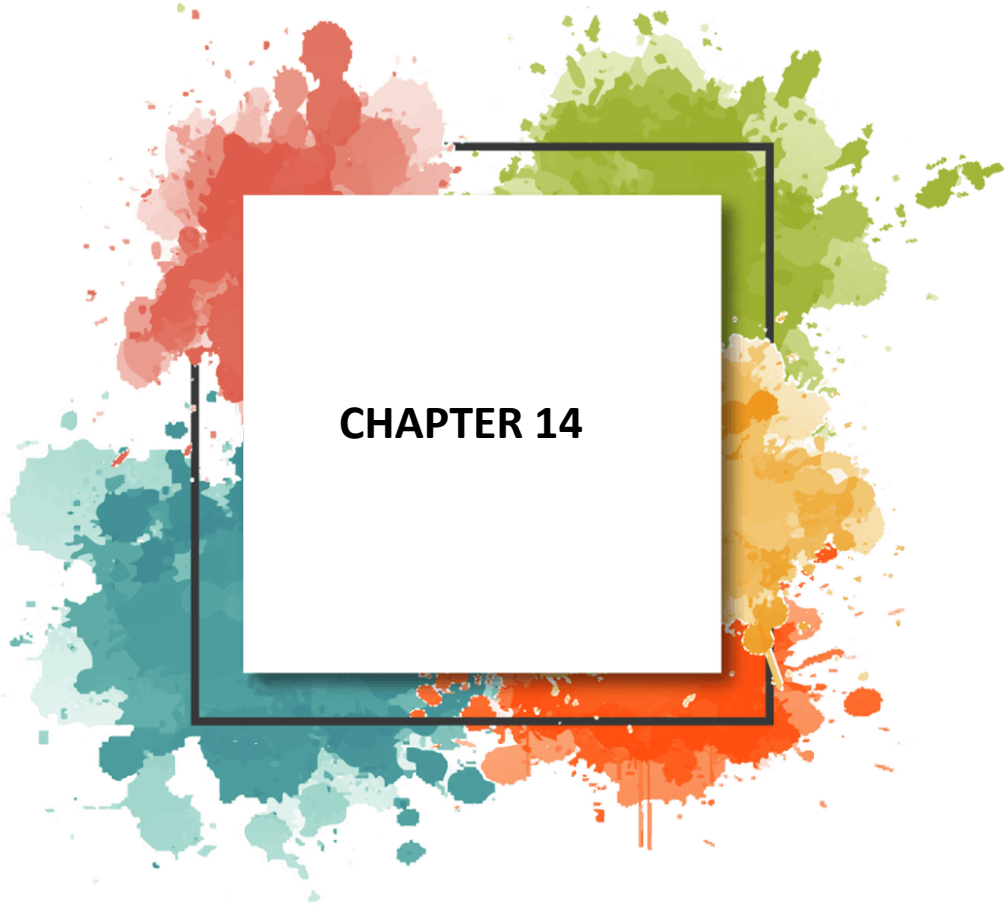
CHAPTER 14

Evaluation of Helium Ion Radiotherapy in Thyroid Cancer Using SRIM/TRIM Simulations

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1. INTRODUCTION

Thyroid cancer is one of the most common types of endocrine malignancies, with an incidence of approximately 40 cases per million people annually, resulting in six deaths on average. Early diagnosis is critical, as delayed detection can significantly impair both quality of life and survival outcomes (American Cancer Society, n.d.; Bozkurt, n.d.). Thyroid cancer comprises several subtypes, including papillary, follicular, medullary, and anaplastic carcinomas, each exhibiting distinct clinical courses and therapeutic responses. Notably, papillary and follicular carcinomas tend to progress slowly, whereas medullary and anaplastic types are associated with more aggressive behavior and poorer prognoses (American Cancer Society, n.d.; National Cancer Institute, n.d.).

Conventional treatment modalities such as surgical intervention, radiotherapy, and chemotherapy are commonly used in the management of thyroid cancer. However, these approaches may not be effective for all patients and are often associated with significant side effects. As a result, alternative therapies such as proton and heavy ion therapy have emerged as promising treatment options. Due to their ability to precisely target tumor cells, these modalities minimize damage to surrounding healthy tissues and provide more effective outcomes with fewer adverse effects (California Protons, n.d.; OncoDaily, n.d.). Among them, boron-based heavy ion therapy shows experimental promise, particularly for radiation-resistant thyroid tumors, owing to its high-energy and targeted therapeutic potential (Frontiers in Oncology, n.d.).

In contemporary particle-based radiotherapy applications, the integration of simulation methods into treatment planning reduces the need for physical experimentation, thereby saving both time and cost. Monte Carlo (MC)-based simulations serve as an indispensable tool, especially when experimental validation is not feasible, enabling patient-specific dose calculations (Battistoni et al., 2016). In this study, the SRIM/TRIM software was utilized. SRIM (Stopping and Range of Ions in Matter) is a free program capable of rapidly computing stopping power, range, and scattering distributions for various ions at different energies (Giri, Khatiwada, & Bista, 2022). Its user-friendly interface and broad accessibility for universities and research institutions make SRIM/TRIM a particularly favored option. Simulations conducted with SRIM/TRIM yield results rapidly and allow the safe modeling of potentially hazardous physical processes within a virtual environment. One study in proton therapy reported a remarkably low calculation error of 0.74%, demonstrating the ability of SRIM/TRIM to generate realistic and validated outcomes (Giri et al., 2022). These advantages underscore the effectiveness, cost-efficiency, and reliability of SRIM/TRIM in both clinical and academic settings.

