EDITORS
**PROF. DR. ENGİN ŞAHNA**
**PROF. DR. HASAN AKGÜL**

# International Perspectives on Contemporary Approaches in HEALTH SCIENCES

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SERÜVEN YAYINEVİ

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# INTERNATIONAL PERSPECTIVES ON CONTEMPORARY APPROACHES IN HEALTH SCIENCES

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**İÇINDEKILER**

**BÖLÜM 1**

THE POTENTIAL ROLE OF OXIDATIVE STRESS AND NEUROINFLAMMATION IN MORPHINE TOLERANCE ..... 1

***Ercan ÖZDEMİR***

**BÖLÜM 2**

MEDICAL HIERARCHY AND PATIENT SAFETY ..... 21

***Mevlut KARADAG***

**BÖLÜM 3**

CRISPR/CAS GENOME EDITING FROM EARLY EXPECTATIONS TO CLINICAL REALITY AND BEYOND ..... 47

***Neslihan Pinar OZATES***

***Cigir BIRAY AVCI***

**BÖLÜM 4**

MATERNAL NUTRITION AND EPIGENETICS ..... 73

***Şule ARSLAN***

***NOOR MANSOOR HASAN ABDULLAH ALSUBARI***

**BÖLÜM 5**

MICROFLUIDICS, HIGH-SPEED IMAGING AND ARTIFICIAL INTELLIGENCE IN DIGITAL HEMORHEOLOGY ..... 97

***Ahmet Alperen SUBAŞI***

***Nurten BAHTİYAR***

***Birsen AYDEMİR***

**BÖLÜM 6**
AN OVERVIEW OF ANALYTICAL METHODS USED IN CELL CULTURE ..... 119
***SALİH ERAT***

**BÖLÜM 7**
THERAPEUTİC POTENTIAL OF ALKALOID-CONTAINING PLANTS IN NEURODEGENERATİVE DİSEASES..... 137
***Nouralyakin MANAFİKHİ***
***Beyza Nur YILMAZ***
***Duygu TAŞKIN***
***Turgut TAŞKIN***

Chapter 1

# THE POTENTIAL ROLE OF OXIDATIVE STRESS AND NEUROINFLAMMATION IN MORPHINE TOLERANCE

*Ercan ÖZDEMİR*[1]

1 Dr., Department of Physiology, Faculty of Medicine, Sivas Cumhuriyet University, eozdemir@cumhuriyet.edu.tr., ercan_ozdemir@hotmail.com, ORCID: 0000-0001-8231-1053

## Introduction

Morphine is a widely used analgesic drug for various severe and chronic pains, such as cancer, due to its potent analgesic effect and rapid relief. However, long-term chronic use of morphine can lead to dependence and tolerance development, along with the potential for some side effects (Ozdemir et al. 2022; Nicola et al. 2026; Liu et al. 2026). Morphine dependence is characterized by neuronal adaptations that occur as a result of chronic morphine exposure. Discontinuation of morphine use or administration of antagonists such as naloxone leads to withdrawal symptoms (Khan et al. 2018; Javadi et al. 2013). Common withdrawal symptoms include cough, loss of appetite, diarrhea, abdominal pain, runny nose, and anxiety (Harris and Gewirtz 2005; Evans and Cahill 2016). In physiological terms, these symptoms significantly reduce a person's quality of life and sometimes cause memory impairment. Experimental studies of morphine dependence in animals have shown withdrawal symptoms such as diarrhea, rearing, jumping, teeth grinding, and trembling in the front paws (Listos et al. 2013; Nicoara et al. 2016). Besides physical dependence, another side effect of chronic morphine use is the development of tolerance to its analgesic effects.

Morphine tolerance is a physiological state characterized by a decrease in analgesic response after repeated, long-term use of the drug. It often necessitates increasing the dose to achieve the desired analgesic effect (Ozdemir et al. 2023; Avci et al. 2022). The analgesic efficacy of morphine is frequently inhibited by side effects such as dependence and tolerance (Wang et al. 2021; Baser et al. 2021). While the analgesic and sedative effects of morphine deteriorate with increased doses, there is an increase in undesirable side effects such as nausea and respiratory depression. Morphine tolerance can occur in three different ways: pharmacodynamic, pharmacokinetic, and learned tolerance. Pharmacokinetic tolerance is tolerance that arises from any changes in the distribution and metabolism of opioids, whether genetically based or resulting from long-term use (Ozdemir et al. 2023; Ozturk et al. 2025). Pharmacodynamic tolerance refers to mechanistic and neuroadaptive changes at the cellular level. In addition, it includes significant changes such as a decrease in receptor number and response due to desensitization. Morphine tolerance can also develop through an overgrowth of intracellular secondary messengers such as nitric oxide synthase (NOS), cyclic guanosine monophosphate (cGMP), and cyclic adenosine monophosphate (cAMP), leading to changes in free intracellular Ca2+ concentration (Khan et al. 2017). Learned tolerance is a reduction in drug effect due to learned feedback mechanisms (Mitra et al. 2004). Behavioral tests such as the tail-dipping test and the hot plate test are frequently used to investigate the effect of drugs on tolerance in animal models of morphine tolerance (Kotlinska et al. 2013; Celerier et al. 2003).

In clinical settings, tolerance and dependence may develop following chronic morphine use, leading to addictive behavior in individuals during the discontinuation process. Uncovering the underlying mechanisms for the development of such conditions is crucial for developing new drug candidates. Therefore, the aim of this review is to elucidate the molecular mechanisms associated with the development and expression of morphine tolerance.

**Opioid receptors**

Morfin bir alkaloiddir ve 7-transmembran G protein bağlı reseptörler üzerinden etki gösterir. Opioid reseptörleri, vücut genelinde merkezi ve periferik sinir sistemlerinde geniş ölçüde dağılım gösterir (Che et al. 2023; Reeves et al. 2022). Opioid reseptörleri aynı zamanda solunum yollarında ve gastrointestinal kanalda tespit edilir ve buradaki reseptörler hareketliliği ve salgıların düzenlenmesini sağlar (Listos et al. 2019; Fan et al. 2024; Lin et al. 2026). Opioid reseptörler beş ana tipe ayrılır: Mu opioid reseptörleri (MOR), kappa opioid reseptörleri (KOR), delta opioid reseptörleri (DOR), Nociceptin/Orphanin FQ (NOP) reseptörleri (NOR) ve zeta (ZOR) reseptörleri (Stein et al. 2003) (Table 1).

MOR receptors are primarily responsible for analgesic effects, respiratory depression, constipation, euphoria, dependence, vasodilation, and effects associated with miosis. MOR agonists increase pyloric muscle tone, inhibit gastric emptying, and cause constipation by delaying intestinal transit. Morphine acts on the respiratory center in the brainstem, causing respiratory depression (James and Williams 2020; Ozdemir 2020). KOR receptors are found in the central and peripheral nervous systems and are responsible for diuresis, dysphoria, and analgesic effects. DOR receptors are similarly concentrated in the brain and spinal cord and produce analgesic effects. Another opioid receptor, NOR receptors, produce analgesia or hyperalgesia. NOP receptors exhibit binding properties to the endogenous peptide nociceptin/orphanin (James and Williams 2020; Cox et al. 2015).

MOR agonists exhibit a wide variety of receptor pharmacodynamics. The nature of opioid signaling allows different agonists to elicit diverse responses. This is further complicated by ligand-dependent signaling events such as modulation by arrestin 1 and 2 (Liu et al. 2026). Ligand-dependent signaling of MOR agonists is a frequently studied research topic. One such example is the opioid agonist fentanyl. Fentanyl has a high affinity for binding to MOR and exerts its analgesic effect through activation of G protein signaling (Zhuang et al. 2024).

| Receptor | Subtypes | G protein subunit | Function | Location |
|---|---|---|---|---|
| mu (μ)<br>MOR | $\mu_1$, $\mu_2$, $\mu_3$ | Gi | **$\mu_1$:**<br>analgesia<br>physical dependence<br>**$\mu_2$:**<br>respiratory depression<br>miosis<br>euphoria<br>reduced GI motility<br>**$\mu_3$:**<br>possible vasodilation | brain<br>spinal cord<br>substantia gelatinosa<br>peripheral sensory neurons<br>intestinal tract |
| delta (δ)<br>DOR | $\delta_1$, $\delta_2$ | Gi | analgesia<br>antidepressant effects<br>convulsant effects<br>dependence<br>respiratory depression | brain<br>peripheral sensory neurons |
| kappa (κ)<br>KOR | $\kappa_1$, $\kappa_2$, $\kappa_3$ | Gi | analgesia<br>anticonvulsant effects<br>depression<br>dissociative/<br>hallucinogenic effects<br>miosis<br>dysphoria<br>sedation | • brain<br>• hypothalamus<br>• periaqueductal grey<br>• spinal cord<br>• substantia gelatinosa<br>• peripheral sensory neurons |
| nociceptin receptor<br>NOR | ORL1 | Gi | • anxiety<br>• depression<br>• appetite<br>• development of tolerance to μ-opioid agonists | • brain<br>• amygdala<br>• hippocampus<br>• hypothalamus<br>• spinal cord |
| zeta (ζ)<br>ZOR | | Gi | • tissue growth<br>• embryonic development<br>• regulation of cancer cell proliferation | • heart<br>• liver<br>• skeletal muscle<br>• kidney<br>• brain<br>• pancreas<br>• fetal tissue |

**Table 1.** *Classification of opioid receptors (Stein et al. 2003)*

Fentanyl has a higher usage rate compared to morphine. This is because fentanyl has been shown to activate β-arrestin-2 less than morphine, which increases its analgesic potency. It also results in fewer side effects such as constipation and addiction (Raehal and Bohn 2011). Endogenous opioids, known as endomorphines, also exhibit interesting signaling properties. They show high selectivity for MOR and produce strong analgesic effects by stimulating the G protein pathway. However, it has been suggested that the endogenous peptide β-endorphin, similar to fentanyl, enhances analgesia through MOR-mediated signaling by reducing the tendency to attract

β-arrestin-2 (Pradhan et al. 2011). Buprenorphine, a partial MOR agonist, has been the subject of extensive research due to its diverse signaling properties. Its partial agonist effect on MOR receptors reduces respiratory depression, improving its safety profile (Khroyan et al. 2015). Furthermore, buprenorphine has been shown to prefer G protein pathways over β-arrestin pathways (Raehal and Bohn 2011). Additionally, various herbal products, such as the alkaloid mitragynin derived from Mitragyna speciosa, have been reported to exhibit strong interactions with MOR. Mitragynin acts as a partial MOR agonist and may also interact with other receptor systems. These characteristics suggest its potential as a novel drug and analgesic agent (Krugel et al. 2016).

**Molecular mechanisms involved in morphine tolerance**

**Receptor desensitization**

Desensitization is the decrease in analgesic response that occurs after prolonged, repeated use of opioids and agonists due to the internalization of receptors or inhibition of MOR receptor activity (Swingler et al. 2025). Repeated doses of morphine reduce cyclic adenosine monophosphate (cAMP) levels and alter the permeability of calcium or potassium ion channels, leading to hyperpolarization and thus a decrease in analgesic effect (Berger and Whistler 2010). The transport of opioid receptors such as MOR is a crucial factor in the development of morphine tolerance. Chronic exposure to morphine has been reported to increase receptor transport, resulting in a decrease in the number of membrane receptors in the cell and consequently the development of morphine tolerance (Williams et al. 2015). Internalization of receptors plays a role in the development of morphine tolerance. However, recent studies have shown that internalization of MORs has a weak effect on the development of tolerance to opioids. Furthermore, morphine tolerance may also involve desensitization to MORs (Allouche et al. 2014).

In morphine tolerance, calcium and potassium ion channels are effective in rapid desensitization. In contrast, enzyme activity is important in chronic desensitization. Increased adenylate cyclase activity and its effect on cAMP response element binding protein (CREB) raise cAMP levels. MOR desensitization may also be linked to dysregulation of the cytoplasmic proteins β-arrestin-1 and β-arrestin-2 (Bohn et al. 2000). β-arrestins bind to MOR receptors and activate serine/threonine kinase, thereby altering their phosphorylation. Signal transduction in the cell is inhibited by β-arrestin activation, and desensitization occurs at the receptor (Al-Hasani et al. 2011). In addition, activation of mitogen-activated protein kinases (MAPKs) may also contribute to opioid receptor desensitization. Long-term morphine use is reported to have a strong effect on extracellular signaling regulatory kinases (ERK1/2) (Nabemoto et al. 2008). Recent evidence suggests that changes

in the gut microbiota also play a role in morphine tolerance (Ghosh et al. 2022). Chronic morphine exposure causes changes in the composition of the gut microbiota, which in turn affects the metabolism of the drug. Tolerance development to other opioid analgesics besides morphine does not follow the same mechanisms as with morphine. Following exposure to fentanyl, a potent and rapid internalization of the MOR leads to downstream signaling and tolerance mechanisms (Anselmi et al. 2013). Opioids with high efficacy, such as fentanyl and etorphine, cause desensitization and internalization of the opioid receptor via β-arrestin proteins, leading to opioid tolerance (Zuo et al. 2005). Furthermore, evidence suggests that fentanyl activates β-arrestin-2 to a lesser extent than morphine. This results in increased analgesic efficacy and significantly reduced side effects (Fan et al. 2003).

**Role of oxidative stress**

Chronic use of morphine can have harmful effects on cells by promoting free radical formation and disrupting the cell's antioxidant defense system. Evidence shows that morphine causes a significant decrease in glutathione (GSH) levels (Skrabalova et al. 2013). Long-term morphine administration to patients with chronic pain resulted in an acute decrease in GSH levels in cerebrospinal fluid (Goudas et al. 1999). Furthermore, an experimental study demonstrated that chronic morphine treatment reduced GSH levels in mouse brain tissue (Abdel-Zaher et al. 2013). In addition, long-term morphine use causes significant changes in glutathione peroxidase (GSHPx), catalase (CAT), and superoxide dismutase (SOD) activities (Skrabalova et al. 2013).

Peroxynitrite ($ONOO^-$) is a potent reactive nitrogen and oxidant molecule effective in opioid-induced analgesia, resulting from the combination of nitric oxide (NO) and superoxide (O2-) produced by immune system cells (Bounds et al. 2024). Nitric oxide synthase (NOS), NADPH-oxidase, and spinal manganese superoxide dismutase (MnSOD) also participate in peroxynitrite formation. Activation of the immune response and the release of interleukin 6, TNF-α, interleukin 1β, as well as the secretion of pro-inflammatory cytokines such as N-methyl-D-aspartate (NMDA) receptors, also play a role in peroxynitrite formation. Peroxynitrite formation enhances the development of morphine tolerance (Salvemini et al. 2009). Furthermore, peroxynitrite is essential for the activation of the NF-κB pathway and indirectly inhibits mitochondrial respiration. Activation of NOS leads to the production of NO and superoxide, while inactivation of MnSOD promotes proxinitrite formation. Evidence suggests that NOS inhibition reduces morphine-induced analgesic tolerance (Skrabalova et al. 2013). Studies demonstrating the role of oxidative stress and neuroinflammation in morphine tolerance are shown in Table 2.

**Table 1.** *The role of oxidative stress and neuroinflammation in morphine tolerance*

| Drugs | Model | Mechanisms | Findings | References |
|---|---|---|---|---|
| NMDA receptor antagonist MK-801 | Rats | Inhibition of NMDA receptor | Reduces morphine tolerance | Trujillo and Akil 1991 |
| D-NAME, L-NAME, L-NMMA | Male Swiss albino mice | Inhibition of NO pathway | Attenuates morphine tolerance and dependence | Dambisya and Lee 1996 |
| L-NAME | Male Wistar albino rats | Inhibition of NO-cGMP pathway | L-NAME reduces the development of tolerance to morphine | Ozdemir et al. 2011 |
| None | Mice C57BL/6 | Modulation of NO/CO-cGMP pathway | The CO/NO-cGMP pathway is up regulated | Liang and Clark 2004 |
| Pioglitazone | C57 mices, KO mice PPARγ deletion | Activation PPAR-γ | Reduces morphine tolerance | de Guglielmo et al. 2014 |
| Suvorexant | Adult male Razi mice | NMDA, AMPA and CREB proteins | Suvorexant alleviates morphine induced tolerance | Esmaili et al. 2021 |
| SB612111 a NOP receptor blocker | Female C57BL/6 J mice | Nociceptin opioid peptide (NOP) receptor | SB612111 reduces morphine induced tolerance | Hao et al. 2021 |
| SR-17018 | C57BL6 mice | SR-17018 exhibits strong binding to morphine G protein. | Reduces morphine tolerance | Grim et al. 2020 |
| Thalidomide | Male NMRI mice | Nitric oxide pathway T98G human glioblastoma cell | Inhibition of NOS Attenuates morphine tolerance | Khan et al. 2017 |
| Bupropion | Male Balb-c mice | Suppression of glutamate, norepinephrine, inflammatory and oxidative status, | Reduces morphine tolerance | Hamdy et al. 2018 |
| Mesoporous polydopamine (MPDA) nanoparticles | Male Wistar rats | Increases endogenous antioxidants and inhibition of inflammation | Attenuates morphine tolerance | Kuthati et al. 2021 |
| Zingerone | Adult male NMRI mice | Suppressing oxidative stress and NLRP3 inflammasome activation | Alleviates morphine tolerance | Molavinia et al. 2024 |
| Amiloride | Adult male C57/BL6 mice | Suppressing microglial activation and inhibits NF-κB expression. | Attenuates morphine tolerance | Gei et al. 2024 |
| T cell immunoglobulin domain and mucin domain-3 (TIM3) | C57BL/6 male and female mice | Microglial neuroinflammation by suppressing the TRAF6/NF-κB pathway | TIM3 attenuates morphine antinociceptive tolerance | Yang et al. 2025 |
| Pinocembrin | Male C57BL/6 mice | Inhibition of STAT3 mediated microglia activation and neuroinflammation. | Pinocembrin alleviates chronic morphine-induced analgesic tolerance and hyperalgesia | Han et al. 2022 |

| | | | | |
|---|---|---|---|---|
| Caveolin-1 Scaffolding Domain Peptides | Sprague–Dawley rats | Inhibits NLRP3 Inflammasome and ERK/c-JUN Pathway | Attenuates morphine antinociceptive tolerance | Liu et al. 2022 |
| IIK7 (melatonin type 2 receptor agonist | Wistar rats | Restoring Nrf2 and HO-1 | IIK7 Attenuates and Reverses Morphine Tolerance in Neuropathic Pain | Kuthati et al. 2024 |

Nitric oxide (NO) is a gaseous molecule synthesized from L-arginine by the action of NOS. Also a neurotransmitter, NO plays important roles in signal transmission between neurons, glial cells, and cerebral blood vessels. Three isoforms of the NOS enzyme have been isolated: iNOS (inducible nitric oxide synthase), nNOS (neuronal nitric oxide synthase), and eNOS (endothelial nitric oxide synthase) (Serreli et al. 2023). Accumulated evidence suggests that changes in NO levels are associated with some neurodegenerative diseases. Degenerative processes occurring in the brain induce glutamate release, leading to increased levels of NO via the nNOS pathway. NO activates protein kinase G (PKG) by increasing cGMP levels via the guanylyl cyclase enzyme and also leads to phosphorylation of synaptophysin. This leads to enhanced glutamatergic signaling (Dzoljic et al. 2015). Recent studies have focused on the effects of NOS inhibitors and NO precursor on the development of morphine tolerance. Morphine tolerance has two distinct phases: induction and expression. The mechanisms underlying morphine tolerance are schematized in Figure 1.