The use of heavy ions as an alternative to conventional radiotherapy methods widely employed in cancer treatment offers significant advantages from both physical and radiobiological perspectives. Under ideal conditions, radiotherapy is expected to minimize toxicity in the entrance channel (normal tissue) while effectively destroying cancer cells within the targeted region (tumor). In this context, ions heavier than protons have demonstrated superior performance compared to conventional X-rays (Park, Paganetti, Schuemann, Jia, & Min, 2021). Despite the potential benefits of heavy ion therapy, the number of theoretical academic studies on the subject remains limited. This study aims to conduct a theoretical analysis of heavy ion therapy using SRIM/TRIM, a Monte Carlo-based and freely available simulation software. Monte Carlo (MC) simulations play a critical role in radiotherapy, particularly in assessing physical parameters that are difficult or impossible to measure directly (Durante, Debus, & Loeffler, 2021). Studies based on such simulations provide researchers with valuable preliminary insights before transitioning to clinical applications and offer important information regarding the practical implications of theoretical findings.

2. MATERIAL AND METHODS

In this study, the TRIM (Transport of Ions in Matter) simulation program was employed to analyze the interactions of helium ions within thyroid tissue. TRIM is a Monte Carlo-based simulation software that is part of the SRIM (Stopping and Range of Ions in Matter) package, and it models the energy deposition and interaction processes of ions within target materials in detail. By simulating the trajectories and energy losses of ions as they pass through different material layers, TRIM provides valuable data that can support radiation therapy planning.

The user interface of the SRIM/TRIM application is shown in Figure 1. At the top of the screen, buttons are available for selecting the ion type and accessing help documentation. The second section allows the user to define the simulation environment, including the physical layers along with their respective density and thickness parameters. On the right side of this panel, a compound dictionary button provides access to a set of predefined materials that can be selected directly. The third section, located at the bottom of the interface, enables users to choose the types of graphs to be saved upon completion of the simulation. Additionally, this section allows users to set the number of ions to be simulated and to launch the simulation using the buttons on the far right. Once the simulation is executed, the ion trajectories can be visualized using the graphical output shown in Figure 2. In this plot, the vertical columns represent the physical layers defined earlier in the central section of Figure 1, while the white lines in the middle indicate the paths followed by ions as they travel from the outside inward.

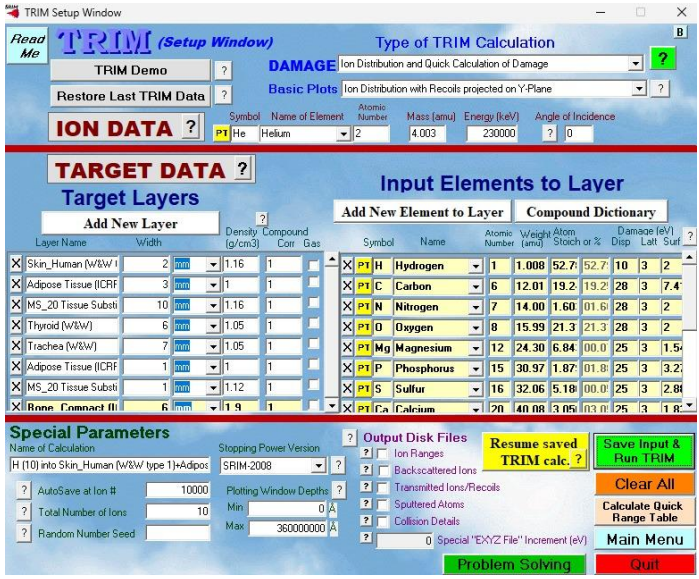


Figure 1. Graphical user interface of the TRIM simulation program, illustrating the sections for ion selection, material configuration, and simulation settings.

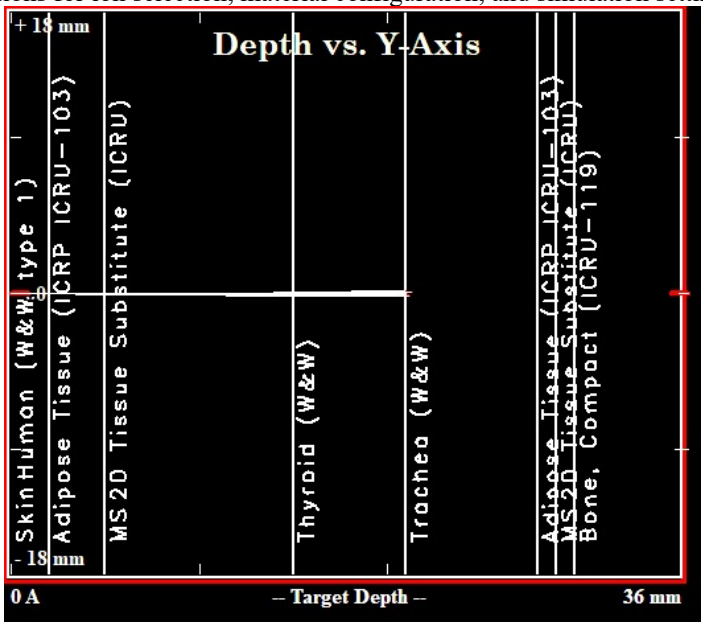


Figure 2. Depth–Y plot illustrating the trajectories of helium ions through different tissue layers in the TRIM simulation.

In this study, helium was selected as the ion type, and a model possessing physical properties similar to human thyroid tissue was used as the target material. The number of incident ions was set at 10,000 to ensure statistical reliability of the data while maintaining computational efficiency. Based on preliminary trials, the ion energy range was determined to be between 170 MeV

and 210 MeV. The simulation results reveal detailed information about the trajectories of helium ions within the tissue, including energy deposition profiles, scattering patterns, and local energy densities. The obtained ion range data demonstrate the average distance traveled by the ions and the depth at which they come to rest. Lateral distribution graphs illustrate how the ions spread across the horizontal plane. Among the simulation outputs, the ionization and phonon values represent distinct modes of energy transfer from the ions to the tissue during their passage.

Anatomically, the thyroid gland is located in the neck and is surrounded by multiple layers of tissue, starting from the skin and progressing to deeper structures. Beneath the most superficial layer—the skin—lies the subcutaneous adipose tissue, followed by the platysma muscle. Deeper still is the superficial cervical fascia, which is covered by the infrahyoid muscles (sternohyoid, sternothyroid, thyrohyoid, and omohyoid muscles). Beneath these muscles lies the pretracheal fascia (middle cervical fascia), which directly envelops the thyroid gland. The thyroid itself is encased in a fibrous capsule that is tightly adherent to the gland's parenchyma. Within the capsule lies the thyroid parenchyma, which contains follicular cells and parafollicular (C) cells. On its posterior surface, the thyroid is connected to the trachea and esophagus via the Berry ligament (lateral thyroid ligament) (Moore et al., 2018; Standring, 2020).

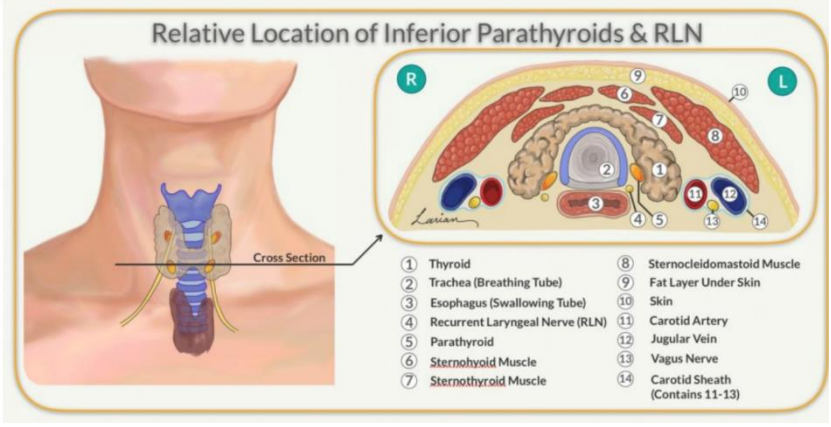


Figure 3. Illustration of the anatomical layers surrounding the thyroid gland, including the skin, subcutaneous fat, musculature, fasciae, and the fibrous capsule enclosing the gland. Source: (HyperparathyroidMD, n.d.)

Skin: The average density of human skin is approximately 1.1 g cm^{-3}, and this value was adopted in the model (Tobin, 2006).

Adipose Tissue: The density of adipose tissue ranges between 0.925 and 0.975 g cm^{-3}. In the model, a density value of 1.0 g cm^{-3} was used (Martin et al., 1994).

Muscle: The density of muscle tissue is approximately 1.0597 g cm^{-3}. A value of 1.05 g cm^{-3} was assumed in the model (Aubert et al., 2005).

Thyroid: The density of the thyroid gland typically ranges from 1.0 to 1.2 g cm^{-3}. In this simulation, a density value of 1.05 g cm^{-3} was adopted (Pankow et al., 1985).

Trachea: The trachea is primarily composed of hyaline cartilage, whose density generally falls between 1.0 and 1.2 g cm^{-3}. It is well-documented in the literature that the elastic modulus of tracheal cartilage varies with age, resulting in inter-individual differences in density **(**Safshekan et al., 2017). The model used a density value of 1.05 g cm^{-3} for this layer.

Compact Bone: The density of healthy human cortical bone is approximately 1.85 g cm^{-3}. In this study, a value of 1.9 g cm^{-3} was applied (Aqua-Calc, n.d.).

The atomic densities, thicknesses, and compositional elements of each tissue layer used in the model are presented in the tables below. These tables were generated using the SRIM/TRIM simulation software.

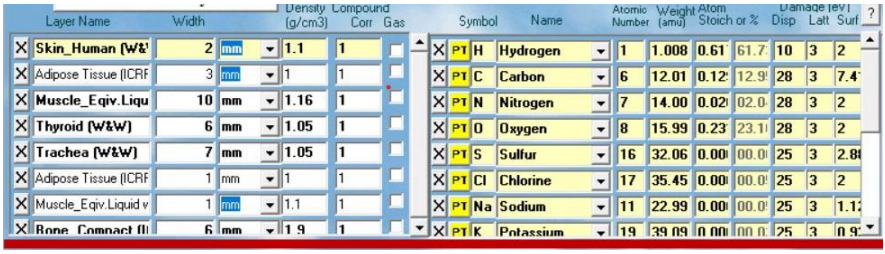


Figure 4. Representation of the skin layer with its structural and density parameters used in the simulation.