Experimental studies show that morphine tolerance is reduced in animals with NOS deficiency (Dambisya and Lee 1996). Furthermore, administration of L-NAME (an NOS inhibitor) has been shown to reduce the development of morphine tolerance in animals using the hot plate test (Hosseinzadeh et al. 2012). The role of cGMP in the development of morphine-induced analgesic tolerance has been found to be closely related to NO levels (Ozdemir et al. 2011). Activation of the NO-cGMP pathway has been found to cause abnormal excitation in neurons in the brain, which is associated with severe pain. Administration of L-arginine can cause chronic pain by activating the NO-cGMP pathway (Liang and Clark 2004). In addition, zingerone, a phenolic compound, has been shown to reduce morphine tolerance by suppressing oxidative stress and NLRP3 inflammatory activation (Molavinia et al. 2024).

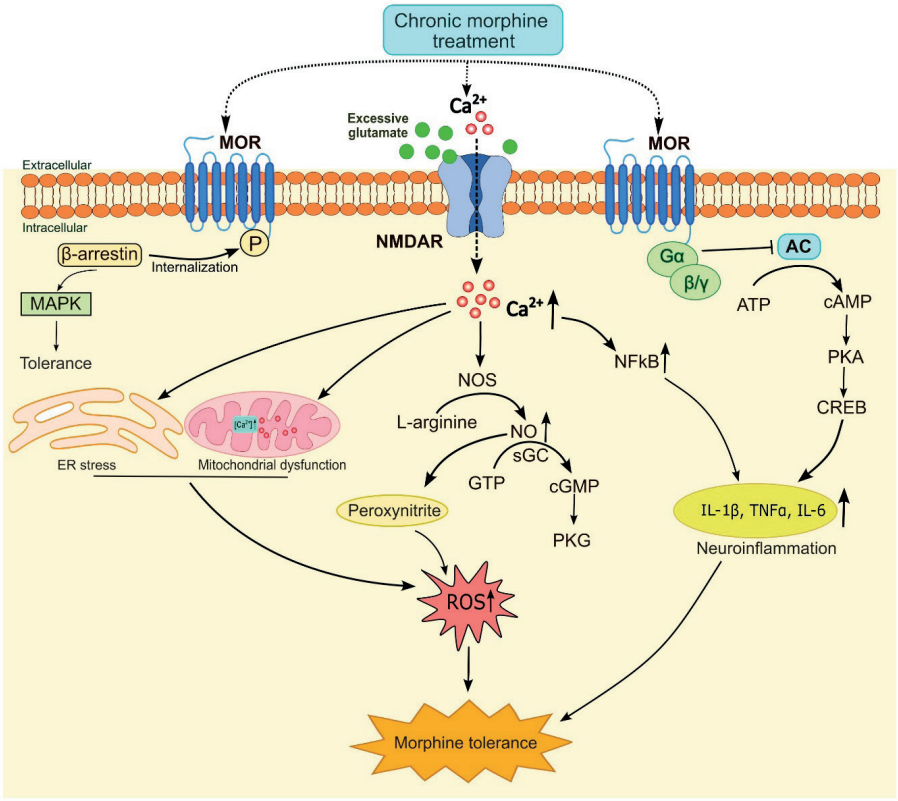



**Figure 1.** Schematic diagram illustrating multiple signaling pathways regulating morphine-induced tolerance. Chronic morphine exposure phosphorylates opioid receptors, leading to inhibition of adenylate cyclase activity and ultimately a decrease in protein kinase A (PKA). NMDA receptor (NMDAR) activation leads to an increase in calcium levels in the postsynaptic neuron. This results in NO formation via calcium nitric oxide synthase (NOS) activation. NO activates soluble guanylate cyclase (GC) and increases cGMP formation. Excess NO produced can damage mitochondria via nitrosation, leading to increased mitochondrial ROS levels. μ-opioid receptors (MOR) also activate the MAPK signaling cascade, leading to NF-kB activation. Through the activation of the NF-kB pathway, morphine causes oxidative stress via its effect on ROS and TNFα pathways.

### Role of neuroinflammation

Accumulated evidence suggests that neuroinflammation plays a critical role in morphine analgesic tolerance (Grace et al. 2016; Shi et al. 2025). Chronic morphine use induces morphine tolerance by causing a significant increase in the concentration of pro-inflammatory cytokines interleukin-1beta (IL-1β), interleukin-6 (IL-6), and interleukin-10 (IL-10), and tumor necrosis factor

alpha (TNF-α) (Mansouri et al. 2018). Long-term administration of morphine activates glial cells, leading to the release of pro-inflammatory cytokines and a neuroinflammatory response (Wu et al. 2025; Cui et al. 2026). The released pro-inflammatory mediators alter neuronal excitability, reducing the analgesic efficacy of opioids and leading to tolerance. Inhibition of microglial TNF-α receptors leads to decreased morphine tolerance (Berta et al. 2012). Simultaneously, suppression of IL-1β in the dorsal root ganglion prolonged the analgesic effect of morphine (Shen et al. 2011). Furthermore, microglial TLR4 regulates TGF-β-activated kinase 1 (TAK1) expression and causes activation of the NOD-like receptor family pyrin domain-containing 3 (NLRP3). This, in turn, induces neuroinflammation, contributing to morphine tolerance (Wang et al. 2021). Evidence suggests that morphine tolerance also involves neuroimmune interactions and activation of toll-like receptor 4 (TLR4) (Watkins et al. 2009). Therefore, morphine can induce morphine tolerance by binding to TLR4, triggering the release of pro-inflammatory cytokines and glutamate (Liu et al. 2011). NLRP3 inflammasome inhibition reduces analgesic tolerance to morphine (Cai et al. 2016), suggesting that NLRP3 inflammasome activation plays a role in morphine tolerance (Liu et al. 2020).

In the pathophysiology of neuronal diseases, oxidative stress and neuroinflammation frequently play roles in the same processes (He et al. 2020). Oxidative stress increases morphine-induced neuroinflammation and subsequently leads to tolerance. For example, curcumin, an antioxidant compound, has been shown to significantly reduce morphine-induced pro-inflammatory cytokine levels (Sheikholeslami et al. 2019). There is considerable evidence that endoplasmic (ER) stress contributes to morphine analgesic tolerance. Experimental studies have reported that ER stress is induced in the frontal cortex, basal ganglia, and occipital cortex (Sil et al. 2018), spinal cord (Liu et al. 2018), and dorsal root ganglia (DRG) (Taskiran and Avci 2021) of animals treated with morphine, leading to morphine tolerance. Morphine-induced ER stress caused damage to cortical neurons and inhibited nerve recovery (Liu et al. 2021). Furthermore, oxidative stress has been shown to have the potential to activate ER stress in vitro (Zeng et al. 2015). Chronically administered morphine can trigger oxidative stress during the development of morphine tolerance, leading to ER stress. ER stress contributes to the development of morphine tolerance due to intracellular cross-interactions between MOR signaling and the unfolded protein response (UPR). While acute low-dose morphine administration produces an analgesic effect due to GSK3β serine 9 phosphorylation, chronic morphine administration suppresses phosphorylation, leading to tolerance (Okuyama et al. 2020). Therefore, targeting ER stress to reduce the development of morphine tolerance may be a meaningful therapeutic strategy. Furthermore, evidence has shown that inhibition of the TRPM2 cation channel in animal

DRG tissue reduces morphine tolerance by preventing ER stress and apoptosis (Ciltas et al. 2025).

Peroxisome proliferator-activated receptor gamma (PPAR-γ) is associated with lipid metabolism, cell death, neural cell differentiation, and neuroinflammation. PPARγ is commonly isolated in the lateral hypothalamic region, controlling dopamine release from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) (Sarruf et al. 2009). PPARγ is a transcription factor essential for cell differentiation and various metabolic processes (Janani and Kumari et al. 2015). Three types of PPAR isotypes are isolated, distributed in most peripheral tissues, neurons in the brain, and glial cells: PPARα, PPARβ, and PPARγ (Ray et al. 2014). Studies on rat spinal cord have shown that pioglitazone reduces morphine tolerance by suppressing neuroinflammation (Charkpour et al. 2015). Morphine tolerance-reducing effects have been demonstrated in studies on rat cerebral cortex (Ghavimi et al. 2015).

NO suppression and the resulting morphological changes in the cell occur as a result of macrophage activation by PPARγ agonists. Therefore, activation of these receptors by PPARγ agonists can cause apoptosis via the NF-kB signaling pathway (Wilson et al. 2000). There is considerable evidence suggesting that PPARγ plays a significant role in the development of morphine tolerance. Experimental studies have shown that pioglitazone reduces morphine tolerance in rats (Ghavimi et al. 2014). Simultaneously, the morphine tolerance-reducing effects of pioglitazone were blocked by GW-9662, a PPAR-γ antagonist. Long-term morphine exposure can lead to activation of glial cells and release of pro-inflammatory cytokines (Liu et al. 2011). Increased pro-inflammatory mediators in neuronal tissue can lead to tolerance by altering excitability and plasticity, thereby reducing the efficacy of morphine.

Depending on the pharmacodynamics of receptor activation, opioids can play both neurodegenerative and neuroprotective roles in the brain. Morphine's neuroprotective effect is achieved by activating the PI3K pathway, which ensures cell survival and prevents apoptosis via Akt. In a later step, NF-kB is activated by phosphorylation of IkB kinase, further enhancing cell survival. Furthermore, NF-kB plays a role in various pathophysiological conditions in the brain, such as brain trauma, stroke, and epilepsy (Rehni et al. 2008). Chronic morphine also increases TNF-α levels, activating macrophages and causing cell proliferation. This, in turn, activates the MAPK signaling cascade by leading to extracellular matrix remodeling and cytokine release. TNF-α also transports NF-kB from astrocytes to the nucleus, leading to increased levels of pro-inflammatory cytokines. Chronic morphine therapy increases oxidative stress in the brain, inducing TNF-α and leading to increased TNF-α

expression in microglia. Additionally, long-term morphine administration phosphorylates p38MAPK, ultimately resulting in the reorganization of TNF-α expression (Sawaya et al. 2009).

It has been shown that activation of tumor necrosis factor-α-induced protein 8-like 2 (TIPE2) in the spinal cord reduces morphine tolerance by decreasing neuronal ROS and suppressing neuronal apoptosis and NF-κB activation (Yang et al. 2023). The mechanisms underlying morphine tolerance are schematized in Figure 1.

**Conclusion and future perspectives**

In conclusion, this review highlights that morphine tolerance is not solely governed by neuronal opioid receptor adaptations; it is significantly triggered by the complex interplay between oxidative stress and neuroinflammation. Chronic morphine administration activates the TLR4 signaling pathway on glial cells, sequentially activating the NF-κB transcription factor. This cascade leads to the overproduction of pro-inflammatory cytokines and reactive oxygen species (ROS), creating a neurotoxic microenvironment that impairs synaptic plasticity and blunts opioid analgesic efficacy. Targeting the TLR4/NF-κB axis and mitigating oxidative stress through antioxidant and anti-inflammatory adjuvant therapies represents a highly promising strategy for preserving the therapeutic benefits of morphine. Ultimately, shifting the therapeutic focus to these non-neuronal mechanisms could prevent dangerous dose escalations, reduce addiction risks, and significantly optimize chronic pain management.

## References

Abdel-Zaher, A. O., Mostafa, M. G., Farghaly, H. S., Hamdy, M. M., & Abdel-Hady, R. H. (2013). Role of oxidative stress and inducible nitric oxide synthase in morphine-induced tolerance and dependence in mice: Effect of alpha-lipoic acid. *Behavioural Brain Research*, *247*, 17–26.

Al-Hasani, R., & Bruchas, M. R. (2011). Molecular mechanisms of opioid receptor-dependent signaling and behavior. *Anesthesiology*, *115*(6), 1363–1381.

Allouche, S., Noble, F., & Marie, N. (2014). Opioid receptor desensitization: Mechanisms and its link to tolerance. *Frontiers in Pharmacology*, *5*, 280.

Anselmi, L., Jaramillo, I., Palacios, M., Huynh J., & Sternini, C. (2013). Ligand-induced μ opioid receptor internalization in enteric neurons following chronic treatment with the opiate fentanyl. *Journal of Neuroscience Research*, *91*(6), 854–860.

Avci, O., Ozdemir, E., Taskiran, A. S., Inan, Z. D. S., & Gursoy, S. (2022). Metformin prevents morphine-induced apoptosis in rats with diabetic neuropathy: A possible mechanism for attenuating morphine tolerance. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *395*(11), 1449–1462.

Baser, T., Ozdemir, E., Filiz, A. K., Taskiran, A. S., & Gursoy, S. (2021). Ghrelin receptor agonist hexarelin attenuates antinociceptive tolerance to morphine in rats. *Canadian Journal of Physiology and Pharmacology*, *99*(5), 461–467.

Berger, A. C., & Whistler, J. L. (2010). How to design an opioid drug that causes reduced tolerance and dependence. *Annals of Neurology*, *67*(5), 559–569.

Berta, T., Liu, T., Liu, Y. C., Xu, Z. Z., & Ji, R. R. (2012). Acute morphine activates satellite glial cells and up-regulates IL-1beta in dorsal root ganglia in mice via matrix metalloprotease-9. *Molecular Pain*, *8*, 18.

Bohn, L. M., Gainetdinov, R. R., Lin, F. T., Lefkowitz, RJ., & Caron, M. G. (2000). μ-Opioid receptor desensitization by β-arrestin-2 determines morphine tolerance but not dependence. *Nature*, *408*(6813), 720–723. https://10.1038/35047086

Bounds, P. L., & Koppenol, W. H. (2024). Peroxynitrite: A tale of two radicals. *Redox Biochemistry and Chemistry*, *10*, 100038.

Cai, Y., Kong, H., Pan, Y. B., Jiang, L., Pan, X. X., Hu, L., ... & Liu, W. T. (2016). Procyanidins alleviates morphine tolerance by inhibiting activation of NLRP3 inflammasome in microglia. *Journal of Neuroinflammation*, *13*(1), 53.

Célérier, E., Yazdi, M. T., Castañé, A., Ghozland, S., Nyberg, F., & Maldonado, R. (2003). Effects of nandrolone on acute morphine responses, tolerance and dependence in mice. *European Journal of Pharmacology*, *465*(1-2), 69–81.

Charkhpour, M., Ghavimi, H., Ghanbarzadeh, S., Yousefi, B., Khorrami, A., Mesgari, M., & Hassanzadeh, K. (2015). Protective effect of pioglitazone on morphine-induced neuroinflammation in the rat lumbar spinal cord. *Journal of Biomedical Science*, *22*, 99.

Che, T., & Roth, B. L. (2023). Molecular basis of opioid receptor signaling. *Cell*, *186*(24), 5203–5219.

Cox, B. B., Christie, M. J., Devi, L., Toll, L., & Traynor, J. R. (2015). Challenges for opioid receptor nomenclature: IUPHAR review 9. *British Journal of Pharmacology*, *172*(2), 317–323.

Cui, S., Kim, W. M., Jang, E. A., Lee, Y. J., Kang, D. H., Lee, H. G., & Choi, J. I. (2026). The role of nucleus accumbens dopamine signaling in the development of morphine tolerance in neuropathic pain. *The Korean Journal of Pain*, *39*(1), 106–115.

Dambisya, Y. M., & Lee, T. L. (1996). Role of nitric oxide in the induction and expression of morphine tolerance and dependence in mice. *British Journal of Pharmacology*, *117*(5), 914–918.

de Guglielmo, G., Kallupi, M., Scuppa, G., Stopponi, S., Demopulos, G., Gaitanaris, G., & Ciccocioppo, R. (2014). Analgesic tolerance to morphine is regulated by PPARγ. *British Journal of Pharmacology*, *171*(23), 5407–5416.

Džoljić, E., Grabatinić, I., & Kostić, V. (2015). Why is nitric oxide important for our brain? *Functional Neurology*, *30*(3), 159–163.

Esmaili-Shahzade-Ali-Akbari, P., Hosseinzadeh, H., & Mehri, S. (2021). Effect of suvorexant on morphine tolerance and dependence in mice: Role of NMDA, AMPA, ERK and CREB proteins. *NeuroToxicology*, *84*, 64–72.

Evans, C. J., & Cahill, C. M. (2016). Neurobiology of opioid dependence in creating addiction vulnerability. *F1000Research*, *5*, 1748.

Fan, X., Zhang, J., Zhang, X., Yue, W., & Ma, L. (2003). Differential regulation of β-arrestin 1 and β-arrestin 2 gene expression in rat brain by morphine. *Neuroscience*, *117*(2), 383–389.

Fan, Y. Z., Duan, Y. L., Chen, C. T., Wang, Y., & Zhu, A. P. (2024). Advances in attenuating opioid-induced respiratory depression: A narrative review. *Medicine*, *103*(29), e38837.

Gei, L., Yan, Y., Xing, W., Li, Q., Chen, X., Yan, F., ... & Chen, D. (2024). Amiloride alleviates morphine tolerance by suppressing ASIC3-dependent neuroinflammation in the spinal cord. *European Journal of Pharmacology*, *963*, 176173.

Ghavimi, H., Charkhpour, M., Ghasemi, S., Mesgari, M., Hamishehkar, H., Hassanzadeh, K., Arami, S., & Hassanzadeh, K. (2015). Pioglitazone prevents morphine antinociceptive tolerance via ameliorating neuroinflammation in rat cerebral cortex. *Pharmacological Reports*, *67*(1), 78–84.

Ghavimi, H., Hassanzadeh, K., Maleki-Dizaji, N., Azarfardian, A., Ghasami, S., Zolali, E., & Charkhpour, M. (2014). Pioglitazone prevents morphine antinociception tolerance and withdrawal symptoms in rats. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *387*(9), 811–821.

Ghosh, N., Kesh, K., Singh, P. K., Sharma, U., Chupikova, I., Ramakrishnan, S., & Roy, S. (2022). Morphine use induces gastric microbial dysbiosis driving gastric inflammation through TLR2 signaling which is attenuated by proton pump inhibitor. *British Journal of Pharmacology*, *180*(11), 1435–1452. https://doi.org/10.1111/bph.16025

Goudas, L. C., Langlade, A., Serrie, A., Matson, W., Milbury, P., Thurel, C., Sandouk, P., & Carr, D. B. (1999). Acute decreases in cerebrospinal fluid glutathione levels after intracerebroventricular morphine for cancer pain. *Anesthesia & Analgesia*, *89*(5), 1209–1215.

Grace, P. M., Strand, K. A., Galer, E. L., Urban, D. J., Wang, X., Baratta, M. V., ... & Watkins, L. R. (2016). Morphine paradoxically prolongs neuropathic pain in rats by amplifying spinal NLRP3 inflammasome activation. *Proceedings of the National Academy of Sciences*, *113*(24), E3441-E3450.