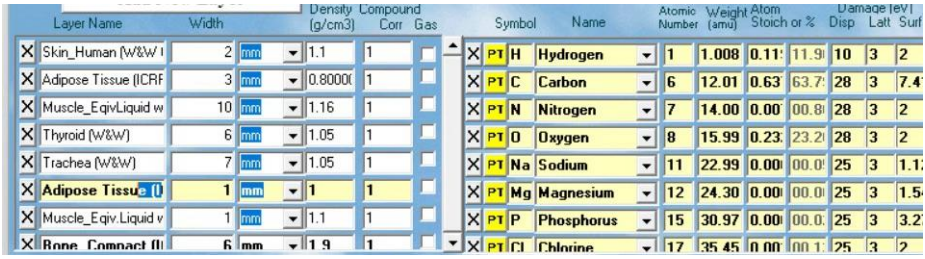


Figure 5. Representation of the adipose tissue layer with its structural characteristics and assigned density in the simulation.

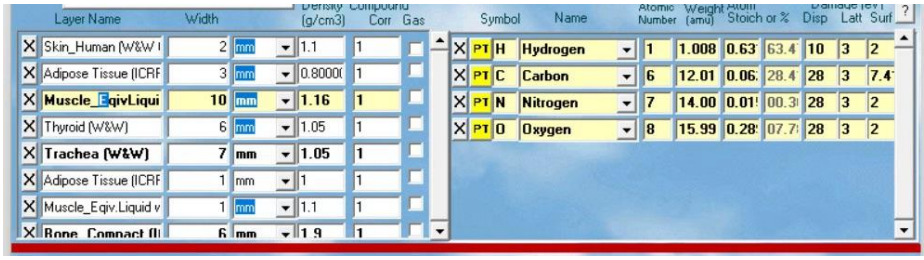


Figure 6. Representation of the muscle layer, illustrating its structural composition and density parameters used in the simulation.

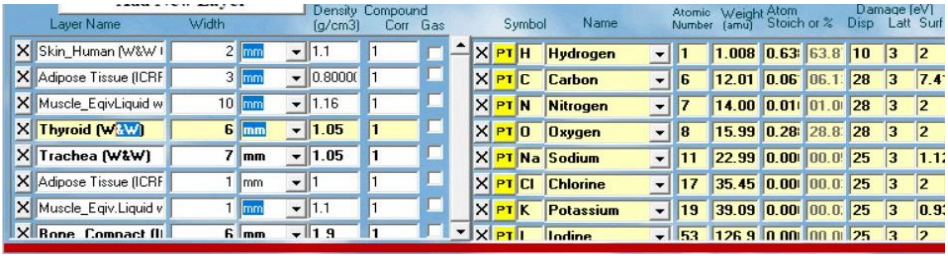


Figure 7. Representation of the thyroid layer, including its anatomical structure and the density value applied in the simulation.

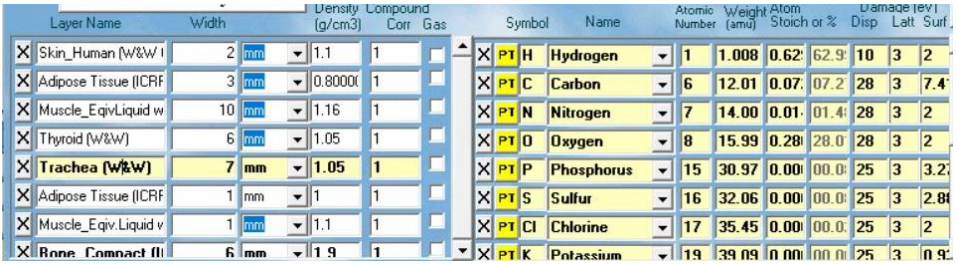


Figure 8. Representation of the trachea layer, highlighting its cartilaginous structure and density parameters used in the simulation.

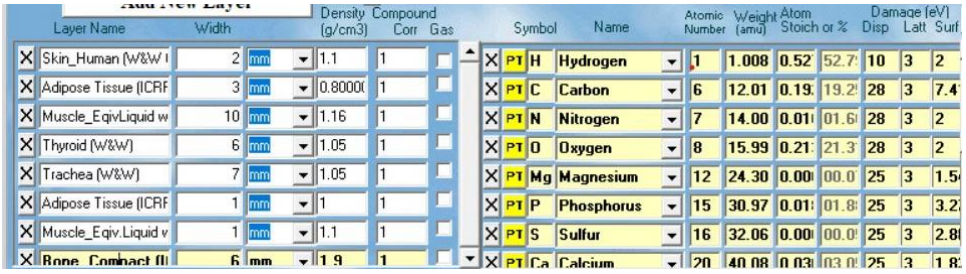


Figure 9. Representation of the compact bone layer, showing its structural characteristics and density used in the simulation model.

3. RESULTS

During the simulation, ions lose energy progressively as they travel through the tissue. The most significant energy transfer occurs at the Bragg peak. For the beam to deliver its entire energy most efficiently to the tumor, the Bragg peak must coincide with the location of the thyroid tissue. When this condition is met, maximum therapeutic impact is achieved on cancerous cells while sparing the surrounding healthy tissue. Accurate localization of energy loss within the intended area enhances treatment success and minimizes collateral damage to healthy tissue. Therefore, ensuring that the beam's energy is deposited precisely within the thyroid tissue is one of the most critical factors for optimizing treatment efficacy. This plays a particularly crucial role in preventing or minimizing damage to healthy structures adjacent to the cancerous region (Park et al., 2021).

The Linear Energy Transfer (LET) curve indicates the amount of energy deposited by ionizing particles per unit distance in the medium and serves as an important indicator of biological effectiveness. According to the data obtained from SRIM/TRIM simulations, the typical behavior of the LET curve begins with a low value, gradually increases with depth, and rises sharply as it approaches the Bragg peak, where it reaches its maximum. After this peak, the curve shows a steep decline. This profile illustrates how ions transfer energy within the tissue and identifies the point of maximum biological effectiveness (Brookhaven National Laboratory, n.d.).

Average range refers to the mean penetration depth of the ions. Average straggling denotes the average lateral deviation of the ions within the tissue. Average vacancy per ion indicates the average number of atomic vacancies (defects) created by each ion. Bragg peaks represent the points of highest energy loss on the LET curve. For energies between 170 and 180 MeV, the average range increases by approximately 1.09% for each 1 MeV increment. Between 180 and 210 MeV, the average range increases by approximately 1.95% for every 2 MeV increment.

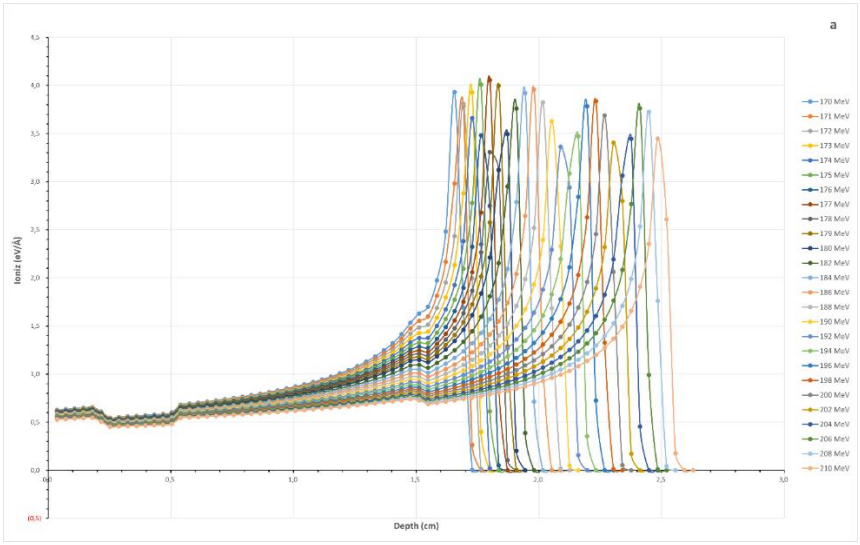


Figure 10. Bragg curves representing the energy deposition profiles of helium ions within the 170–180 MeV energy range.

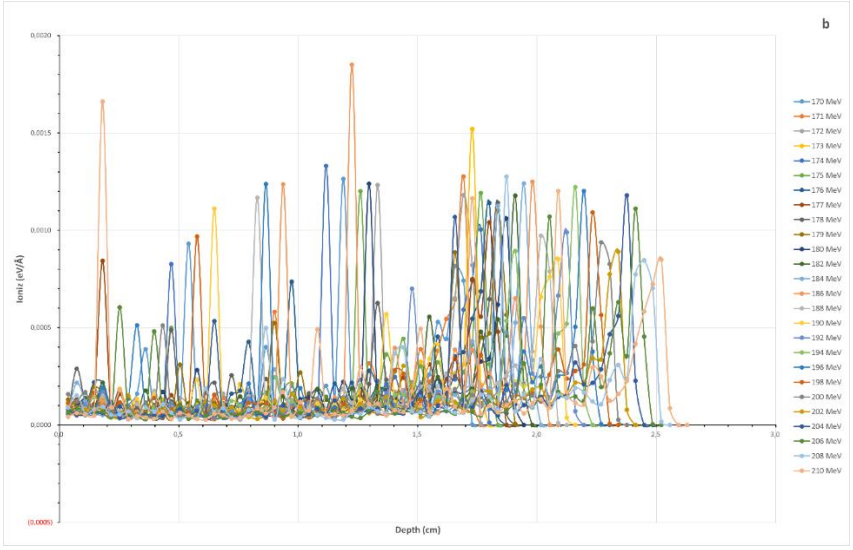
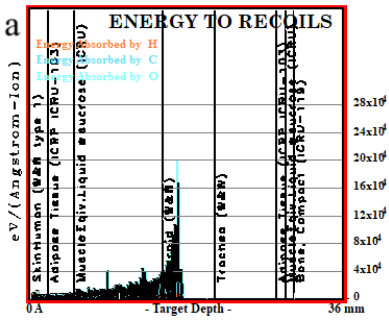


Figure 11. Recoil energy distributions of helium ions within the 170–180 MeV energy range, illustrating the variations in energy transferred to the target atoms during collisions.