Grim, T. W., Schmid, C. L., Stahl, E. L., Pantouli, F., Ho, J. H., AcevedoCanabal, A., Kennedy, N. M., Cameron, M. D., Bannister, T. D., & Bohn, L. M. (2020). A G protein signaling-biased agonist at the μ-opioid receptor reverses morphine tolerance while preventing morphine withdrawal. *Neuropsychopharmacology*, *45*(2), 416–425.

Hamdy, M. M., Elbadr, M. M., & Barakat, A. (2018). Bupropion attenuates morphine tolerance and dependence: Possible role of glutamate, norepinephrine, inflammation, and oxidative stress. *Pharmacological Reports*, *70*(5), 955–962.

Han, D., Dong, W., & Jiang, W. (2022). Pinocembrin alleviates chronic morphine-induced analgesic tolerance and hyperalgesia by inhibiting microglial activation. *Neurological Research*, *44*(10), 946–955.

Hao, X. Q., Wang, Z. Y., Chen, J. M., Wu, N., & Li, J. (2021). Involvement of the nociceptin opioid peptide receptor in morphine-induced antinociception, tolerance and physical dependence in female mice. *Metabolic Brain Disease*, *36*(8), 2243–2253.

Harris, A. C., & Gewirtz, J. C. (2005). Acute opioid dependence: Characterizing the early adaptations underlying drug withdrawal. *Psychopharmacology*, *178*(4), 353–366.

He, J., Zhu, G., Wang, G., & Zhang, F. (2020). Oxidative stress and neuroinflammation potentiate each other to promote progression of dopamine neurodegeneration. *Oxidative Medicine and Cellular Longevity*, *2020*, 6137521.

Hosseinzadeh, H., Imenshahidi, M., Hosseini, M., & Razavi, B. M. (2012). Effect of linalool on morphine tolerance and dependence in mice. *Phytotherapy Research*, *26*(9), 1399–1404.

James, A., & Williams, J. (2020). Basic opioid pharmacology—an update. *British Journal of Pain*, *14*(2), 115–121.

Janani, C., & Kumari, B. R. (2015). PPAR gamma gene–a review. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*, *9*(1), 46–50.

Javadi, S., Ejtemaeimehr, S., Keyvanfar, H. R., Moghaddas, P., Aminian, A., Rajabzadeh, A., Mani, A. R., & Dehpour, A. R. (2013). Pioglitazone potentiates development of morphine-dependence in mice: Possible role of NO/cGMP pathway. *Brain Research*, *1510*, 22–37.

Khan, M. I., Momeny, M., Ostadhadi, S., Jahanabadi, S., EjtemaeiMehr, S., Sameem, B., Zarrinrad, G., & Dehpour, A. R. (2018). Thalidomide attenuates development of morphine dependence in mice by inhibiting PI3K/Akt and nitric oxide signaling pathways. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *82*, 39–48.

Khan, M. I., Ostadhadi, S., Mumtaz, F., Momeny, M., Moghaddaskho, F., Hassanipour, M., Ejtemaei-Mehr, S., & Dehpour, A. R. (2017). Thalidomide attenuates the development and expression of antinociceptive tolerance to μ-opioid agonist morphine through l-arginine-iNOS and nitric oxide pathway. *Biomedicine & Pharmacotherapy*, *85*, 493–502.

Khroyan, T. V., Wu, J., Polgar, W. E., Cami-Kobeci, G., Fotaki, N., Husbands, S. M., & Toll, L. (2015). BU08073 a buprenorphine analogue with partial agonist activity at μ-receptors in vitro but long-lasting opioid antagonist activity in vivo in mice. *British Journal of Pharmacology*, *172*(2), 668–680.

Kotlinska, J., Gibula-Bruzda, E., Witkowska, E., Chung, N., Schiller, P., & Izdebski, J. (2013). Antinociceptive effects of two deltorphins analogs in the tail-immersion test in rats. *Peptides*, *39*, 103–110.

Kruegel, A. C., Gassaway, M. C., Kapoor, A., Váradi, A., Majumdar, S., Filizola, M., Javitch, J. A., & Sames, D. (2016). Synthetic and receptor signaling explorations of the mitragyna alkaloids: Mitragynine as an atypical molecular framework for opioid receptor modulators. *Journal of the American Chemical Society*, *138*(21), 6754–6764.

Kuthati, Y., & Wong, C. S. (2024). The melatonin type 2 receptor agonist IIK7 attenuates and reverses morphine tolerance in neuropathic pain rats through the suppression of neuroinflammation in the spinal cord. *Pharmaceuticals*, *17*(12), 1638.

Kuthati, Y., Busa, P., Tummala, S., Rao, V. N., Davuluri, V. N. G., Ho, Y. P., & Wong, C. S. (2021). Mesoporous polydopamine nanoparticles attenuate morphine tolerance in neuropathic pain rats by inhibition of oxidative stress and restoration of the endogenous antioxidant system. *Antioxidants*, *10*(2), 195.

Liang, D. Y., & Clark, J. D. (2004). Modulation of the NO/CO-cGMP signaling cascade during chronic morphine exposure in mice. *Neuroscience Letters*, *365*(2), 73–77.

Lin, Y., Lu, P., Ding, Q., Tao, X., Tan, Q., & Liu, M. (2026). Opioid-induced constipation: Mechanistic insights, experimental models, and future perspectives. *Biomedicines*, *14*(5), 995.

Listos, J., Baranowska-Bosiacka, I., Talarek, S., Listos, P., Orzelska, J., Fidecka, S., Gutowska, I., Kolasa, A., Rybicka, M., & Chlubek, D. (2013). The effect of perinatal lead exposure on dopamine receptor D2 expression in morphine dependent rats. *Toxicology*, *310*, 73–83.

Listos, J., Łupina, M., Talarek, S., Mazur, A., Orzelska-Górka, J., & Kotlińska, J. (2019). The mechanisms involved in morphine addiction: An overview. *International Journal of Molecular Sciences*, *20*(17), 4302.

Liu, C. H., Cherng, C. H., Lin, S. L., Yeh, C. C., Wu, C. T., Tai, Y. H., & Wong, C. S. (2011). N-methyl-D-aspartate receptor antagonist MK-801 suppresses glial pro-inflammatory cytokine expression in morphine-tolerant rats. *Pharmacology Biochemistry and Behavior*, *99*(3), 371–380.

Liu, J., Yi, S., Shi, W., Zhang, G., Wang, S., Qi, Q., ... & metni kesilen yazarlar (2021). The pathology of morphine-inhibited nerve repair and morphine-induced nerve damage is mediated via endoplasmic reticulum stress. *Frontiers in Neuroscience*, *15*, 618190.

Liu, Q., Su, L. Y., Sun, C., Jiao, L., Miao, Y., Xu, M., ... & metni kesilen yazarlar (2020). Melatonin alleviates morphine analgesic tolerance in mice by decreasing NLRP3 inflammasome activation. *Redox Biology*, *34*, 101560.

Liu, W., Jiang, P., & Qiu, L. (2022). Blocking of caveolin-1 attenuates morphine-induced inflammation, hyperalgesia, and analgesic tolerance via inhibiting NLRP3 inflammasome and ERK/c-JUN pathway. *Journal of Molecular Neuroscience*, *72*(5), 1047–1057.

Liu, X., Song, Q., Wang, S., Hou, R., Jia, H., Zhang, Z., & Liang, Y. (2026). Therapeutic potential of biased μ-opioid receptor agonists for delaying opioid tolerance in chronic pain management. *Neurochemistry International*, 106162.

Mansouri, M. T., Naghizadeh, B., Ghorbanzadeh, B., Alboghobeish, S., Amirgholami, N., Houshmand, G., ... & metni kesilen yazarlar (2018). Venlafaxine prevents morphine antinociceptive tolerance: The role of neuroinflammation and the l-arginine-nitric oxide pathway. *Experimental Neurology*, *303*, 134–141.

Mitra, S., Sinatra, R. S., & Warltier, D. C. (2004). Perioperative management of acute pain in the opioid-dependent patient. *Anesthesiology*, *101*(1), 212–227.

Molavinia, S., Nikravesh, M., Pashmforoosh, M., Vardanjani, H. R., & Khodayar, M. J. (2024). Zingerone alleviates morphine tolerance and dependence in mice by reducing oxidative stress-mediated NLRP3 inflammasome activation. *Neurochemical Research*, *49*(2), 415–426.

Nabemoto, M., Mashimo, M., Someya, A., Nakamura, H., Hirabayashi, T., Fujino, H., Kaneko, M., Okuma, Y., Saito, T., & Yamaguchi, N. (2008). Release of arachidonic acid by 2-arachidonoyl glycerol and HU210 in PC12 cells; roles of Src, phospholipase C and cytosolic phospholipase A2α. *European Journal of Pharmacology*, *590*(1-3), 1–11.

Nicoara, D., Zhang, Y., Nelson, J. T., Brewer, A. L., Maharaj, P., DeWald, S. N., Shirachi, D. Y., & Quock, R. M. (2016). Hyperbaric oxygen treatment suppresses withdrawal signs in morphine-dependent mice. *Brain Research*, *1648*, 434–437.

Nicola, M. A., Hefny, F. H., Morsy, R. M., Abdel-Maksoud, F. M., Gamea, M. G., & Hamdy, M. M. (2026). Modulation of morphine tolerance and dependence by pyrroloquinoline quinone in mice: Insights into mechanistic bridge from NMDARs-CaMKII signaling to oxidative neuroinflammation. *The Journal of Pharmacology and Experimental Therapeutics*, 104956.

Okuyama, Y., Jin, H., Kokubun, H., & Aoe, T. (2020). Pharmacological chaperones attenuate the development of opioid tolerance. *International Journal of Molecular Sciences*, *21*(20), 7575.

Ozdemir, E., Avcı, O., Inan, Z. D. S., Taskiran, A. S., Gunes, H., & Gursoy, S. (2023). Aspirin attenuates morphine antinociceptive tolerance in rats with diabetic

neuropathy by inhibiting apoptosis in the dorsal root ganglia. *Metabolic Brain Disease*, *38*(6), 2145–2158.

Ozdemir, E., Bagcivan, I., Durmus, N., Altun, A., & Gursoy, S. (2011). The nitric oxide–cGMP signaling pathway plays a significant role in tolerance to the analgesic effect of morphine. *Canadian Journal of Physiology and Pharmacology*, *89*(2), 89–95.

Ozdemir, E., Baser, T., & Taskiran, A. S. (2022). Blockade of orexin receptor type-1 by SB-334867 and activation of orexin receptor type-2 attenuate morphine tolerance in rats. *Physiology International*, *109*(4), 457–474.

Ozdemir, E. (2020). The role of the cannabinoid system in opioid analgesia and tolerance. *Mini-Reviews in Medinal Chemistry*, *20*(10), 875–885.

Ozturk, A., Ozdemir, E., Ozkaraca, M., Taskiran, A. S., & Altun, A. (2025). TRPV1 channel antagonist capsazepine alleviates morphine tolerance and morphine-induced neurotoxicity by preventing mitochondrial damage and apoptosis: An in vivo and in vitro study. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *398*(12), 17773–17791.

Pradhan, A. A., Befort, K., Nozaki, C., Gavériaux-Ruff, C., & Kieffer, B. L. (2011). The delta opioid receptor: An evolving target for the treatment of brain disorders. *Trends in Pharmacological Sciences*, *32*(10), 581–590.

Raehal, K. M., & Bohn, L. M. (2011). The role of beta-arrestin2 in the severity of antinociceptive tolerance and physical dependence induced by different opioid pain therapeutics. *Neuropharmacology*, *60*(1), 58–65.

Ray, L. A., Roche, D. J., Heinzerling, K., & Shoptaw, S. (2014). Opportunities for the development of neuroimmune therapies in addiction. *International Review of Neurobiology*, *118*, 381–401.

Reeves, K. C., Shah, N., Muñoz, B., & Atwood, B. K. (2022). Opioid receptor-mediated regulation of neurotransmission in the brain. *Frontiers in Molecular Neuroscience*, *15*, 919773.

Rehni, A. K., Bhateja, P., Singh, T. G., & Singh, N. (2008). Nuclear factor-κ-B inhibitor modulates the development of opioid dependence in a mouse model of naloxone-induced opioid withdrawal syndrome. *Behavioral Pharmacology*, *19*(3), 265–269.

Salvemini, D., & Neumann, W. L. (2009). Peroxynitrite: A strategic linchpin of opioid analgesic tolerance. *Trends in Pharmacological Sciences*, *30*(4), 194–202.

Sarruf, D. A., Yu, F., Nguyen, H. T., Williams, D. L., Printz, R. L., Niswender, K. D., & Schwartz, M. W. (2009). Expression of peroxisome proliferator-activated receptor-γ in key neuronal subsets regulating glucose metabolism and energy homeostasis. *Endocrinology*, *150*(2), 707–712.

Sawaya, B. E., Deshmane, S. L., Mukerjee, R., Fan, S., & Khalili, K. (2009). TNF alpha production in morphine-treated human neural cells is NF-κB-dependent. *Journal of Neuroimmune Pharmacology*, *4*(1), 140–149.

Serreli, G., & Deiana, M. (2023). Role of dietary polyphenols in the activity and expression of nitric oxide synthases: A review. *Antioxidants*, *12*(1), 147.

Sheikholeslami, M. A., Parvardeh, S., Ghafghazi, S., Moini Zanjani, T., & Sabetkasaei, M. (2019). The attenuating effect of curcumin on morphine dependence in rats: The involvement of spinal microglial cells and inflammatory cytokines. *Iranian Journal of Pharmaceutical Research*, *18*(Suppl. 1), 198–207.

Shen, C. H., Tsai, RY., Shih, M. S., Lin, S. L., Tai, Y. H., Chien, C. C., ... & metni kesilen yazarlar (2011). Etanercept restores the antinociceptive effect of morphine and suppresses spinal neuroinflammation in morphinetolerant rats. *Anesthesia & Analgesia*, *112*(2), 454–459.

Shi, R., Jin, T., Xu, W., & Liang, Y. (2025). The therapeutic potential of phytochemicals in morphine tolerance: Targeting microglia-mediated neuroinflammation. *Frontiers in Pharmacology*, *16*, 1669257.

Sil, S., Periyasamy, P., Guo, M. L., Callen, S., & Buch, S. (2018). Morphine-mediated brain region-specific astrocytosis involves the ER stress-autophagy axis. *Molecular Neurobiology*, *55*(8), 6713–6733.

Skrabalova, J., Drastichova, Z., & Novotny, J. (2013). Morphine as a potential oxidative stress-causing agent. *Mini-Reviews in Organic Chemistry*, *10*(4), 367–372.

Stein, C., Schäfer, M., & Machelska, H. (2003). Attacking pain at its source: New perspectives on opioids. *Nature Medicine*, *9*(8), 1003–1008.

Swingler, M., Donadoni, M., Unterwald, E. M., Maggirwar, S. B., & Sariyer, I. K. (2025). Molecular and cellular basis of mu-opioid receptor signaling: Mechanisms underlying tolerance and dependence development. *Frontiers in Neuroscience*, *19*, 1597922.

Taskiran, A. S., & Avci, O. (2021). Effect of captopril, an angiotensin-converting enzyme inhibitor, on morphine analgesia and tolerance in rats, and elucidating the inflammation and endoplasmic reticulum stress pathway in this effect. *Neuroscience Letters*, *741*, 135504.

Trujillo, K. A., & Akil, H. (1991). Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. *Science*, *251*(4989), 85–87.

Wang, H., Huang, M., Wang, W., Zhang, Y., Ma, X., Luo, L., ... & Xu, T. (2021). Microglial TLR4-induced TAK1 phosphorylation and NLRP3 activation mediates neuroinflammation and contributes to chronic morphine-induced antinociceptive tolerance. *Pharmacological research*, *165*, 105482.

Watkins, L. R., Hutchinson, M. R., Rice, K. C., & Maier, S. F. (2009). The "toll" of opioid-induced glial activation: Improving the clinical efficacy of opioids by targeting glia. *Trends in Pharmacological Sciences*, *30*(11), 581–591.

Williams, J. T., Ingram, S. L., Henderson, G., Chavkin, C., von Zastrow, M., Schulz, S., Koch, T., Evans, C. J., & Christie, M. J. (2013). Regulation of μ-opioid receptors: Desensitization, phosphorylation, internalization, and tolerance. *Pharmacological Reviews*, *65*(1), 223–254.

Willson, T. M., Brown, P. J., Sternbach, D. D., & Henke, B. B. (2000). The PPARs: From orphan receptors to drug discovery. *Journal of Medicinal Chemistry*, *43*(4), 527–550.

Wu, W., Li, Q., & Yang, F. (2025). The actions of morphine on microglia and the underlying effects on associated adverse effects. *Psychopharmacology*, 1–20. Advanced online publication.

Yang, H., Shen, T., Qi, Z., Yang, Z., Zhang, M., Jiang, J., ... & Lu, C. (2025). TIM3 attenuates morphine antinociceptive tolerance and microglial neuroinflammation by suppressing the TRAF6/NF-κB pathway in male mice. *Neuropharmacology*, 110638.

Yang, Z., Zhang, F., Abdul, M., Jiang, J., Li, Y., Li, Y., ... & metni kesilen yazarlar (2023). Tumor necrosis factor-α-induced protein 8-like 2 alleviates morphine antinociceptive tolerance through reduction of ROS-mediated apoptosis and MAPK/NF-κB signaling pathways. *Neuropharmacology*, *238*, 109667.

Zeng, X. S., Jia, J. J., & Ma, L. F. (2015). Gensenoside Rb1 protects rat PC12 cells from oxidative stress-induced endoplasmic reticulum stress: The involvement of thioredoxin-1. *Molecular and Cellular Biochemistry*, *410*(1–2), 239–246.

Zhuang, Y., Wang, Y., He, B., He, X., Zhou, X. E., Guo, S., ... & Xu, H. E. (2022). Molecular recognition of morphine and fentanyl by the human μ-opioid receptor. *Cell*, *185*(23), 4361–4375.

Zuo, Z. J. A. (2005). The role of opioid receptor internalization and β-arrestins in the development of opioid tolerance. *Anesthesia & Analgesia*, *101*(3), 728–734.

Chapter 2

# MEDICAL HIERARCHY AND PATIENT SAFETY

*Mevlut KARADAG*[1]

1 Assoc. Prof., Department of Medical Data Processing, Vocational School of Health Services, Lokman Hekim University, Ankara 06510, Türkiye mevlut.karadag@lokmanhekim.edu.tr, Orcid.org/0000-0001-7288-1925

## 1. Introduction

Healthcare services are complex sociotechnical systems that require a high level of expertise and have a low tolerance for error. In an operating room, the surgeon, anesthesia team, operating room nurse, technician, and support staff; in an intensive care unit, the intensivist, nurse, physiotherapist, pharmacist, dietitian, and consulting physician; and in an emergency department, the triage nurse, emergency physician, paramedic, security personnel, and registration staff all work interdependently to protect patient safety. Within this complexity, the distribution of roles, seniority, expertise, and boundaries of responsibility inevitably produces a certain degree of hierarchy. Therefore, rather than labeling medical hierarchy directly as "good" or "bad," it is necessary to explain under which conditions it supports patient safety and under which conditions it becomes a risk.