When the initial energy was increased from 171 MeV to 180 MeV, a noticeable upward trend was observed in the displacement damage profiles (DPA – Displacements Per Atom) induced by particles penetrating the tissue. This indicates that as the energy increases, particles interact more strongly with the material, thereby gaining a greater potential to displace atoms from their original positions.

Table 1. Summary of the minimum and maximum recoil (scattering) energies corresponding to each initial ion energy, along with the respective depths at which maximum recoil energy was observed in the simulated tissue model.

Initial Energy (MeV)	**Recoil Energy Range (eV Å^{-1})**	**Mode (eV Å^{-1})**	**Depth at Which the Mode Occurs (mm)**
171	2.29×10^{-7}- 3.52×10^{-3}	3.52×10^{-3}	16.92
172	2.69×10^{-5}- 2.57×10^{-3}	2.57×10^{-3}	17.28
173	2.79×10^{-7}- 3.72×10^{-3}	3.72×10^{-3}	17.28
174	4.78×10^{-5}- 2.94×10^{-3}	2.94×10^{-3}	17.64
175	1.04×10^{-6}- 3.22×10^{-3}	3.22×10^{-3}	17.64
176	8.82×10^{-5}- 3.24×10^{-3}	3.24×10^{-3}	18.00
177	1.20×10^{-6}- 2.85×10^{-3}	2.85×10^{-3}	18.00
178	8.63×10^{-5}- 3.33×10^{-3}	3.33×10^{-3}	18.36
179	1.33×10^{-5}- 2.77×10^{-3}	2.77×10^{-3}	18.36
180	8.74×10^{-5}- 3.27×10^{-3}	3.27×10^{-3}	18.72


a
ENERGY TO RECOILS
eV/(Angstrom-Ion)
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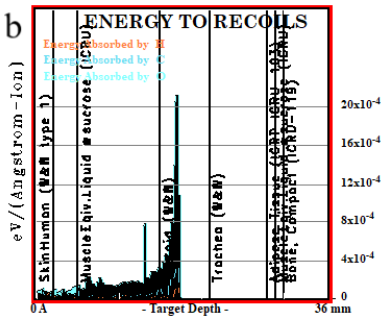
- Target Depth -
36 mm


b
ENERGY TO RECOILS
eV/(Angstrom-Ion)
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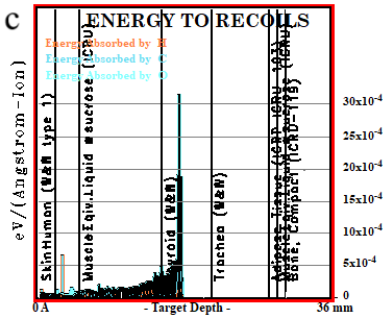
- Target Depth -
36 mm


c
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eV/(Angstrom-Ion)
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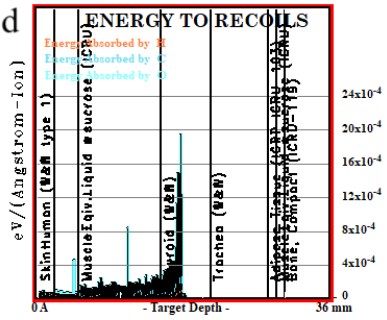
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ENERGY TO RECOILS
eV/(Angstrom-Ion)
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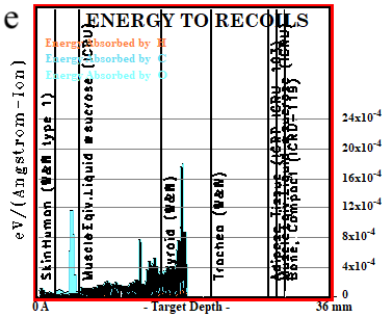
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eV/(Angstrom-Ion)
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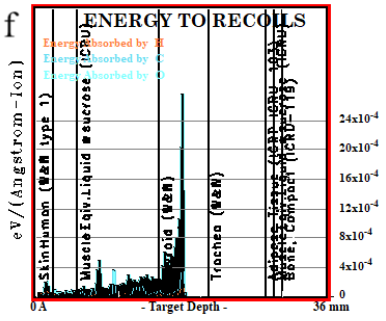
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ENERGY TO RECOILS
eV/(Angstrom-Ion)
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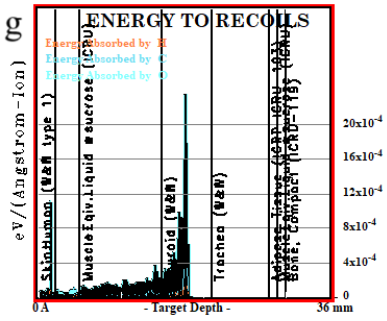
36 mm


g
ENERGY TO RECOILS
eV/(Angstrom-Ion)
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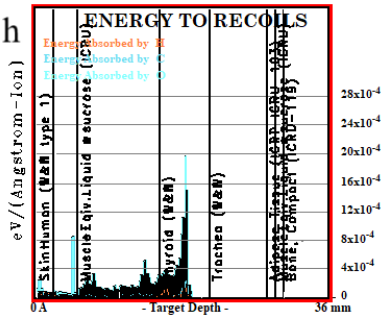
36 mm


h
ENERGY TO RECOILS
eV/(Angstrom-Ion)
0 A
- Target Depth -
36 mm

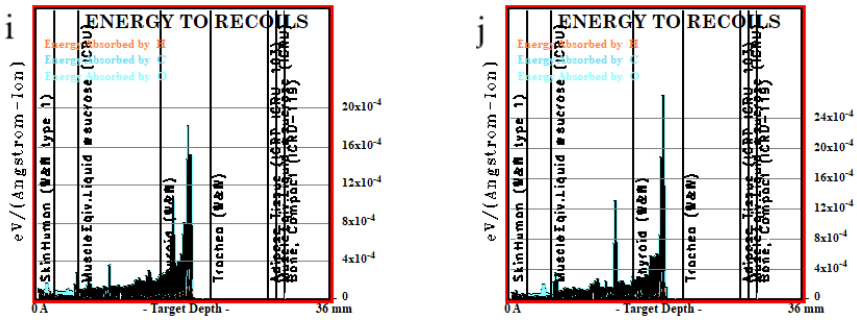


Figure 12. Recoil energy distribution graphs for recoil atoms are presented. Subfigures (a)–(j) visually illustrate the recoil energy distributions corresponding to each energy level from 171 MeV to 180 MeV, respectively. The horizontal axis represents depth (mm), while the vertical axis shows recoil energy (eV $Å^{-1}$).

According to Table 2, the depth at which maximum recoil energy occurs shows a gradual increase with rising initial energy levels from 171 MeV to 180 MeV. Specifically, the depth increases from approximately 16.9 mm at the lowest energy to about 18.7 mm at the highest energy level. The mode values of recoil energy exhibit slight fluctuations at intermediate energy levels, reaching the highest density within that range, while showing a mild decrease at both the lower and upper ends of the energy spectrum.

Table 2. Analysis of radiation-induced damage based on Displacements Per Atom (DPA) profiles for varying initial helium ion energies.

Initial Energy (MeV)	DPA Range (atom/atom)	Maximum DPA (atom/atom)	Depth at Which Maximum Occurs (mm)
171	$0–5.04\times10^{-7}$	5.04×10^{-7}	169.2
172	$0–3.93\times10^{-7}$	3.93×10^{-7}	172.8
173	$0–4.93\times10^{-7}$	4.93×10^{-7}	172.8
174	$0–4.34\times10^{-7}$	4.34×10^{-7}	176.4
175	$0–4.54\times10^{-7}$	4.54×10^{-7}	176.4
176	$0–4.70\times10^{-7}$	4.70×10^{-7}	180.0
177	$0–4.06\times10^{-7}$	4.06×10^{-7}	180.0
178	$0–4.91\times10^{-7}$	4.91×10^{-7}	183.6
179	$0–3.54\times10^{-7}$	3.54×10^{-7}	183.6
180	$0–4.95\times10^{-7}$	4.95×10^{-7}	187.2

Although the value observed at 180 MeV is very close to that at 171 MeV, the overall trend—despite intermediate fluctuations—indicates that higher initial energies can lead to higher DPA (Displacements Per Atom) values. A notable observation is that at certain energies (e.g., 172, 177, and 179 MeV), the maximum DPA values are relatively low. This suggests that particle interactions depend not only on energy, but also on parameters such as the density, composition, and nature of the interaction medium.

This finding demonstrates that high-energy particles can penetrate deeper into tissue and produce maximum damage at greater depths. Such information is critically important in applications like radiation therapy or materials testing, where the beam energy must be adjusted according to the desired penetration depth.

As energy increases, both a general rise in maximum DPA values and a shift of the damage peak to deeper regions are observed. These data reveal that the damage potential of radiation within tissue is directly correlated with energy, and that specific energy ranges may be more suitable for achieving effective or safer

dosing. Moreover, variations in DPA profiles represent important physical insights that should be considered in material selection, shielding calculations, and treatment planning.

4. DISCUSSION

In this study, the physical interactions of helium ions accelerated within the 170–210 MeV energy range with thyroid and surrounding tissues were modeled using SRIM/TRIM simulations. Key parameters such as energy loss, ion range, Bragg peak profile, and lateral scattering were thoroughly analyzed. According to the Displacements Per Atom (DPA) analysis, as the initial energy increased, the maximum DPA values ranged from 5.04×10^{-7} to 4.95×10^{-7}, occurring at depths between 169.2 mm and 187.2 mm. These results indicate that helium ions are capable of delivering energy effectively to deep tissues in a controlled manner. Within the selected energy range, each increase in ion energy resulted in a range extension of approximately 1.09% to 1.95%, directly influencing the depth of energy deposition in the target tissue. Accurate modeling of energy loss is therefore critical in maximizing therapeutic effects on cancerous tissue while minimizing damage to surrounding healthy structures, ultimately improving treatment success (Krishnan & Ranjith, 2023). Most existing studies in the literature use water phantoms or soft tissue-equivalent biomaterials, which often fall short in accurately reflecting tissue-specific energy interactions (Ekinci et al., 2023; Mahalesh & Kumar, 2015). Our study addresses this limitation by modeling thyroid tissue based on its actual compositional properties.