One of the most important transformations in the patient safety literature over the past two decades has been the recognition that errors cannot be reduced solely to the carelessness of individual clinicians. Reason's (2000) systems approach showed that safety depends not only on individuals behaving correctly, but also on organizations designing systems that detect errors, learn from them, and create layers of defense. Current evidence indicates that patient safety remains a central issue for health systems worldwide. Bates et al. (2023) demonstrated that adverse events in inpatient care remain common and that a substantial proportion are preventable. Levine et al. (2024) showed that adverse events are also more visible than expected in ambulatory care. These findings emphasize that safety is not limited to high-risk areas such as intensive care units or operating rooms, but is a quality domain that must be managed across the entire healthcare chain.

Medical hierarchy is one of the hidden yet powerful determinants of this chain. If a healthcare worker recognizes an incorrect medication dose but cannot challenge a senior physician; if a nurse senses deterioration in a patient but fears being accused of "overreacting"; if a resident observes a breach of sterility in the surgical field but remains silent; or if a patient is worried that a wrong procedure may be performed on their body but cannot ask a question, the problem is not merely a lack of individual courage. The problem is how hierarchy codes certain voices as legitimate, others as unnecessary, certain questions as threats, and certain warnings as disrespectful.

The World Health Organization's Global Patient Safety Action Plan 2021–2030 addresses the reduction of avoidable harm as one of the core goals of health systems and emphasizes safety culture, patient and family engagement, learning systems, and leadership responsibility (World Health Organization [WHO], 2021). The spirit of this plan suggests that patient safety

cannot be achieved merely by writing protocols, but also by transforming communication patterns and power relations within institutions. A protocol produces safety only when there is a culture in which people can speak up when the protocol is violated.

The central argument of this study is as follows: Medical hierarchy is functional insofar as it organizes clinical expertise and decision-making responsibility; however, it becomes a patient safety risk when it prevents speaking up, questioning, incident reporting, and mutual learning. Therefore, the aim of healthcare management should not be to eliminate hierarchy completely, but to transform it into a safety-oriented, accountable, psychologically safe, and communication-friendly structure. This transformation should be addressed not only through training, but also through leadership, measurement, reporting, team structure, performance systems, and organizational justice mechanisms.

## 2. Conceptual Framework of Medical Hierarchy

Medical hierarchy refers to the unequal distribution of knowledge, authority, status, seniority, profession, expertise, and decision-making responsibility in healthcare organizations. Hierarchy is not limited to who reports to whom on an organizational chart. It also concerns who speaks during ward rounds, whose words are not interrupted in meetings, whose warnings are taken seriously in the operating room, who is blamed in incident reporting, and how seriously a patient's objection is considered. In this sense, medical hierarchy is both a formal and an informal structure.

Hierarchy has a functional dimension. In moments of clinical uncertainty, there is a need for someone who can make rapid decisions and assume responsibility. In situations such as trauma management in the emergency department, unexpected bleeding in the operating room, sudden arrest in the intensive care unit, or fetal distress in the delivery room, ambiguity in task allocation may weaken patient safety. Experience and expertise are particularly important in the management of complex clinical decisions. Therefore, from a patient safety perspective, the problem is not the existence of hierarchy, but the production of one-way obedience, unquestionable authority, and status-based silence by hierarchy (King et al., 2008; Leonard et al., 2004; Manser, 2009; Nembhard & Edmondson, 2006; Okuyama et al., 2014).

Classical sociology of the professions explains the professional authority of medicine through information asymmetry, social prestige, and professional autonomy. Historically, medicine has held a higher status than other health professions, and the authority to diagnose, plan treatment, and make clinical decisions has largely been concentrated at the center of the medical profession.

Although this arrangement played an important role in the development of modern healthcare, a one-directional model of authority is insufficient in today's systems, where team-based care has become widespread. Fields such as chronic disease management, older adult care, intensive care, oncology, rehabilitation, and palliative care require not only physician decision-making, but also interdisciplinary coordination (Bookey-Bassett et al., 2017; Freidson, 1970; Freidson, 1988; Mitchell et al., 2012; Starr, 1982; World Health Organization, 2010).

Hierarchy is also related to power distance. Power distance is a concept that describes the extent to which lower-status individuals accept inequality between themselves and authority as natural and unquestionable (Hofstede, 2001; Hofstede et al., 2010; Kim et al., 2020; Lee & Lee, 2024). In healthcare organizations, high power distance may manifest itself through unwritten rules such as "the senior person knows better," "a resident does not speak when the professor is present," "a nurse does not object to a physician," or "a student is disrespectful if they ask questions." Lee and Lee (2024) showed that hierarchical unit culture and power distance orientation are associated with silence behavior among nurses. This finding suggests that silence is not only individual timidity, but also a behavioral pattern produced by unit culture and status relations.

The scoping review by Essex et al. (2023) shows that hierarchy in healthcare organizations affects not only communication, but also how care is prioritized, how employees position themselves, and how professional relationships are negotiated. Therefore, hierarchy is not merely an interpersonal issue; it is a fundamental dimension of organizational order that intersects with patient safety.

Another dimension of medical hierarchy is "seniority hierarchy." Hierarchy also exists within the same profession: specialist physician-resident physician, senior resident-junior resident, charge nurse-clinical nurse, academic-student, chief physician-clinical chief, and similar relationships. Seniority hierarchy may facilitate learning and the transfer of experience; however, when it is combined with humiliating language, mobbing, belittlement, punishment, or a harsh culture regarded as a "natural part of training," it weakens safety and professional development. Therefore, hierarchy should be evaluated not only in terms of patient safety, but also in terms of healthcare worker well-being and professional sustainability.

### 3. Hierarchy from the Perspective of Patient Safety

Patient safety is the totality of systems, cultures, and practices designed to reduce preventable harm and make healthcare processes safe. Hierarchy touches many points of this totality. The first point of contact is communication. In healthcare, critical information is often transferred between professions,

shifts, and institutions. Hierarchy determines the direction and openness of this transfer. If information flows only from top to bottom within a team, warnings from below may be delayed or filtered.

The second point of contact is decision-making processes. Medical decisions depend on the accurate interpretation of patient-related data and the integration of observations from different professionals. A nurse may notice subtle changes in a patient's behavior; a pharmacist may detect a drug interaction; a physiotherapist may assess mobilization risk; and a family member may recognize an unusual condition in the patient. If hierarchy includes this information in the decision-making process, it improves safety; if it excludes it, it creates a clinical blind spot.

The third point of contact is incident reporting and learning. In institutions where error reporting is punitive, staff may avoid reporting incidents. In such cases, while the organization tries to identify the individual who made the error, it may fail to see the system conditions that made the error possible. AHRQ's SOPS Hospital Survey 2.0 demonstrates that patient safety culture can be measured through components such as communication openness, communication about errors, event reporting, response to errors, management support, teamwork, and handoffs (Agency for Healthcare Research and Quality [AHRQ], 2024). A substantial proportion of these components are directly related to hierarchical communication.

The fourth point of contact is clinical prioritization. In hierarchical cultures, the opinion of the highest-status person may always be treated as the most accurate opinion. Yet patient safety requires decisions to be based not on status, but on evidence, observation, standards, and collective reasoning. In a hierarchical team, correcting a wrong decision may be more difficult because the cost of objection increases when the person who made the wrong decision has high status. For this reason, safe teams must combine decision-making responsibility with questionability.

The fifth point of contact is patient engagement. Traditional medical hierarchy positions the patient as a passive recipient of information and the physician as the main owner of the decision. However, the modern patient safety approach regards the patient and family as safety partners. Patients should be able to ask questions about the procedure to be performed, verify their medications, participate in identity verification, and express their concerns. Therefore, transforming hierarchy concerns not only interprofessional relationships, but also the patient-professional relationship.

## 4. Culture of Silence and Speaking Up

One of the most visible consequences of hierarchy from a patient safety perspective is the inability of healthcare workers to express risks they observe. This behavior, referred to in the literature as "speaking up," means that a healthcare professional clearly communicates a concern to someone who has the power to intervene when they notice a situation that threatens, or has the potential to threaten, patient safety (Kane et al., 2023). In this context, speaking up is not merely talking or expressing an opinion; it is a professional responsibility behavior aimed at making clinical risk visible, adding safety information to the decision-making process, and preventing possible harm.

In healthcare, silence often stems not from a lack of knowledge, but from the social and institutional cost of speaking. A nurse may realize that a medication dose may be incorrect; a resident may think that a senior physician's decision involves an incomplete assessment; a student may observe a breach of sterility; or a technician may sense a safety risk related to a device. However, these individuals may fear being perceived as "disrespectful," "incompetent," "slowing down the work," "challenging authority," or "creating problems for the team" if they raise a warning. Therefore, silence cannot be interpreted merely as a lack of individual courage; it is an organizational behavioral pattern produced by hierarchical culture, power distance, previous experiences, leadership style, and the institution's responses after incident reporting.

Medical hierarchy affects speaking behavior at three levels in particular. The first is the individual level. The employee may not be confident in their own knowledge, may fear raising a false alarm, or may think that their clinical experience is insufficient. This is especially evident among students, new staff members, interns, residents, and junior nurses. The second is the relational level. Negative past experiences with a senior team member, demeaning language, anger, ridicule, or the failure to take warnings seriously reduce subsequent speaking behavior. The third is the organizational level. Failure to protect people who report incidents, lack of feedback after reporting, evaluating errors through individuals rather than systems, and managers' failure to support open communication institutionalize silence. van Dongen et al. (2024) stated that the factors influencing speaking-up behavior in hospitals should be addressed at the individual, relational, contextual, and organizational levels.

A culture of silence becomes particularly pronounced in clinical environments characterized by high power distance. Power distance refers to the extent to which lower-status individuals accept inequality between themselves and authority as natural and unquestionable. In healthcare organizations, this may appear through unwritten rules such as "the senior

person knows better," "a resident does not speak when the professor is present," "a nurse does not object to a physician," or "a student is disrespectful if they asks questions." Lee and Lee (2024) showed that hierarchical unit culture and power distance orientation are associated with silence behavior among nurses. This finding is critical for patient safety because the earliest warning signals in clinical teams are often noticed by those closest to the patient.

A culture of silence affects not only the person who does not speak, but the entire care process. Clinical decisions become safer when observations and knowledge from different professional groups are brought together. A nurse may notice subtle changes in the patient's clinical condition; a pharmacist may detect a drug interaction; a physiotherapist may evaluate mobilization risk; a resident may notice a critical change in a new laboratory result; and a family member may recognize unusual behavior in the patient. When hierarchical culture blocks the upward flow of such information, the team makes decisions with incomplete data. This may lead to delayed intervention, incorrect treatment, repeated errors, late recognition of patient deterioration, and lost opportunities for learning after an event (Dayton & Henriksen, 2007; Henriksen & Dayton, 2006; Massey et al., 2017; Nembhard & Edmondson, 2006; Okuyama et al., 2014; Zaij et al., 2023).

Speaking up often requires social courage as much as technical knowledge. A staff member may know clinically what is risky, but may remain silent because they cannot predict how their concern will be received. Questions such as "What if I am misunderstood?", "Will the senior person become angry with me?", "Will I be excluded from the team?", "Will I be invited to this operation or ward round again?", or "Will they think I am ignorant?" make it difficult to express patient safety concerns. Therefore, the call to "speak up when you see a risk" is not sufficient on its own. Institutional trust must be established that what the employee says will be heard, taken seriously, and not punished.

The scoping review by Kim et al. (2020) on speaking-up training showed that most interventions in this area focus on the individual's communication skills. However, in hierarchical structures, the core problem that makes speaking difficult is not merely that individuals do not know the right sentence. Even if an employee knows the correct SBAR format (Situation, Background, Assessment, Recommendation), that is, an appropriate warning statement or escalation step including the current situation, background, assessment, and recommendation, they may remain silent if they think they will be humiliated, punished, or ignored when they speak. Therefore, speaking up is a multilevel patient safety behavior that must be addressed not only through communication training, but also together with psychological safety, just culture, inclusive leadership, and reliable incident reporting mechanisms.

The most dangerous aspect of a culture of silence is that it is often invisible. A low number of incident reports in a unit does not always mean that safe care is being delivered; it may also mean that staff are not reporting, that reports do not lead to results, or that people who report have had negative experiences. Similarly, the absence of objections in meetings may not mean that decisions are correct; it may indicate that team members fear the consequences of objecting. Therefore, managers should interpret silence not as the absence of a problem, but as a potential safety signal.

From the perspective of healthcare managers, the central question should not be limited to "Why do employees not speak?" A more appropriate question is: "Which conditions in this institution make speaking risky?" To answer this question, incident reporting rates, the distribution of reports by professional group, near-miss reports, staff safety culture surveys, psychological safety measurements, patient complaints, handoff errors, and unit-level feedback should be evaluated together. In particular, the absence of reporting by certain professional groups, the silence of newcomers in meetings, residents' inability to express lack of supervision, or the systematic failure to document nurses' warnings should be monitored as indicators of hierarchical silence.

For this reason, it may be useful to conduct regular "silence audits" in institutions. A silence audit is a managerial assessment that seeks to make visible not only the number of incidents, but also what remains unsaid. Within this scope, the following questions may be asked: In which units are no near-miss reports submitted? Which professional groups do not speak in safety meetings? In which clinical processes does the same risk recur despite the absence of reporting? How are employees received when they object to a senior person? Do newcomers know to whom they should communicate safety concerns? Can patients and family members express their concerns? These questions bring the hidden effects of hierarchy on patient safety onto the management agenda.

In conclusion, a culture of silence is one of the most critical risk mechanisms through which medical hierarchy affects patient safety. Hierarchy is functional insofar as it organizes clinical expertise and responsibility; however, it creates safety gaps when it prevents staff from expressing risks, reporting errors, asking questions, and contributing to decision-making processes. Therefore, speaking-up behavior cannot be left to individual courage. Institutions must clearly demonstrate that speaking up is a legitimate, expected, protected, and professionally valuable behavior for patient safety. The foundation of this transformation is psychological safety, which is discussed in the next section.

## 5. Psychological Safety: The Mechanism That Transforms Hierarchy into a Safety Culture

Psychological safety means that team members can engage in interpersonal risk-taking behaviors such as asking questions, admitting errors, expressing ideas, seeking help, and voicing concerns related to patient safety without fear of punishment, exclusion, or humiliation. Edmondson's (1999) classic study showed that psychological safety is a fundamental condition for learning teams. In healthcare, psychological safety is not a substitute for technical expertise; rather, it is the social foundation that enables technical expertise to be used safely. A team may have a high level of clinical knowledge, advanced technology, and detailed protocols; however, if team members cannot express risks, hesitate to report errors, or see senior decisions as unquestionable, patient safety remains fragile.

The relationship between medical hierarchy and psychological safety is bidirectional. Hierarchy may be functional in terms of clinical expertise, task allocation, and decision-making responsibility; however, when it becomes rigid and unquestionable, it closes down speaking. Psychological safety does not aim to eliminate hierarchy, but to make open communication, mutual learning, and safety-oriented dissent possible within hierarchy. In safe teams, the leadership role continues; however, the leader regards listening to different observations before making decisions, discussing uncertainties, and taking team members' warnings seriously as part of their responsibility. Therefore, psychological safety produces not an "authority-free team," but a "questionable and learning-oriented authority" (Edmondson, 1999, 2003; Edmondson & Lei, 2014; Grailey et al., 2021; Nembhard & Edmondson, 2006; O'Donovan & McAuliffe, 2020).

O'Donovan and McAuliffe (2020) examined the factors that enable psychological safety in healthcare teams within the framework of prioritizing patient safety, learning orientation, leadership support, familiarity among team members, inclusiveness, and status/hierarchy relations. These findings show that psychological safety cannot be explained solely by individual goodwill or personal communication skills. Team members knowing one another, clarifying roles, the leader inviting questions and objections, not punishing the person who reports an incident, and using feedback for learning purposes are key determinants in the institutionalization of psychological safety.

Psychological safety cannot be developed sustainably through a one-off training session, poster campaign, or short-term awareness activity. The intervention review by O'Donovan et al. (2020) shows that interventions designed to improve psychological safety and speaking behavior require multicomponent and sustained approaches to be more effective. Therefore, in

healthcare organizations, psychological safety should be addressed together with daily leadership behaviors, meeting structures, debriefing routines, responses to incident reporting, disciplinary processes, performance appraisal systems, and the culture of feedback within teams.

The systematic review by Montgomery et al. (2025) states that the relationship between psychological safety and objective patient safety outcomes has so far been assessed in a limited number of studies, although some studies have shown significant associations between psychological safety and reported medical errors or specific safety outcomes. This is important in two respects. First, psychological safety is not a "soft" or secondary dimension of patient safety, but a system capacity that must be measured and managed. Second, unit-level follow-up studies, intervention research, and measurements linked to safety indicators are needed to demonstrate more strongly the effect of psychological safety on patient outcomes.

From the perspective of healthcare managers, psychological safety should be treated not as an abstract cultural goal, but as a monitorable and improvable management domain. For this purpose, institutions should evaluate safety culture surveys, speaking behavior measures, incident reporting data, the distribution of reports by professional group, near-miss reports, staff feedback, and patient complaints together. In particular, how nurses, residents, students, technicians, and newly hired staff experience the speaking climate should be analyzed separately. High average safety culture scores may mask the silence of low-status or vulnerable groups.

Clinical leaders play a critical role in developing psychological safety. It is not enough for a leader to ask, "Do you have any objections?" What matters more is how the leader responds when a team member actually raises an objection. Leadership styles that force the person who gives a warning to defend themselves, belittle them, or portray them as someone who "slows down the work" increase silence. In contrast, responses such as "This warning was valuable for patient safety," "Let us check this together," or "We will address this report at the system level" legitimize speaking behavior. Therefore, psychological safety is built not by the leader's discourse alone, but through the real experiences team members have after speaking up.

In conclusion, psychological safety is the fundamental mechanism that transforms medical hierarchy in favor of patient safety. In rigid hierarchies, information gains value according to status; in teams with high psychological safety, information is evaluated according to its contribution to safety. This approach does not remove the clinical responsibility of the senior professional; however, it makes decision-making processes more open, more inclusive, and

more learning-oriented. From a patient safety perspective, the goal is not the absence of hierarchy, but a culture in which hierarchy does not close down speaking, does not punish feedback, and makes team members active subjects of the safety process.

**6. Hierarchy in Clinical Settings: Operating Room, Intensive Care Unit, and Emergency Department**

The effect of medical hierarchy on patient safety is most visible in high-risk clinical settings.

The operating room is one of the clinical areas where medical hierarchy is most visible. Surgical teams must simultaneously cope with time pressure, technical complexity, anesthesia management, sterility, bleeding risk, team turnover, and unexpected complications. In this environment, the surgeon, anesthesia team, operating room nurse, technician, and support staff must rapidly integrate information from different fields of expertise. Arad et al. (2022) showed that deficiencies in teamwork in operating rooms are associated with the implementation of safety standards in surgical processes. This finding demonstrates that operating room safety depends not only on the technical skill of the surgeon, but also on the team's shared situational awareness, communication quality, and safety-oriented coordination.

In the operating room, hierarchy often forms around the technical leadership of the surgeon. This leadership is functional in terms of surgical responsibility; however, it creates a safety risk if it makes the observations of other professionals in the team secondary. An operating room nurse may notice missing equipment, an incorrect count, a breach of sterility, a risk in patient positioning, or a discrepancy during the time-out. The anesthesia team may observe physiological changes before they become visible in the surgical field. A technician may detect a critical detail related to device or material safety. Delaying or disregarding such information because of status creates a weak link in the surgical safety chain.

Skråmm et al. (2021) showed that operating room nurses experience team communication as one of the essential non-technical skills for patient safety. Vikan et al. (2024) emphasized that safe surgical teams depend not only on technical expertise, but also on team members knowing one another, role clarity, open communication, and mutual trust. Therefore, the mere existence of checklists is not sufficient to strengthen surgical safety in operating rooms. For a checklist to be effective, all team members must actively participate in the process, the time-out procedure must not turn into a formality, and every team member must know that they can speak up when they see a risk.

From a managerial perspective, three practices are priorities for reducing hierarchy-related risks in operating rooms. First, a brief and structured briefing should be held before every list or procedure. In this meeting, patient identity, surgical site, expected risks, team roles, special equipment requirements, and possible complication scenarios should be shared. Second, closed-loop communication and standard warning phrases should be used for critical alerts during the procedure. Third, a short debriefing should be conducted after the procedure so that the team can evaluate what went well, what failed, and what should be changed in the next procedure. These practices do not weaken surgical leadership; on the contrary, they transform surgical leadership into safety-oriented leadership capable of using team knowledge.

The intensive care unit is another area in which the effect of hierarchy on patient safety emerges in a different form. Intensive care patients are generally monitored for multiple organ failure, complex medication regimens, invasive interventions, rapid physiological changes, and high mortality risk. Therefore, patient safety depends not only on the accuracy of medical decisions, but also on the early recognition of subtle changes in the patient and timely escalation. Because intensive care nurses are in continuous contact with patients, they are often the professionals who first notice physiological changes, behavioral differences, pain responses, changes in sedation level, or deviations in response to treatment. Nevertheless, interprofessional hierarchy may prevent nurses' warnings from being taken seriously and in a timely manner. If a nurse notices that a patient is deteriorating but hesitates because they may be perceived as "raising unnecessary alarms" or "interfering with the physician's decision," escalation may be delayed. Similarly, a resident may wait in a situation that requires consulting a senior physician because of anxiety about "appearing incompetent." Such delays may have critical consequences for patient safety in intensive care. Therefore, hierarchy management in intensive care is not merely a matter of interprofessional respect, but also a matter of early warning and timely intervention.

In intensive care teams, safe hierarchy is possible through shared situational awareness that includes each professional group's knowledge of the patient in the decision-making process. When the nurse's observation, the physician's diagnostic assessment, the pharmacist's medication safety warning, the physiotherapist's mobilization assessment, and the dietitian's nutrition plan are brought together, the patient can be monitored more holistically. Therefore, multidisciplinary mini-rounds, structured handoffs, SBAR-based escalation, closed-loop communication, and clear consultation pathways should be regarded as essential patient safety practices in intensive care.

From a managerial perspective, intensive care units should clearly define under which circumstances, to whom, and how nurses' warnings and junior physicians' consultations should be communicated. For example, direct escalation protocols may be created for specific changes in vital signs, changes in consciousness, decreased urine output, new arrhythmia, ventilator dyssynchrony, or unexpected laboratory deviations. These protocols should not remain only on paper; they should be reinforced through shift-based training, simulations, and case reviews. In this way, the message is strengthened that "raising a warning" is not an exceptional behavior requiring personal courage, but a natural part of the standard care process.

Handoff processes in intensive care are also critical from the perspective of hierarchy. During handoff, not only laboratory values and treatment plans, but also "areas of concern" should be communicated. Information such as which patient is expected to deteriorate, which treatment response is uncertain, which patient has generated different views within the team, and what needs to be reassessed should be shared explicitly. This type of structured communication makes the intuitive observations of lower-status team members visible and prevents hierarchy from filtering information flow.

The emergency department is another clinical area characterized by uncertainty, time pressure, and high patient volume. Triage decisions, diagnostic uncertainty, crowding, risk of violence, pressure from family members, resource constraints, and interrupted workflow make communication fragile in the emergency department. In this environment, hierarchy may be functional for rapid decision-making and task allocation; however, overly authoritarian communication patterns may prevent team members from expressing early safety signals. A delayed warning in the emergency department may lead to incorrect triage categorization, delayed treatment, or a missed critical diagnosis.

In the emergency department, information critical for safety does not reside solely with the physician. The triage nurse may notice the patient's initial appearance, pain level, behavior, and changes in the waiting area. The paramedic may carry clinical information from the prehospital process. Security personnel or registration staff may even be the first to observe sudden changes in a patient's behavior. A junior physician may draw attention to a laboratory or imaging result that was overlooked because of the workload of a senior physician. Therefore, safe decision-making in emergency departments depends on the rapid and structured inclusion of information from different positions into the decision-making process.

Standardized communication tools are particularly important for reducing the negative effects of hierarchy in the emergency department. The SBAR format can be used for rapid and clear information transfer. In the

handoff of critically ill patients, the patient's current situation, brief clinical background, assessment, and recommendation should be communicated explicitly. Closed-loop communication confirms that an instruction has been heard and implemented correctly. Especially in high-risk situations such as resuscitation, trauma, stroke, sepsis, acute coronary syndrome, and pediatric emergencies, safety is strengthened when the team leader clearly assigns roles, invites team members to raise warnings, and shares critical decisions aloud.

Short briefing and debriefing practices are also useful in the emergency department. At the beginning of a shift, patient volume, staffing status, critical area capacity, high-risk patients waiting, and anticipated bottlenecks can be shared briefly. A short debriefing after critical events should evaluate not only the clinical outcome, but also team communication and hierarchical behaviors. Questions such as "Who was able to speak?", "Which information arrived late?", "Which warning could have been heard earlier?", and "What will we change in the next similar event?" strengthen the learning capacity of emergency department teams.

Emergency departments are also settings in which hierarchical tensions are intensely experienced in communication with patients and family members. Waiting time, uncertainty, pain, anxiety, and the risk of violence affect not only communication within the team, but also the patient-professional relationship. When a patient or family member notices clinical deterioration, they should be able to communicate it to the team. However, treating the patient's voice as an "ordinary complaint" may cause safety signals to be missed. Therefore, emergency departments should establish clear channels, visible information processes, and safe feedback mechanisms through which patients and families can express their concerns.

**7. Educational Hierarchy, Residents, and Nurses**

Medical hierarchy becomes more pronounced in educational institutions. Medical schools, research hospitals, and specialty training programs are not only institutions that provide services; they also produce professional identity. In these institutions, students and residents learn not only clinical knowledge, but also tacit rules such as "how to speak," "when to remain silent," "whom not to challenge," "which errors are acceptable," and "which behaviors are normal." Therefore, medical hierarchy shapes not only today's patient safety, but also the clinical culture of the future.

Residents occupy a dual position with regard to safety. On the one hand, they carry a major burden of clinical service and assume critical roles in patient care. On the other hand, they are professionals who are being trained, evaluated, and dependent on the approval of senior physicians. This

dependence may make it difficult for residents to ask questions, say "I don't know," or object to a senior decision. When the educational culture reinforces the myth of the "good physician who never makes mistakes," residents may tend to hide their uncertainties. A safe educational environment, however, should teach that making an error is not the main risk; hiding an error is.

For nurses, hierarchy operates both at the interprofessional and intra-institutional levels. Nursing care includes continuous patient observation, medication administration, care planning, patient education, and safe handoff processes. Nevertheless, in some institutions, nurses' contributions to clinical decision-making processes are not sufficiently visible. In a hierarchical culture, a nurse's warning may be perceived as "interfering with the physician's decision." This perception is a serious loss for patient safety because nurses are often the professionals who first detect small but meaningful changes in a patient's condition.

Students and newcomers are also vulnerable to the effects of hierarchy. In clinical practice, students are among the most vulnerable groups in terms of speaking up when they notice an error or risk. They may not be confident in their level of knowledge and may also fear evaluation, exclusion, or humiliation. Therefore, educational institutions should establish not a culture of "the student remains silent and observes," but a culture of "the student asks questions and learns safely." Speaking-up training should go beyond teaching technical communication phrases and should create a structure that makes students and young professionals feel that their voice is legitimate.

**8. Transforming Hierarchy in Favor of Patient Safety: Leadership and Culture**

From a patient safety perspective, the critical question is how hierarchy is managed. Approaches that completely reject hierarchy may overlook the realities of expertise and responsibility in healthcare. However, approaches that regard hierarchy as natural and immutable may normalize silence and insecurity. Therefore, the goal for healthcare organizations is not an "authority-free team," but "questionable authority." Questionable authority refers to a culture in which the leader retains decision-making responsibility, but information is evaluated according to its contribution to safety rather than according to status. The culture of silence and the problem of psychological safety defined in previous sections are transformed in this section into areas for managerial intervention.

The first element in this transformation is inclusive leadership. An inclusive leader is not merely someone who gives orders, but someone who invites the contributions of team members, legitimizes questions, does not punish people

who report errors, and includes the knowledge of different professions in the decision-making process. van Dongen et al. (2024) emphasize that leadership approach holds an important place among organizational factors influencing speaking behavior. It is often not enough for the leader to ask, "Do you have any objections?" What is more decisive is how team members are treated when they actually raise an objection.

The second element is just culture. Just culture is a safety approach that does not automatically blame the person who made an error, but distinguishes between intentional negligence, risky behavior, and learnable system errors. Reason's (2000) systems approach states that human error is inevitable and that organizations must design defense systems that can detect errors. Just culture gives employees the message not that "you will be punished if you report an error," but that "if you report an error, the system will learn; however, knowingly violating safety also entails responsibility."

The third element is the learning organization. A patient safety culture is not limited to conducting root cause analysis only after an event occurs. Near misses, uncertainty, minor disruptions, staff concerns, and patient complaints are also sources of learning. In hierarchical institutions, learning is often seen as top-down training; in safe institutions, however, learning flows in every direction. Information flow from nurse to physician, technician to management, patient to team, and student to senior is possible.

The fourth element is measurement. A culture that is not measured cannot be managed. AHRQ's patient safety culture tools make it possible to monitor areas such as communication openness, communication about errors, event reporting, management support, teamwork, and handoffs (AHRQ, 2024). However, measurement should not be conducted merely to produce survey scores. Measurement results should be shared with units, translated into action plans, and reassessed at regular intervals. Otherwise, surveys may create in staff the feeling that "they asked again, but nothing changed."

**9. Intervention Tools: TeamSTEPPS, SBAR, Briefing, and Debriefing**

Structured team communication is one of the leading tools used to transform hierarchy in favor of patient safety. TeamSTEPPS 3.0, updated by AHRQ, is an evidence-based team approach that focuses on communication, leadership, situation monitoring, and mutual support skills to improve team performance and patient outcomes (AHRQ, 2023). The importance of TeamSTEPPS lies in transforming communication from a matter of individual politeness into a learnable and applicable team skill.

The qualitative systematic review by Gleeson et al. (2023) shows that interprofessional communication in hospital settings may be complicated

by differences in education, roles, status, and professional culture, but that it remains essential for safe care. Dietl et al. (2023) demonstrated that interprofessional communication training can strengthen mechanisms related to psychological safety and perceptions of patient safety.

SBAR is one of the most widely used structured communication tools in healthcare. This method, which consists of the headings Situation, Background, Assessment, and Recommendation, facilitates brief, clear, and clinically focused communication, particularly among team members with differences in seniority. A nurse saying to a physician "the patient is deteriorating" may sometimes be perceived as vague; however, saying "Situation: The patient's oxygen saturation has dropped to 88% in the last 10 minutes. Background: The patient is hospitalized with COPD and pneumonia. Assessment: Respiratory distress is increasing, and the current oxygen support is insufficient. Recommendation: I recommend that you assess the patient now and request an intensive care consultation if necessary" makes the clinical decision-making process safer.

Briefing is a short team meeting held before a procedure or shift. Its purpose is to share the plan, roles, risks, specific patient conditions, and expected problems with the team. Briefing prevents hierarchy from producing one-way orders because it gives team members the opportunity to speak before the procedure begins. Examples include a time-out in the operating room, a shift-start meeting in the intensive care unit, preparation of the trauma team in the emergency department, or a team discussion before a high-risk delivery in the delivery room.

Debriefing is a short learning conversation after an event or procedure. It is conducted around questions such as "What went well?", "What was difficult?", and "What will we change next time?" If debriefing is non-punitive, it can open a learning space even in hierarchical teams. However, if debriefing turns into a meeting in which only the senior person criticizes others, it damages psychological safety. Safe debriefing invites the contribution of the whole team and evaluates the process rather than the person.

Simulation-based training is also a powerful tool for making hierarchy visible. Simulation does not only teach technical skills; it shows how team members communicate under stress, who speaks, who remains silent, how the leader responds to warnings, and how role ambiguity emerges. However, the quality of debriefing is decisive for simulation to be effective. Training should not remain at the level of "say the right sentence"; it should also discuss the cultural and structural factors that prevent speaking within the team.

**10. Positioning Patients and Families Not Outside Hierarchy, but Within Safety**

Discussions of medical hierarchy often focus on relationships among healthcare workers. However, patients and families are also within this hierarchy. In the traditional model, the patient is positioned outside medical knowledge and at the lower level of the decision-making process. This model becomes more pronounced in situations such as low health literacy, language barriers, older age, disability, or migration. A patient may hesitate to ask questions; even if they notice that the wrong medication is being administered, they may remain silent by thinking "the doctor/nurse knows better."

The patient safety approach requires that the patient and family be regarded as safety partners in care. Patients can participate in the verification of their own identity, notice inconsistencies in their medication list, confirm the surgical site, ask questions when they do not understand discharge instructions, and inform the team about changes in their clinical condition. However, for this to occur, the institution must clearly communicate to patients the message that "asking questions is your right and part of your safety."

Patient engagement does not mean reversing hierarchy. The physician and healthcare team retain their clinical knowledge and responsibility; however, the patient is accepted as an expert in their own experience and values. This approach is particularly important in chronic disease management, cancer care, intensive care decisions, palliative care, and discharge planning. In hierarchical cultures, patient engagement often remains at the level of "obtaining a signature on an informed consent form." Genuine engagement, however, requires that the patient understand the options, discuss risks, and be meaningfully included in care decisions.

For this reason, tools such as the teach-back method, plain language, multilingual materials, patient safety cards, medication reconciliation forms, and patient and family advisory councils may be used in patient safety management. Institutions should regard patient complaints not as threats to reputation, but as safety signals that hierarchy may fail to see. A patient complaint is often not merely dissatisfaction; it may also indicate a communication gap, a problem of respect, a care coordination deficit, or a safety risk.

**11. Digitalization, Artificial Intelligence, and New Forms of Hierarchy**

Digitalization in healthcare adds a new dimension to the debate on medical hierarchy. Electronic health records, clinical decision support

systems, artificial intelligence applications, remote monitoring technologies, patient portals, and automated alert systems are not merely tools that accelerate workflow; they also produce new power arrangements that affect whose knowledge becomes more visible, whose knowledge is regarded as more legitimate, and whose decision becomes more decisive. Traditional medical hierarchy is mostly built on seniority, profession, expertise, and institutional position, whereas in the digital age, algorithms, data dashboards, risk scores, and electronic record systems have also become important actors in clinical decision-making processes. Therefore, digitalization should not be regarded as a neutral technological field that eliminates hierarchy, but as a new sociotechnical order that must be managed within a patient safety culture (Sutton et al., 2020; Wachter, 2015).

Digital systems have the potential to strengthen patient safety. Clinical decision support systems may contribute significantly to detecting drug interactions, reducing dosing errors, making critical laboratory results visible, identifying the risk of patient deterioration early, and supporting clinical decisions with evidence-based information. Sutton et al. (2020) state that clinical decision support systems can improve patient safety and quality of care when designed appropriately; however, expected benefits may be limited when these systems are not integrated properly into clinical workflow, create alert fatigue, or weaken user trust. Therefore, the safety contribution of digital tools depends not only on technological accuracy, but also on clinical context, user experience, team communication, and organizational safety culture.

Artificial intelligence applications have a similarly dual effect on patient safety. Choudhury and Asan (2020) show that artificial intelligence can support patient safety in diagnostic support, risk prediction, error reduction, medication management, and patient monitoring. Nevertheless, high technical performance alone is not sufficient for artificial intelligence systems to produce clinical impact. Kelly et al. (2019) draw attention to key challenges in transferring artificial intelligence applications into real clinical settings, including data quality, external validation, integration into clinical workflow, ethical responsibility, explainability, and user trust. In this context, artificial intelligence should be positioned not as an independent authority that replaces clinical judgment, but as a tool that supports human expertise and strengthens team decision-making.

One of the most critical hierarchy-related aspects of digitalization is the risk of algorithmic authority. Whereas the most decisive authority within the clinical team was previously the senior professional or manager, digital systems may create a new "data authority." A risk score, algorithmic alert, or clinical decision support recommendation may influence the direction of decision-

making within the team. In some respects, this is beneficial because the clinical concern of a low-status team member may become more visible when supported by an objective digital alert. For example, if a nurse's observation that a patient is deteriorating is supported by an early warning score, it may become easier for the clinical team to take that warning seriously. However, if algorithms become unquestionable authorities, clinicians' critical judgment, their capacity to evaluate the patient context, and the space for team discussion may weaken. Therefore, digital tools should be designed not as systems that "make the decision," but as systems that make decisions discussable and safer.

Another risk of algorithmic systems is their potential to produce bias or reproduce existing inequalities in invisible ways. Obermeyer et al. (2019) showed that an algorithm used in healthcare could produce systematic bias for certain patient groups. This finding demonstrates that digital systems cannot always be assumed to be neutral and objective. If an algorithm has learned inequalities embedded in historical data, the clinical decision support system may also reproduce these inequalities. Therefore, when using artificial intelligence and algorithmic decision support systems, healthcare organizations should focus not only on accuracy rates, but also on which patient groups the system performs better or worse for, which data it was trained on, in which clinical contexts it is valid, and which decisions it influences.

Electronic health records also carry both opportunities and risks for patient safety. Electronic record systems may strengthen continuity of care, access to data, medication reconciliation, and interdisciplinary information sharing. However, when these systems are poorly designed, they may create a heavy documentation burden for healthcare workers. Sinsky et al. (2016) showed that physicians devote a substantial proportion of their working time to electronic records and desk work. This is not merely an efficiency problem; reduced clinical time, divided attention, burnout, and mechanically completed records may also create risks for patient safety. If management focuses only on record completion rates but does not monitor how these records contribute to clinical decision-making, team coordination, and patient safety, the electronic system may cease to be a safety tool and become part of bureaucratic hierarchy.

Digital systems also transform the patient-professional hierarchy. Patient portals, online laboratory results, personal health records, and digital appointment/messaging systems may increase patients' access to information. Irizarry et al. (2015) state that patient portals carry important potential for patient engagement; however, their use is closely related to patients' digital literacy, access opportunities, and system design. Therefore, patient portals should be evaluated not merely as technical platforms that provide information, but as tools that enable patients to become more active and safer subjects in the care process.