The simulation results demonstrate that helium ions, due to their sharp Bragg peak and limited lateral scattering, have high therapeutic potential for thyroid cancer by reducing the risk of damage to critical organs adjacent to the thyroid (Petersson et al., 2019). Compared to protons, helium ions exhibit reduced lateral scattering, and compared to carbon ions, they offer a more homogeneous LET profile. While carbon ions are effective, their application in clinical settings is limited by high infrastructure costs (Mohamad et al., 2017). In contrast, helium ions provide therapeutic precision with moderate LET values and stand out as a more accessible alternative due to lower infrastructure requirements (Krämer et al., 2016; Tommasino & Durante, 2015). Regarding dose conformity, helium ions have shown high compatibility with FLUKA Monte Carlo calculations, with deviations of less than 1% in dose distributions (Mein et al., 2018). In one study, patients treated with EBRT reported severe acute toxicities (e.g., dermatitis, dysphagia), whereas proton therapy was associated with a lower toxicity profile (Jeans et al., 2022; Moreno et al., 2019; van den End et al., 2025). This is particularly important for thyroid cancer patients in reducing quality-of-life issues arising from long-term treatments (Winter et al., 2024). Indeed, the

National Cancer Institute (NCI) notes that particle therapies tend to provide better long-term quality-of-life outcomes.

Current simulation studies predominantly focus on proton and carbon ion therapies targeting tumors in organs such as the liver, lung, brain, and prostate. However, ion-based simulations targeting small but vital organs like the thyroid remain relatively scarce. One study reported that increasing ion mass enhances recoil effects and alters dose distribution, with recoil contributing up to 69% of the total dose in helium ion therapy (Ekinci et al., 2023) The high vascularization and small volume of the thyroid demand high precision in dose calculations, and minimizing scattering into adjacent tissues is critical for clinical success (Takao et al., 2019). LET profiles generated by SRIM/TRIM show that ions gradually lose energy through tissue and reach peak biological effectiveness at the Bragg peak. Geant4-DNA also accurately estimates Bragg peak positions while offering higher-resolution modeling of molecular energy distribution and DNA damage in high-LET regions (Incerti et al., 2009). FLUKA, although less detailed at the microscopic level than SRIM/TRIM, provides accurate LET curves through its comprehensive macroscopic modeling (Battistoni et al., 2007). Fast Monte Carlo simulations such as MonteRay also show high consistency with SRIM/TRIM data in the 170–210 MeV range, with 1.09%–1.95% increases in ion range, offering sufficient accuracy for clinical applications (Krämer & Scholz, 2000). The energy-dependent increase in average ion range is clearly observed in SRIM/TRIM data, indicating deeper tissue penetration with increasing energy; this trend is generally consistent with results from Geant4-DNA and FLUKA (Battistoni et al., 2007; Incerti et al., 2009). Geant4 models this increase in a nonlinear but stable manner (Paganetti, 2012), while FLUKA may show minor variations in range due to factors such as secondary particle production (Battistoni et al., 2007). Regarding average straggling, SRIM/TRIM provides detailed statistical distributions of energy spread, and Geant4-DNA and FLUKA exhibit similar trends, supporting the reliability of these tools for treatment planning (Battistoni et al., 2007; Paganetti, 2012).

5. CONCLUSION

This study models the physical interactions of helium ions accelerated within the 170–210 MeV energy range in thyroid tissue and surrounding anatomical structures using SRIM/TRIM simulations. The developed tissue model is based on real anatomical and histological parameters, incorporating structures such as skin, adipose tissue, muscle, trachea, and bone to construct a clinically realistic geometry. As a result, the simulations generated highly reliable data critical for radiotherapy applications, including ion energy loss (Bragg peak), range distribution, lateral scattering, and microscopic damage (DPA and recoil).

Simulation results revealed that the penetration range of helium ions increases with energy by approximately 1.09% to 1.95%, the Bragg peak can be precisely positioned within the thyroid tissue, and adjacent healthy tissues can be effectively spared. Furthermore, the low degree of lateral scattering and the homogeneity of the linear energy transfer (LET) profile enable accurate and safe dose distributions, particularly for thyroid tumors located near vital structures such as the trachea and esophagus. Recoil energy curves and DPA analyses demonstrated that the extent of microscopic structural damage is energy-dependent, with maximum DPA occurring at depths ranging from 169.2 mm to 187.2 mm for energies between 171 and 180 MeV.

These findings suggest that helium ions provide a sharper and more conformal dose distribution compared to protons, and a more cost-effective treatment alternative compared to carbon ions, while still maintaining favorable biological effectiveness (Mohamad et al., 2017; Tommasino & Durante, 2015; Krämer et al., 2016**).** The consistency of SRIM/TRIM simulation outputs with those of other Monte Carlo frameworks such as Geant4-DNA and FLUKA further supports the reliability and clinical relevance of this approach (Battistoni et al., 2007; Incerti et al., 2009; Krämer & Scholz, 2000).

In conclusion, this research demonstrates that helium ions represent a powerful and feasible therapeutic option for thyroid cancer. Due to their favorable physical and biological properties, helium ions offer the potential to maximize therapeutic outcomes while minimizing collateral damage to healthy tissue, particularly in small and highly vascularized organs like the thyroid (Petersson et al., 2019; Takao et al., 2019). The data generated herein provide a solid theoretical foundation for future clinical investigations and may serve as a basis for integration into AI-assisted treatment planning systems, facilitating personalized and patient-specific therapies. In this respect, the study holds not only scientific value but also transformative potential for clinical practice.

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