Nevertheless, digitalization may not strengthen patient engagement equally for every patient group. Grossman et al. (2019) emphasize the importance of interventions to increase patient portal use among vulnerable populations. Older adults, individuals with low health literacy, patients with disabilities, people facing language barriers, socioeconomically disadvantaged groups, and those with limited access to technology may benefit less from digital health services. In this case, digitalization may create a new form of digital exclusion rather than reduce the patient-professional hierarchy. Therefore, digital patient safety strategies should focus not only on the existence of technology, but also on who can use it, who is left outside, and to what extent patients can understand the information they obtain and reflect it in care decisions.

For healthcare managers, the core principle in the era of digitalization is this: algorithms, dashboards, electronic records, and artificial intelligence systems do not replace patient safety culture; they become meaningful only within a safe culture. If team members do not trust one another, hesitate to report errors, digital alerts are not taken seriously, or clinical concerns cannot be expressed because of hierarchical pressure, even the most advanced digital system will provide only limited safety benefits. By contrast, in teams with high psychological safety, openness to learning, shared use of data for decision-making, and the ability to evaluate digital tools together with human expertise, technology can become a powerful patient safety instrument. Therefore, digitalization is not a solution that automatically eliminates medical hierarchy; rather, when managed properly, it is a field of transformation that can make hierarchy more transparent, more questionable, and more safety-oriented.

## 12. Conclusion

Medical hierarchy is a structure inherent in healthcare and necessary to a certain extent. The distribution of expertise, seniority, experience, and responsibility may be functional for clinical decision-making, leadership in crises, and team coordination. However, when hierarchy prevents speaking, asking questions, objecting, reporting incidents, and making the voices of patients or staff visible, it becomes an important risk area for patient safety. Therefore, the aim should not be to eliminate hierarchy completely, but to transform it into a safety-oriented structure through psychological safety, open communication, just culture, inclusive leadership, and patient-family engagement.

This study shows that the effect of medical hierarchy on patient safety cannot be explained solely by individual attitudes. A culture of silence, power distance, punitive approaches to error, closed communication, and unquestionable authority may prevent critical information held by clinical

teams from being reflected in decision-making processes. By contrast, in teams with high psychological safety, staff can express risks earlier, errors can become opportunities for learning, and the knowledge of different professional groups can be incorporated more effectively into patient care.

The structure required for patient safety is not an "authority-free team," but "questionable authority." In this approach, leadership and clinical responsibility continue; however, the leader's authority acquires a function that invites team members' contributions, takes warnings seriously, and evaluates safety information independently of status rather than producing silence. In this way, hierarchy ceases to be merely a top-down power relationship and becomes a manageable safety variable that supports open communication and shared learning.

The main implication for healthcare organizations is that the effect of hierarchy on patient safety must be actively managed. For this purpose, inclusive leadership behaviors should be developed, incident reporting should be removed from punitive approaches, speaking-up behavior should be supported, structured communication tools such as TeamSTEPPS, SBAR, briefing, and debriefing should be embedded in daily practice, and safety culture should be measured regularly. In addition, the participation of patients and families in the safety process should be strengthened, and digital systems should be designed as tools that support human judgment and team communication.

In conclusion, medical hierarchy is neither entirely harmful nor entirely beneficial for patient safety in itself. What is decisive is how hierarchy is constructed, experienced, and managed. Safe healthcare organizations do not leave hierarchy as an invisible mechanism of pressure; rather, they reorganize it within an accountable, learning-oriented, psychologically safe, and patient-centered culture.

## References

Agency for Healthcare Research and Quality. (2023). TeamSTEPPS 3.0. Retrieved from https://www.ahrq.gov/teamstepps-program/index.html

Arad, D., Finkelstein, A., Rozenblum, R., & Magnezi, R. (2022). Patient safety and staff psychological safety: A mixed methods study on aspects of teamwork in the operating room. Frontiers in Public Health, 10, Article 1060473. doi:10.3389/fpubh.2022.1060473

Bates, D. W., Levine, D. M., Salmasian, H., Syrowatka, A., Shahian, D. M., Lipsitz, S., ... Mort, E. A. (2023). The safety of inpatient health care. New England Journal of Medicine, 388(2), 142–153. doi:10.1056/NEJMsa2206117

Bookey-Bassett, S., Markle-Reid, M., McKey, C. A., & Akhtar-Danesh, N. (2017). Understanding interprofessional collaboration in the context of chronic disease management for older adults living in communities: A concept analysis. Journal of Advanced Nursing, 73(1), 71–84. doi:10.1111/jan.13162

Dayton, E., & Henriksen, K. (2007). Communication failure: Basic components, contributing factors, and the call for structure. Joint Commission Journal on Quality and Patient Safety, 33(1), 34–47. doi:10.1016/S1553-7250(07)33005-5

Dietl, J. E., Derksen, C., Keller, F. M., & Lippke, S. (2023). Interdisciplinary and interprofessional communication intervention: How psychological safety fosters communication and increases patient safety. Frontiers in Psychology, 14, Article 1164288. doi:10.3389/fpsyg.2023.1164288

Dukhanin, V., Dy, S. M., Sharma, R., Vass, M., Zhang, A., Bass, E. B., & Rosen, M. A. (2023). Patient and family engagement: Rapid response. In Making healthcare safer IV: A continuous updating of patient safety harms and practices. Rockville, MD: Agency for Healthcare Research and Quality. doi:10.23970/AHRQEPC_MHS4ENGAGE

Edmondson, A. C. (1999). Psychological safety and learning behavior in work teams. Administrative Science Quarterly, 44(2), 350–383. doi:10.2307/2666999

Edmondson, A. C. (2003). Speaking up in the operating room: How team leaders promote learning in interdisciplinary action teams. Journal of Management Studies, 40(6), 1419–1452. doi:10.1111/1467-6486.00386

Edmondson, A. C., & Lei, Z. (2014). Psychological safety: The history, renaissance, and future of an interpersonal construct. Annual Review of Organizational Psychology and Organizational Behavior, 1, 23–43. doi:10.1146/annurev-orgpsych-031413-091305

Essex, R., Kennedy, J., Miller, D., & Jameson, J. (2023). A scoping review exploring the impact and negotiation of hierarchy in healthcare organisations. Nursing Inquiry, 30(4), Article e12571. doi:10.1111/nin.12571

Freidson, E. (1970). Professional dominance: The social structure of medical care. New York, NY: Atherton Press.

Freidson, E. (1988). Profession of medicine: A study of the sociology of applied knowledge. Chicago, IL: University of Chicago Press. Original work published 1970

Gleeson, L. L., O'Brien, G. L., O'Mahony, D., & Byrne, S. (2023). Interprofessional communication in the hospital setting: A systematic review of the qualitative literature. Journal of Interprofessional Care, 37(2), 203–213. doi:10.1080/1356 1820.2022.2028746

Grailey, K. E., Murray, E., Reader, T. W., & Brett, S. J. (2021). The presence and potential impact of psychological safety in the healthcare setting: An evidence synthesis. BMC Health Services Research, 21, Article 773. doi:10.1186/s12913-021-06740-6

Henriksen, K., & Dayton, E. (2006). Organizational silence and hidden threats to patient safety. Health Services Research, 41(4 Pt 2), 1539–1554. doi:10.1111/j.1475-6773.2006.00564.x

Hofstede, G. (2001). Culture's consequences: Comparing values, behaviors, institutions, and organizations across nations (2nd ed.). Thousand Oaks, CA: Sage.

Hofstede, G., Hofstede, G. J., & Minkov, M. (2010). Cultures and organizations: Software of the mind: Intercultural cooperation and its importance for survival (3rd ed.). New York, NY: McGraw-Hill.

Kane, J., Munn, L., Kane, S. F., & Srulovici, E. (2023). Defining speaking up in the healthcare system: A systematic review. Journal of General Internal Medicine, 38(15), 3406–3413. doi:10.1007/s11606-023-08322-0

Kim, S., Appelbaum, N. P., Baker, N., Bajwa, N. M., Chu, F., Pal, J. D., ... Bochatay, N. (2020). Patient safety over power hierarchy: A scoping review of healthcare professionals' speaking-up skills training. Journal for Healthcare Quality, 42(5), 249–263. doi:10.1097/JHQ.0000000000000257

King, H. B., Battles, J., Baker, D. P., Alonso, A., Salas, E., Webster, J., ... Salisbury, M. (2008). TeamSTEPPS™: Team strategies and tools to enhance performance and patient safety. In K. Henriksen, J. B. Battles, M. A. Keyes, & M. L. Grady (Eds.), Advances in patient safety: New directions and alternative approaches (Vol. 3: Performance and tools). Rockville, MD: Agency for Healthcare Research and Quality.

Lee, S. E., & Lee, J. W. (2024). Effects of hierarchical unit culture and power distance orientation on nurses' silence behavior: The roles of perceived futility and hospital management support for patient safety. Journal of Nursing Management, 2024, Article ID 6564570. doi:10.1155/jonm/6564570

Leonard, M., Graham, S., & Bonacum, D. (2004). The human factor: The critical importance of effective teamwork and communication in providing safe care. Quality and Safety in Health Care, 13(Suppl. 1), i85–i90. doi:10.1136/qshc.2004.010033

Levine, D. M., Syrowatka, A., Salmasian, H., Shahian, D. M., Lipsitz, S., Zebrowski, J. P., ... Bates, D. W. (2024). The safety of outpatient health care: Review of electronic health records. Annals of Internal Medicine, 177(6), 738–748. doi:10.7326/M23-2063

Manser, T. (2009). Teamwork and patient safety in dynamic domains of healthcare: A review of the literature. Acta Anaesthesiologica Scandinavica, 53(2), 143–151. doi:10.1111/j.1399-6576.2008.01717.x

Massey, D., Chaboyer, W., & Anderson, V. (2017). What factors influence ward nurses' recognition of and response to patient deterioration? An integrative review of the literature. Nursing Open, 4(1), 6–23. doi:10.1002/nop2.53

Mitchell, P., Wynia, M., Golden, R., McNellis, B., Okun, S., Webb, C. E., ... Von Kohorn, I. (2012). Core principles & values of effective team-based health care. NAM Perspectives. doi:10.31478/201210c

Montgomery, A., Chalili, V., Lainidi, O., Mouratidis, C., Maliousis, I., Paitaridou, K., & Leary, A. (2025). Psychological safety and patient safety: A systematic and narrative review. PLOS ONE, 20(4), Article e0322215. doi:10.1371/journal.pone.0322215

Nembhard, I. M., & Edmondson, A. C. (2006). Making it safe: The effects of leader inclusiveness and professional status on psychological safety and improvement efforts in health care teams. Journal of Organizational Behavior, 27(7), 941–966. doi:10.1002/job.413

O'Donovan, R., & McAuliffe, E. (2020a). A systematic review exploring the content and outcomes of interventions to improve psychological safety, speaking up and voice behaviour. BMC Health Services Research, 20, Article 101. doi:10.1186/s12913-020-4931-2

O'Donovan, R., & McAuliffe, E. (2020b). A systematic review of factors that enable psychological safety in healthcare teams. International Journal for Quality in Health Care, 32(4), 240–250. doi:10.1093/intqhc/mzaa025

Okuyama, A., Wagner, C., & Bijnen, A. B. (2014). Speaking up for patient safety by hospital-based health care professionals: A literature review. BMC Health Services Research, 14, Article 61. doi:10.1186/1472-6963-14-61

Reason, J. (2000). Human error: Models and management. BMJ, 320(7237), 768–770. doi:10.1136/bmj.320.7237.768

Skråmm, S. H., Smith Jacobsen, I. L., & Hanssen, I. (2021). Communication as a non-technical skill in the operating room: A qualitative study. Nursing Open, 8(4), 1822–1828. doi:10.1002/nop2.830

Starr, P. (1982). The social transformation of American medicine. New York, NY: Basic Books.

Tyler, E. R., Yalden, O., Fan, L., Ji, S., Kirchner, J., Yount, N. D., & Sorra, J. (2024). Surveys on Patient Safety Culture® (SOPS®) Hospital Survey 2.0: 2024 user database report (AHRQ Publication No. 25-0006). Rockville, MD: Agency for Healthcare Research and Quality.

Umoren, R., Kim, S., Gray, M. M., Best, J. A., & Robins, L. (2022). Interprofessional model on speaking up behaviour in healthcare professionals: A qualitative study. BMJ Leader, 6(1), 15–19. doi:10.1136/leader-2020-000407

van Dongen, D., Guldenmund, F., Grossmann, I., & Groeneweg, J. (2024). Classification of influencing factors of speaking-up behaviour in hospitals: A systematic review. BMC Health Services Research, 24, Article 1657. doi:10.1186/s12913-024-12138-x

Vikan, M., Haugen, A. S., Bøyum, S., & Wiig, S. (2024). The anatomy of safe surgical teams: An interview-based qualitative study. Patient Safety in Surgery, 18, Article 12. doi:10.1186/s13037-024-00389-w

World Health Organization. (2010). Framework for action on interprofessional education and collaborative practice. Geneva, Switzerland: Author.

World Health Organization. (2021). Global patient safety action plan 2021–2030: Towards eliminating avoidable harm in health care. Geneva, Switzerland: Author.

Zaij, S., Pereira Maia, K., Leguelinel-Blache, G., Roux-Marson, C., Kinowski, J. M., & Richard, H. (2023). Intervention of pharmacist included in multidisciplinary team to reduce adverse drug event: A qualitative systematic review. BMC Health Services Research, 23, Article 927. doi:10.1186/s12913-023-09512-6

Chapter 3

# CRISPR/CAS GENOME EDITING FROM EARLY EXPECTATIONS TO CLINICAL REALITY AND BEYOND

*Neslihan Pinar OZATES*[1]
*Cigir BIRAY AVCI*[2]

1 Research Asistant, PhD, Harran University Faculty of Medicine, Medical Biology Department, ORCID: 0000-0001-6856-4644.
2 Professor, Ege University Faculty of Medicine, Medical Biology Department, ORCID: 0000-0001-8251-4520

## 1. INTRODUCTION

The ability to precisely manipulate the genome has long represented one of the central ambitions of molecular medicine. Earlier genome-editing platforms, particularly zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), demonstrated that targeted genetic modification was technically feasible. However, their reliance on complex protein engineering, limited scalability, and substantial technical barriers restricted broader translational applicability(Gaj et al., 2013; Jinek et al., 2012). The emergence of clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) systems fundamentally altered this landscape by introducing a programmable, RNA-guided approach to genome editing that was comparatively simple, adaptable, and experimentally accessible. Once Cas9 was shown to be programmable through guide RNA redesign, CRISPR/Cas rapidly evolved from a bacterial adaptive immune mechanism into a transformative biotechnology platform spanning basic research, agriculture, diagnostics, and medicine(Cong et al., 2013; Mali et al., 2013).

Few biomedical technologies have generated expectations comparable to those associated with CRISPR/Cas systems. Following the demonstration of programmable genome editing in 2012 and its rapid implementation in mammalian cells, the possibility of directly correcting disease-causing mutations appeared increasingly realistic. The awarding of the 2020 Nobel Prize in Chemistry to Jennifer na and Emmanuelle Charpentier further reinforced the perception that genome editing had entered a new therapeutic era(Doudna, 2020; Xu & Li, 2020).

Yet, as CRISPR technologies progressed from proof-of-concept studies toward clinical implementation, the translational reality proved considerably more complex than early optimism suggested. Although landmark milestones-including the approval of the first CRISPR-based therapy for severe hemoglobinopathies and the emergence of in vivo genome editing trials-demonstrated that therapeutic genome editing was no longer theoretical, substantial biological and technical limitations also became apparent. Concerns regarding off-target activity, unpredictable repair outcomes, immune responses, and persistent delivery barriers soon revealed that precision editing remained constrained by biological complexity. At the same time, ethical controversies, particularly surrounding heritable genome editing, exposed tensions between technological capability and societal readiness(Angelini Stewart et al., 2025; Caplan et al., 2015; Modell et al., 2021).

Importantly, the evolution of the field has not been defined solely by its limitations but also by its capacity for adaptation. In response to concerns associated with classical double-strand break-mediated editing, increasingly

sophisticated approaches-including high-fidelity nucleases, base editing, prime editing, transient delivery systems, and programmable regulatory technologies-have emerged in an effort to improve precision and safety. These developments fundamentally changed both the possibilities and limitations of genome editing(Modell et al., 2021).

This chapter critically examines the trajectory of CRISPR/Cas genome editing from scientific breakthrough to clinical implementation. Particular attention is given to technological progress, therapeutic translation, unresolved barriers, safety concerns, ethical considerations, and emerging complementary approaches that may extend beyond classical CRISPR-based editing.

## 2. FROM DISCOVERY TO TRANSLATIONAL GENOME EDITING

The emergence of CRISPR/Cas genome editing represents one of the fastest translational advances in modern biomedicine. Within little more than a decade, a bacterial defense mechanism evolved into a clinically actionable therapeutic platform capable of modifying disease-associated genes in human cells. Its development was closely linked to efforts aimed at overcoming the practical limitations of earlier genome-engineering platforms.

Although repetitive CRISPR loci were first identified in bacterial genomes in the late 1980s, their biological significance remained unclear for many years. Subsequent work established CRISPR-Cas systems as adaptive immune mechanisms in bacteria and archaea, while the identification of tracrRNA clarified key aspects of guide RNA biology and laid the foundation for programmable editing(Barrangou et al., 2007). Unlike ZFNs and TALENs, which required target-specific protein redesign, CRISPR enabled retargeting through simple guide RNA modification. This shift dramatically reduced technical barriers to genome editing and accelerated adoption across diverse biological disciplines. Within a year, CRISPR-Cas9-mediated editing had been demonstrated in mammalian cells. The apparent simplicity, flexibility, and scalability of the system fundamentally altered expectations regarding what genome editing might realistically achieve(Barrangou, 2015).

Despite its transformative potential, classical Cas9 editing soon revealed important limitations. The system relies primarily on the induction of double-strand DNA breaks (DSBs), followed by endogenous DNA repair pathways such as non-homologous end joining (NHEJ) or homology-directed repair (HDR). Although effective for gene disruption or correction under selected conditions, DSB-mediated editing introduced substantial unpredictability. Repair outcomes varied considerably between cell types and genomic contexts, while unintended consequences, including large deletions and structurally unpredictable repair outcomes. In parallel, concerns regarding

off-target cleavage raised questions about the feasibility of translating genome editing into routine therapeutic settings(Xue & Greene, 2021).

These limitations stimulated diversification within the CRISPR toolbox. Alternative nucleases, including Cas12a and smaller Cas9 orthologs, expanded targeting flexibility and improved delivery compatibility, while RNA-targeting systems such as Cas13 introduced the possibility of reversible transcriptome editing without permanent genomic modification(Abudayyeh et al., 2016; Zetsche et al., 2015).

Perhaps the most consequential transition involved the movement away from DSB-dependent editing toward precision genome modification. Base editing, first introduced in 2016, enabled targeted nucleotide substitutions without generating double-strand breaks, substantially reducing dependence on error-prone repair pathways. Cytidine and adenine base editors subsequently expanded the range of editable pathogenic variants, particularly for single-nucleotide disorders. More recently, prime editing further broadened editing possibilities by enabling precise substitutions, insertions, and deletions through a "search-and-replace" mechanism that combines Cas nickase activity with reverse transcription. These advances marked a shift from genome cutting toward increasingly controlled genome rewriting(Anzalone et al., 2019; Komor et al., 2016).

At the same time, advances in specificity engineering and delivery technologies became central to translational progress. High-fidelity Cas variants and computational guide optimization improved editing precision, while ex vivo and in vivo delivery strategies gradually expanded therapeutic feasibility. Nevertheless, broad tissue accessibility remained limited, highlighting that successful genome editing depends not only on editing chemistry but also on effective biological delivery(R. Zhao et al., 2026).

Collectively, the evolution of CRISPR reflects a broader shift in genome engineering-from proof-of-concept editing toward clinically oriented precision intervention. Yet, as technical capabilities expanded, expectations surrounding safety, deliverability, and therapeutic applicability also intensified, raising an increasingly important question: to what extent has CRISPR fulfilled its early promise?

## 3. WHAT WERE THE EXPECTATIONS OF CRISPR/CAS GENOME EDITING?

The emergence of CRISPR/Cas systems as programmable genome-editing tools generated an unusual degree of scientific optimism. Unlike many biomedical technologies that develop gradually through incremental

innovation, CRISPR entered the field with exceptional momentum. The rapid transition from mechanistic discovery to functional editing in mammalian cells, combined with the relative simplicity of guide RNA programming, fostered expectations that genome engineering might become faster, more precise, and therapeutically transformative. Importantly, these expectations were not merely products of speculative enthusiasm or media attention, but were grounded in legitimate scientific observations regarding the apparent flexibility and efficiency of CRISPR-mediated editing(Doudna & Charpentier, 2014; Hsu et al., 2014).

Among the earliest expectations surrounding CRISPR/Cas systems was programmability. Earlier genome-editing technologies, particularly zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), required target-specific protein redesign, limiting scalability and broader experimental adoption. By contrast, CRISPR-Cas9 introduced a fundamentally different framework in which editing specificity could be redirected through guide RNA modification. This apparent simplicity generated expectations that genome editing would become broadly accessible across research settings, enabling rapid expansion in functional genomics, disease modeling, biotechnology, and synthetic biology. In many respects, these expectations were fulfilled remarkably quickly, as CRISPR technologies became integrated into diverse areas of biomedical research. At the same time, expanding accessibility revealed an important distinction between technical usability and biological reproducibility. Editing outcomes frequently remained dependent on chromatin accessibility, repair pathway activity, cellular context, and guide efficiency, complicating assumptions regarding universal reproducibility (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013).

Beyond programmability, another major expectation concerned precision and safety. Because CRISPR-mediated cleavage relies on sequence complementarity, early perceptions often framed the technology as a highly selective molecular tool capable of correcting pathogenic mutations with minimal unintended consequences. However, this expectation soon proved more complex in practice. Editing outcomes depended not only on target recognition but also on endogenous cellular repair mechanisms, introducing biological variability that complicated assumptions regarding perfectly precise genome modification. Increasingly, precision came to be understood not simply as accurate target recognition, but as the capacity to achieve biologically predictable outcomes with acceptable therapeutic risk(Hsu et al., 2013; Tsai & Joung, 2016).

Beyond technical accessibility and precision, expectations increasingly expanded toward therapeutic transformation. Equally important was the

expectation that genome editing might provide durable therapeutic benefit, particularly for inherited disorders lacking effective treatment options. The prospect of correcting disease-causing mutations through a single intervention generated considerable enthusiasm, raising the possibility that genome editing could move medicine beyond lifelong symptom management toward more sustained biological correction. This expectation gained momentum as CRISPR technologies rapidly progressed from preclinical models toward clinical trials in inherited disorders, metabolic diseases, retinal conditions, and cancer immunotherapy. At the same time, early optimism often underestimated the biological, logistical, and regulatory challenges associated with therapeutic implementation, particularly regarding safe delivery and large-scale clinical feasibility(Cox et al., 2015; Doudna, 2020; Musunuru & Urnov, 2025).

A further expectation concerned broad biological applicability. Once programmable editing became technically feasible, it appeared reasonable to assume that CRISPR systems could eventually be delivered across diverse tissues and disease settings. In practice, however, delivery rapidly emerged as one of the major translational bottlenecks, shaped by immune barriers, tissue accessibility, and vector-related limitations. This realization substantially reshaped expectations surrounding therapeutic universality, making it increasingly evident that successful editing depends not only on identifying pathogenic targets, but also on whether particular tissues are biologically accessible to therapeutic intervention. Nevertheless, significant advances in lipid nanoparticles, viral vectors, and transient delivery systems demonstrated that in vivo genome editing is technically achievable in selected contexts. Yet, the gap between proof-of-concept success and universally deployable therapeutic editing remains substantial(Gillmore et al., 2021; Lino et al., 2018).

Collectively, the expectations surrounding CRISPR/Cas genome editing reflected both scientific possibility and technological optimism. While programmability and research accessibility were realized rapidly, expectations regarding precision, broad delivery, and universal therapeutic applicability proved considerably more complex. This distinction ultimately shaped the evolving debate surrounding whether CRISPR fulfilled the transformative promise associated with its scientific rise(Alariqi et al., 2025; Feierman et al., 2026).

## 4. SCIENTIFIC AND TECHNOLOGICAL ADVANCES SINCE THE NOBEL ERA

The years following the widespread adoption of CRISPR-Cas9 were marked by rapid technological refinement. While early applications revealed important limitations-including off-target activity, unpredictable repair outcomes, and delivery challenges-these barriers accelerated the development of increasingly sophisticated editing strategies. In many respects, the post-

Nobel evolution of CRISPR can be understood as an effort to improve precision, safety, and therapeutic feasibility.

Among the earliest barriers to clinical translation was editing specificity. Although guide RNA-directed targeting enabled unprecedented programmability, studies soon demonstrated that Cas9-mediated cleavage could occur at partially homologous genomic sites. In therapeutic settings, where unintended genomic alterations may carry long-term consequences, even infrequent off-target events became important safety concerns. This realization shifted attention toward improving editing precision through active engineering and empirical validation (Hsu et al., 2014).

In response, increasingly refined nuclease systems were developed to improve specificity without compromising editing efficiency. High-fidelity variants, including eSpCas9, SpCas9-HF1, and HypaCas9, reduced nonspecific DNA interactions and improved mismatch discrimination. At the same time, genome-wide mapping approaches such as GUIDE-seq, CIRCLE-seq, and CHANGE-seq transformed specificity assessment from theoretical prediction into empirical measurement. Although off-target activity was reduced substantially, it became increasingly clear that editing outcomes remained influenced by genomic context and individual genetic variation. Importantly, advances in genome-wide off-target mapping substantially reshaped how editing safety was evaluated. Rather than relying solely on computational prediction, platforms such as GUIDE-seq, CIRCLE-seq, and CHANGE-seq enabled systematic empirical assessment of editing outcomes across diverse genomic settings. As a result, specificity came to be understood less as an intrinsic property of CRISPR systems and more as a measurable characteristic shaped by guide design, chromatin accessibility, and individual genomic variation. This transition contributed to the growing emphasis on variant-aware editing strategies capable of accounting for patient-specific genomic diversity (J. S. Chen et al., 2017; Huang et al., 2022; Lazzarotto et al., 2020; Slaymaker et al., 2016; Tsai et al., 2015, 2017).

At the same time, another important limitation of classical CRISPR-Cas9 became increasingly apparent: its dependence on double-strand DNA breaks (DSBs). Although DSB formation enabled efficient genome disruption, reliance on endogenous repair pathways frequently introduced unpredictable outcomes and variable editing efficiency across biological contexts. As a result, the field gradually shifted away from a "cut-and-repair" paradigm toward strategies capable of more controlled genome modification(Gaudelli et al., 2017).

This transition became particularly evident with the emergence of base editing technologies. By coupling catalytically modified Cas proteins with nucleotide deaminases, base editors enabled targeted nucleotide conversion

without generating double-strand breaks. This approach substantially expanded the therapeutic scope of genome editing, particularly for diseases caused by single-nucleotide variants, while reducing reliance on error-prone repair mechanisms(Rees & Liu, 2018).

Nevertheless, base editing introduced new challenges of its own. Editing within defined activity windows raised concerns regarding unintended bystander modifications, while deaminase-associated off-target effects affecting DNA and RNA substrates also emerged as important considerations. These limitations reinforced the need for continued refinement rather than representing a definitive solution to precision editing. Importantly, these findings also reshaped broader expectations regarding precision genome engineering. Rather than representing a universally corrective technology, base editing increasingly came to be viewed as a context-dependent intervention whose therapeutic suitability depends on mutational architecture, tissue biology, and clinically acceptable safety thresholds. In this regard, the emergence of base editing illustrated a recurring pattern within CRISPR development: technological refinement frequently solved one limitation while simultaneously revealing new layers of biological complexity(Xue & Greene, 2021).

A further advance emerged with the introduction of prime editing in 2019. Unlike base editing, which is largely restricted to transition mutations, prime editing expanded the range of editable variants by enabling targeted substitutions, insertions, and deletions without requiring donor templates or complete double-strand DNA cleavage. By combining Cas9 nickase activity with reverse transcription, prime editing introduced a more flexible framework for precision genome modification. The broader therapeutic interest surrounding prime editing emerged largely from its expanded mutational flexibility. Because the system does not depend on donor DNA templates and can theoretically address a wider spectrum of pathogenic variants than earlier editing platforms, prime editing was increasingly viewed as a potential bridge between experimental precision and broader therapeutic applicability. Although still at relatively early translational stages, recent clinical observations suggest that highly controlled genome rewriting may be approaching practical feasibility (Anzalone et al., 2019; P. J. Chen & Liu, 2023).

Despite its versatility, prime editing also introduced practical challenges. Editing efficiency remained variable across genomic contexts, and the larger molecular architecture complicated delivery compared with earlier editing systems. Nevertheless, recent clinical reports suggest that precision genome rewriting is beginning to transition from experimental possibility toward therapeutic application(Hyeon et al., 2025).

As editing systems became more sophisticated, delivery emerged as one of the principal challenges in genome engineering. Precise DNA modification alone proved insufficient if editing machinery could not be transported safely and efficiently to target tissues at therapeutically meaningful levels(Lino et al., 2018).

Distinct delivery strategies gradually emerged to support therapeutic genome editing. Ex vivo approaches enabled controlled editing of patient-derived cells prior to reinfusion, while in vivo strategies increasingly relied on lipid nanoparticles and viral vectors optimized for selected tissues. Although these advances substantially improved therapeutic feasibility, broad tissue accessibility remained limited, highlighting delivery as a persistent barrier to wider clinical implementation(Gillmore et al., 2021; Lino et al., 2018; Yin et al., 2017).

At the same time, efforts increasingly focused on improving delivery precision and temporal control. Transient expression systems, organ-targeted nanoparticles, and optimized vector engineering aimed to reduce prolonged nuclease exposure while improving tissue specificity and therapeutic safety(Alsaiari et al., 2024).

Alongside experimental advances, computational biology became increasingly integrated into genome-editing design. Guide RNA selection progressively shifted from empirical testing toward predictive modeling informed by large-scale genomic datasets. Machine learning approaches improved prediction of guide efficiency, off-target activity, and repair outcomes, while emerging evidence that human genetic variation influences editing behavior accelerated interest in variant-aware genome-editing strategies. Increasingly, genome editing began to rely not only on molecular engineering but also on predictive biology. The integration of computational learning approaches into guide optimization, repair prediction, and patient-specific variant interpretation raised the possibility that future editing strategies may become progressively individualized. In many respects, computational modeling emerged as an important complement to experimental genome engineering, helping transform CRISPR from an experimentally accessible tool into a more biologically informed therapeutic platform(Du et al., 2026; Saeed et al., 2026; Q. Zhao et al., 2026).

Collectively, these developments transformed CRISPR from an experimentally accessible nuclease system into a more sophisticated therapeutic platform. Advances in specificity engineering, precision editing, delivery technologies, and computational optimization substantially expanded the scope of genome modification while simultaneously reshaping expectations regarding what genome editing could realistically achieve. These

advances laid the foundation for the next critical question: to what extent has technological progress translated into meaningful clinical success?

## 5. CLINICAL TRANSLATION: HAS CRISPR DELIVERED?

The transition of CRISPR/Cas systems from experimental genome engineering to therapeutic intervention represents one of the most closely watched developments in modern medicine. Although early expectations suggested that programmable editing might rapidly enable curative therapies across diverse diseases, clinical translation has proven more selective than initially anticipated. Therapeutic success emerged unevenly across disease contexts, revealing that the feasibility of genome editing depends not only on editing technology but also on disease biology, tissue accessibility, and delivery constraints(Doudna, 2020; Musunuru & Urnov, 2025).

The clearest evidence that CRISPR has delivered on part of its therapeutic promise comes from hematological disorders. Diseases affecting the hematopoietic system proved particularly suitable for genome editing because target cells can be isolated, modified ex vivo, characterized under controlled conditions, and subsequently reinfused into patients. This workflow substantially reduced uncertainties surrounding delivery and editing outcomes, making blood disorders especially favorable for translational implementation(Frangoul et al., 2021).

A major milestone in this context was the regulatory approval of exagamglogene autotemcel (exa-cel; CASGEVY), the first CRISPR-based therapy approved for severe sickle cell disease (SCD) and transfusion-dependent β-thalassemia (TDT). By reactivating fetal hemoglobin production through targeted disruption of the erythroid enhancer of *BCL11A*, the therapy demonstrated that genome editing could achieve clinically meaningful and durable benefit in human disease. More broadly, CASGEVY marked a historic transition from experimental genome engineering toward therapeutic reality(Singh et al., 2024).

Importantly, the success of CASGEVY reflects more than editing precision alone. Hematopoietic stem cells represent a comparatively accessible therapeutic target, while clinically meaningful benefit can often be achieved without correcting every affected cell. In many respects, hematological disorders provided an ideal biological setting for demonstrating the feasibility of genome-editing medicine.

Nevertheless, the success of ex vivo editing also highlights important limitations. Current therapies remain technically demanding, resource intensive, and dependent on individualized manufacturing workflows.

In addition, myeloablative conditioning continues to impose substantial treatment burden, raising important questions regarding scalability, affordability, and broader accessibility.

Beyond ex vivo applications, increasing attention has focused on the feasibility of in vivo genome editing, in which editing systems are delivered directly into affected tissues. Among early efforts, liver-directed interventions produced some of the most encouraging results, supported by the relative accessibility of hepatic tissue and favorable delivery characteristics. The first systemic in vivo CRISPR trials targeting transthyretin amyloidosis (ATTR) demonstrated that lipid nanoparticle-mediated delivery could achieve durable suppression of disease-associated proteins following a single administration. Similar early findings in hereditary angioedema further suggested that therapeutic editing may be achievable in selected settings where tissue targeting is biologically favorable. Together, these studies provided important proof-of-concept evidence that genome editing is not confined to ex vivo manipulation(*ATTR Amyloidosis MAGNITUDE Clinical Trials Temporarily on Hold*, n.d.; *News: CRISPR Trial Update: NTLA-2002 For Hereditary Angioedema - CRISPR Medicine*, n.d.; Gillmore et al., 2021; Massone et al., 2012; Musunuru & Urnov, 2025).

At the same time, these achievements also revealed an important limitation: successful in vivo editing remained concentrated in biologically accessible tissues, particularly the liver. Extending comparable therapeutic efficiency to organs such as skeletal muscle, lung, heart, and the central nervous system continues to represent a considerably greater challenge(Sahel et al., 2023).

Applications in oncology present a more nuanced picture. CRISPR-based approaches have increasingly been explored in engineered immune-cell therapies, particularly through modifications intended to enhance tumor targeting and immune persistence. Although early CRISPR-modified T-cell studies demonstrated feasibility, clinical outcomes remain variable, reflecting the biological complexity and heterogeneity of malignant disease(Rehman & Abbas, 2026; Stadtmauer et al., 2020).

Similarly, applications involving neurological, muscular, and inherited retinal disorders remain at earlier translational stages. While localized editing strategies demonstrated proof-of-concept feasibility in selected settings, broader correction across complex tissues continues to be constrained by delivery barriers, tissue heterogeneity, and safety concerns(R. Zhao et al., 2026).

Collectively, current clinical evidence suggests that CRISPR has achieved meaningful success, but in a highly context-dependent manner. The strongest outcomes have emerged in biologically tractable settings, particularly ex vivo

hematological editing and selected liver-directed interventions where delivery challenges are comparatively manageable. By contrast, expectations regarding universal therapeutic applicability remain substantially unmet. In this sense, the clinical history of CRISPR may be understood not as a story of failure, but of selective success shaped by biological feasibility.

## 6. WHY HAVE EXPECTATIONS NOT BEEN FULLY MET?

The extraordinary expectations surrounding CRISPR/Cas genome editing emerged from legitimate scientific achievements. Yet, despite remarkable technological progress, therapeutic implementation proved more selective and gradual than initially anticipated. Importantly, this gap does not necessarily reflect technological failure. Rather, it highlights an increasingly evident reality: genome editing proved easier to conceptualize than to implement within the complexity of living biological systems(Deokar et al., 2024; Doudna, 2020).

One of the earliest and most persistent concerns involved off-target editing. Although CRISPR systems are designed to recognize specific genomic sequences, imperfect target discrimination may result in unintended cleavage at partially homologous sites. While engineering advances substantially reduced this risk, complete elimination remained difficult because editing outcomes depend not only on sequence complementarity but also on chromatin structure, genomic context, and cellular state(Lopes & Prasad, 2023).

Equally important, growing evidence suggested that even correctly targeted editing may generate unanticipated genomic consequences. Rather than guaranteeing predictable outcomes, editing at intended loci occasionally produced structurally complex repair events and biologically variable outcomes. These findings substantially reshaped how precision came to be understood in genome engineering, shifting emphasis from accurate cleavage alone toward predictable therapeutic outcomes(Aida et al., 2020; Hunt et al., 2023).

A related concern involved the cellular consequences of DNA damage itself. Because classical CRISPR-Cas9 editing frequently relies on double-strand DNA breaks, endogenous stress-response pathways may be activated following editing. Particular attention focused on p53 signaling, raising concerns that DNA damage responses could influence editing efficiency or potentially favor biologically undesirable cellular outcomes. Although the long-term clinical relevance remains debated, these findings reinforced the need to evaluate genome editing within broader cellular contexts(Aussel et al., 2025; Haapaniemi et al., 2018; Ihry et al., 2018; Kosicki et al., 2018).

Beyond genomic concerns, immunogenicity emerged as another important translational challenge. Because several CRISPR nucleases originate from bacterial species commonly encountered by humans, pre-existing immune responses against Cas proteins may influence both safety and therapeutic efficacy. These concerns appear particularly relevant for systemic and repeated in vivo applications, where immune recognition may complicate sustained treatment(Charlesworth et al., 2019; Mehta & Merkel, 2019).

Nevertheless, perhaps the most consequential limitation has proven to be biological delivery. Even highly precise editing systems offer limited therapeutic value if they cannot be transported safely and efficiently to target tissues. Although ex vivo approaches achieved considerable success in hematological disorders, broader in vivo implementation remained restricted by tissue accessibility, vector limitations, biodistribution challenges, and immune constraints. Increasingly, the major obstacle facing genome-editing medicine shifted from editing chemistry itself toward the problem of clinically effective delivery(Lino et al., 2018; Rostami et al., 2024).

Practical considerations further complicated broader implementation. Genome-editing therapies remain technically demanding, expensive, and dependent on specialized manufacturing pipelines. While early clinical successes demonstrated therapeutic feasibility, questions regarding affordability, scalability, and equitable access continue to limit widespread implementation (Shi et al., 2026).

Ethical concerns also continue to shape the future of genome editing. Although somatic editing is increasingly accepted in severe disease settings, heritable germline modification remains deeply controversial. More broadly, ongoing debates surrounding long-term safety, informed consent, equitable access, and regulatory oversight underscore that responsible implementation must evolve alongside technological capability(Ortiz-Bueno et al., 2026).

Taken together, the limitations preventing CRISPR from fully meeting early expectations reflect an important shift in perspective. Initial concerns focused primarily on whether genome editing could be achieved at all; contemporary challenges instead center on whether editing can be implemented safely, predictably, and at clinically meaningful scale. In many respects, genome editing advanced faster than the biological systems required to support its widespread therapeutic application. Understanding how the field responded to these barriers is therefore essential for evaluating whether current limitations represent temporary engineering obstacles or more fundamental biological constraints.

## 7. BEYOND CLASSICAL CRISPR: EMERGING ALTERNATIVES AND COMPLEMENTARY TECHNOLOGIES

As genome-editing technologies matured, it became increasingly clear that no single platform would be universally optimal across all biological contexts. Rather than replacing CRISPR, recent developments expanded the genome-engineering landscape into a more diverse ecosystem of complementary approaches tailored to different therapeutic needs(Doudna, 2020).

Earlier genome-editing systems, particularly TALENs and zinc-finger nucleases (ZFNs), continue to retain relevance in selected applications. Although largely overshadowed by CRISPR because of greater engineering complexity, these platforms may still offer advantages in highly specific targeting contexts and remain clinically relevant in selected cell-therapy settings(Gaj et al., 2013).

At the same time, RNA-targeting systems such as Cas13 introduced the possibility of reversible molecular intervention without permanent genomic modification. Such approaches may prove particularly valuable in settings where transient therapeutic effects are preferable or irreversible DNA editing raises safety concerns(Abudayyeh et al., 2016; Zetsche et al., 2015).

Additional strategies, including CRISPR interference (CRISPRi), CRISPR activation (CRISPRa), and epigenome editing, further expanded the therapeutic landscape by enabling modulation of gene expression without altering DNA sequence itself. These approaches may prove especially valuable in disorders driven by dysregulated gene expression rather than discrete pathogenic mutations(Chavez et al., 2015; Hilton et al., 2015; Qi et al., 2013; Seem et al., 2024). Recent discoveries continue to expand this landscape even further. For example, the characterization of RNA-triggered cell killing mediated by CRISPR–Cas12a2 demonstrates that newly identified CRISPR-associated systems may enable functions extending well beyond conventional genome editing, highlighting the continuing diversification of programmable CRISPR biology (Scholz et al., 2026).

Emerging technologies-including CRISPR-associated transposases (CAST systems) and programmable recombination platforms-suggest that the future of genome engineering may extend beyond conventional editing toward increasingly flexible forms of targeted genome modification. Although many remain at early developmental stages, these systems illustrate the continued diversification of programmable biology(Strecker et al., 2019).

Ultimately, the emergence of complementary technologies does not suggest that CRISPR failed to meet expectations. Rather, it reflects a broader realization that no single editing platform is likely to address the full diversity

of biological and clinical challenges. As genome engineering matured, the emphasis gradually shifted from identifying one dominant technology toward selecting the most appropriate tool for a given therapeutic context. This transition raises an important question: should CRISPR be evaluated solely according to its original expectations, or also by the broader technological ecosystem it helped create?

## 8. HAVE EXPECTATIONS BEEN MET?

Determining whether CRISPR/Cas genome editing fulfilled expectations requires distinguishing between scientific possibility, technological implementation, and clinical reality. From the outset, CRISPR generated unusually ambitious expectations, driven by the prospect that programmable genome editing might fundamentally transform medicine. More than a decade after its emergence as a therapeutic platform, it is increasingly possible to assess which of these expectations have been realized, which remain only partially fulfilled, and which proved more complex than initially anticipated(Doudna, 2020; X. Zhang et al., 2025).

In several important respects, CRISPR clearly met or even exceeded expectations. Perhaps the most transformative achievement concerns programmability and accessibility. Genome engineering, once dependent on highly specialized protein design, became experimentally accessible to a far broader scientific community. Functional genomics, disease modeling, biotechnology, and therapeutic discovery accelerated dramatically, fulfilling expectations that genome editing could become a widely usable research platform.

Expectations regarding therapeutic benefit have also been partially realized. The approval of CRISPR-based therapies for severe hemoglobinopathies, together with successful early-stage in vivo editing in selected diseases, demonstrated that genome editing can achieve meaningful clinical outcomes under favorable biological conditions. Importantly, these advances established proof-of-principle that programmable editing can function as a therapeutic modality rather than solely an experimental technology(Kabrah, 2025; Navarro et al., 2025).

Yet, many expectations were fulfilled only partially. Early perceptions frequently framed CRISPR as a highly precise molecular tool capable of selectively correcting pathogenic mutations. Although major advances substantially improved editing control, precision proved more biologically contingent than initially expected. Off-target activity, repair unpredictability, delivery barriers, and interindividual genomic variability revealed that successful editing depends not only on molecular targeting, but also on broader biological context(Alariqi et al., 2025).

Expectations regarding universal therapeutic applicability also evolved considerably. Clinical success proved strongest in biologically tractable settings, particularly ex vivo editing and selected tissue-specific interventions where delivery challenges were comparatively manageable. By contrast, diseases requiring widespread systemic correction or access to complex tissues remain substantially more difficult to address(Chakraborty et al., 2026; Ma & Qi, 2024).

Perhaps most importantly, CRISPR did not simply fulfill expectations-it reshaped them. Early narratives often envisioned genome editing as a singular solution centered on increasingly precise DNA cutting. Yet, the field instead evolved into a broader ecosystem involving precision editors, RNA editing, epigenome engineering, and programmable gene regulation. In retrospect, one of CRISPR's greatest achievements may lie not only in what it accomplished directly, but also in how profoundly it transformed the landscape of precision medicine. At the same time, important expectations remain unresolved. Universal tissue delivery, predictable editing across diverse biological settings, affordability, long-term safety, and equitable access continue to limit broader therapeutic implementation. Increasingly, the remaining barriers appear biological and logistical rather than purely technological (Alariqi et al., 2025).

Ultimately, whether CRISPR fulfilled expectations depends largely on how those expectations are defined. If judged against early assumptions that genome editing would rapidly become universally safe, broadly accessible, and applicable across virtually all genetic diseases, the answer remains incomplete. However, if evaluated according to whether CRISPR fundamentally transformed biomedical science, enabled previously unimaginable therapeutic strategies, and established genome editing as a clinically viable discipline, the answer is unequivocally affirmative.

## 9. FUTURE PERSPECTIVES AND CONCLUDING REMARKS

The trajectory of CRISPR/Cas genome editing over the past decade illustrates both the extraordinary pace of technological innovation and the complexity of translating molecular advances into clinical medicine. What began as a bacterial defense mechanism rapidly evolved into one of the most influential biotechnological platforms in modern biomedicine. Yet, as this chapter has highlighted, the evolution of CRISPR has been shaped as much by its limitations as by its successes(Azeez et al., 2024; Iyer et al., 2025).

Looking forward, the future of genome editing will likely depend less on whether DNA can be modified and more on how safely, predictably, and selectively such interventions can be implemented. Although advances in precision editing substantially improved specificity and flexibility,

challenges involving delivery, long-term safety, and reproducibility across diverse biological settings remain substantial barriers to broader therapeutic implementation(Ciganek et al., 2026).

One of the most important future directions concerns the expansion of in vivo precision editing beyond biologically favorable tissues. While liver-directed interventions demonstrated that systemic editing is clinically achievable, extending comparable efficiency to more complex organs-including muscle, lung, heart, and the central nervous system-remains considerably more difficult. Future progress will likely depend as much on delivery innovation as on editing chemistry itself (K. Zhang et al., 2024).

At the same time, genome engineering is increasingly moving beyond permanent DNA modification toward more controllable forms of intervention. RNA editing, epigenome engineering, reversible regulation, and programmable gene modulation suggest that future precision medicine may rely on multiple complementary strategies tailored to specific biological contexts(Pacesa et al., 2024; R. Zhao et al., 2026).

Advances in artificial intelligence and predictive biology may further improve therapeutic precision. Increasingly accurate models of guide RNA performance, repair outcomes, and patient-specific genomic variation raise the possibility that genome editing may become progressively more individualized and biologically predictable(R. Zhao et al., 2026).

Nevertheless, scientific progress alone will not determine the long-term impact of CRISPR-based medicine. Ethical governance, affordability, equitable access, regulatory oversight, and long-term monitoring will remain essential components of responsible implementation(Deokar et al., 2024; Ortiz-Bueno et al., 2026).

Whether CRISPR ultimately fulfills all of its original expectations remains uncertain. Yet, its influence on biomedical science is already undeniable. More than a technological breakthrough, CRISPR initiated a broader transition toward programmable medicine-one in which therapeutic intervention depends not only on understanding biology, but increasingly on learning how to rewrite it with precision, restraint, and responsibility.

## REFERENCES

Abudayyeh, O. O., Gootenberg, J. S., Konermann, S., Joung, J., Slaymaker, I. M., Cox, D. B. T., Shmakov, S., Makarova, K. S., Semenova, E., Minakhin, L., Severinov, K., Regev, A., Lander, E. S., Koonin, E. V., & Zhang, F. (2016). C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science (New York, N.Y.)*, *353*(6299). https://doi.org/10.1126/SCIENCE.AAF5573

Aida, T., Wilde, J. J., Yang, L., Hou, Y., Li, M., Xu, D., Lin, J., Qi, P., Lu, Z., & Feng, G. (2020). Prime editing primarily induces undesired outcomes in mice. *BioRxiv*, 2020.08.06.239723. https://doi.org/10.1101/2020.08.06.239723

Alariqi, M., Ramadan, M., Yu, L., Hui, F., Hussain, A., Zhou, X., Yu, Y., Zhang, X., & Jin, S. (2025). Enhancing Specificity, Precision, Accessibility, Flexibility, and Safety to Overcome Traditional CRISPR/Cas Editing Challenges and Shape Future Innovations. *Advanced Science*, *12*(28), e2416331. https://doi.org/10.1002/ADVS.202416331

Alsaiari, S. K., Eshaghi, B., Du, B., Kanelli, M., Li, G., Wu, X., Zhang, L., Chaddah, M., Lau, A., Yang, X., Langer, R., & Jaklenec, A. (2024). CRISPR–Cas9 delivery strategies for the modulation of immune and non-immune cells. *Nature Reviews Materials 2024 10:1*, *10*(1), 44–61. https://doi.org/10.1038/s41578-024-00725-7

Angelini Stewart, A., Ahrens-Nicklas, R. C., Tsai, S. Q., Musunuru, K., Giannikopoulos, P., & Clelland, C. D. (2025). Measurement and clinical interpretation of CRISPR off-targets. *Nature Genetics*, *58*(1), 20. https://doi.org/10.1038/S41588-025-02428-3

Anzalone, A. V., Randolph, P. B., Davis, J. R., Sousa, A. A., Koblan, L. W., Levy, J. M., Chen, P. J., Wilson, C., Newby, G. A., Raguram, A., & Liu, D. R. (2019). Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature*, *576*(7785), 149–157. https://doi.org/10.1038/S41586-019-1711-4

*ATTR Amyloidosis MAGNITUDE Clinical Trials Temporarily on Hold*. (n.d.). Retrieved June 17, 2026, from https://arci.org/magnitudehold/

Aussel, C., Cathomen, T., & Fuster-García, C. (2025). The hidden risks of CRISPR/Cas: structural variations and genome integrity. *Nature Communications 2025 16:1*, *16*(1), 7208-. https://doi.org/10.1038/s41467-025-62606-z

Azeez, S. S., Hamad, R. S., Hamad, B. K., Shekha, M. S., & Bergsten, P. (2024). Advances in CRISPR-Cas technology and its applications: revolutionising precision medicine. *Frontiers in Genome Editing*, *6*, 1509924. https://doi.org/10.3389/FGEED.2024.1509924/FULL

Barrangou, R. (2015). Diversity of CRISPR-Cas immune systems and molecular machines. *Genome Biology*, *16*(1), 247. https://doi.org/10.1186/S13059-015-0816-9

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D. A., & Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science (New York, N.Y.)*, *315*(5819), 1709–1712. https://doi.org/10.1126/SCIENCE.1138140

Caplan, A. L., Parent, B., Shen, M., & Plunkett, C. (2015). No time to waste—the ethical challenges created by CRISPR: CRISPR/Cas, being an efficient, simple, and cheap technology to edit the genome of any organism, raises many ethical and regulatory issues beyond the use to manipulate human germ line cells. *EMBO Reports*, *16*(11), 1421. https://doi.org/10.15252/EMBR.201541337

Chakraborty, C., Bhattacharya, M., Das, A., Agoramoorthy, G., & Lee, S. S. (2026). CRISPR-Cas9-mediated therapeutics: Current clinical trials and therapy approval landscape to treat human diseases. *Molecular Therapy. Nucleic Acids*, *37*(1), 102859. https://doi.org/10.1016/J.OMTN.2026.102859

Charlesworth, C. T., Deshpande, P. S., Dever, D. P., Camarena, J., Lemgart, V. T., Cromer, M. K., Vakulskas, C. A., Collingwood, M. A., Zhang, L., Bode, N. M., Behlke, M. A., Dejene, B., Cieniewicz, B., Romano, R., Lesch, B. J., Gomez-Ospina, N., Mantri, S., Pavel-Dinu, M., Weinberg, K. I., & Porteus, M. H. (2019). Identification of preexisting adaptive immunity to Cas9 proteins in humans. *Nature Medicine 2019 25:2*, *25*(2), 249–254. https://doi.org/10.1038/s41591-018-0326-x

Chavez, A., Scheiman, J., Vora, S., Pruitt, B. W., Tuttle, M., P R Iyer, E., Lin, S., Kiani, S., Guzman, C. D., Wiegand, D. J., Ter-Ovanesyan, D., Braff, J. L., Davidsohn, N., Housden, B. E., Perrimon, N., Weiss, R., Aach, J., Collins, J. J., & Church, G. M. (2015). Highly efficient Cas9-mediated transcriptional programming. *Nature Methods*, *12*(4), 326–328. https://doi.org/10.1038/NMETH.3312

Chen, J. S., Dagdas, Y. S., Kleinstiver, B. P., Welch, M. M., Sousa, A. A., Harrington, L. B., Sternberg, S. H., Joung, J. K., Yildiz, A., & Doudna, J. A. (2017). Enhanced proofreading governs CRISPR-Cas9 targeting accuracy. *Nature*, *550*(7676), 407–410. https://doi.org/10.1038/NATURE24268

Chen, P. J., & Liu, D. R. (2023). Prime editing for precise and highly versatile genome manipulation. *Nature Reviews. Genetics*, *24*(3), 161–177. https://doi.org/10.1038/S41576-022-00541-1

Ciganek, I., Delehedde, C., Galy, A., Cogné, M., Rameix, N., Bordeianu, C., & Pichon, C. (2026). Delivering the future of immunotherapy: A state-of-the-art review of gene editing in immune cells with lipid nanoparticles. *Materials Today Bio*, *39*, 103334. https://doi.org/10.1016/J.MTBIO.2026.103334

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L. A., & Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science (New York, N.Y.)*, *339*(6121), 819–823. https://doi.org/10.1126/SCIENCE.1231143

Cox, D. B. T., Platt, R. J., & Zhang, F. (2015). Therapeutic genome editing: prospects and challenges. *Nature Medicine*, *21*(2), 121–131. https://doi.org/10.1038/NM.3793

Deokar, R., Bhad, V., Bhalerao, G., Deokar, R., Bhad, V., Bhalerao, G., Deokar, R., Bhad, V., & Bhalerao, G. (2024). CRISPR-CAS 9 In Gene Editing: Innovations, Applications and Ethical Challenges. *International Journal of Scientific Research and Technology*, 59–71. https://doi.org/10.5281/ZENODO.14161891

Doudna, J. A. (2020). THE PROMISE AND CHALLENGE OF THERAPEUTIC GENOME EDITING. *Nature*, *578*(7794), 229. https://doi.org/10.1038/S41586-020-1978-5

Doudna, J. A., & Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. *Science*, *346*(6213). https://doi.org/10.1126/SCIENCE.1258096;WEBSITE:WEBSITE:AAAS-SITE;JOURNAL:JOURNAL:SCIENCE;WGROUP:STRING:PUBLICATION

Du, W., Zhang, T., Guo, L., Zheng, Y., Zhang, H., Zhu, X., Tang, D., Hu, H., Chen, L., & Liu, C. (2026). Deep learning–driven prediction of on-target activity, off-target risk, and repair outcomes in CRISPR/Cas9: current landscape and multi-scale perspectives. *Journal of Translational Medicine 2026 24:1*, *24*(1), 769-. https://doi.org/10.1186/S12967-026-08175-1

Feierman, E. R., Whittaker, M. N., Quigley, A., Brooks, D. L., McVeigh, P., Nan, A. X., Hsu, A., Said, H., Soliman, O. Y., Giovenco, R., Davidson, B. L., Alameh, M. G., Liu, D. R., Wang, X., Musunuru, K., & Ahrens-Nicklas, R. C. (2026). Implications of the FDA's new plausible mechanism framework for the development of a personalized in vivo prime editing platform. *American Journal of Human Genetics*, *113*(5), 891–899. https://doi.org/10.1016/j.ajhg.2026.03.018

Frangoul, H., Altshuler, D., Cappellini, M. D., Chen, Y.-S., Domm, J., Eustace, B. K., Foell, J., de la Fuente, J., Grupp, S., Handgretinger, R., Ho, T. W., Kattamis, A., Kernytsky, A., Lekstrom-Himes, J., Li, A. M., Locatelli, F., Mapara, M. Y., de Montalembert, M., Rondelli, D., … Corbacioglu, S. (2021). CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β-Thalassemia. *New England Journal of Medicine*, *384*(3), 252–260. https://doi.org/10.1056/NEJMOA2031054;ISSUE:ISSUE:DOI

Gaj, T., Gersbach, C. A., & Barbas, C. F. (2013). ZFN, TALEN and CRISPR/Cas-based methods for genome engineering. *Trends in Biotechnology*, *31*(7), 397. https://doi.org/10.1016/J.TIBTECH.2013.04.004

Gaudelli, N. M., Komor, A. C., Rees, H. A., Packer, M. S., Badran, A. H., Bryson, D. I., & Liu, D. R. (2017). Programmable base editing of T to G C in genomic DNA without DNA cleavage. *Nature*, *551*(7681), 464–471. https://doi.org/10.1038/NATURE24644;TECHMETA

Gillmore, J. D., Gane, E., Taubel, J., Kao, J., Fontana, M., Maitland, M. L., Seitzer, J., O'Connell, D., Walsh, K. R., Wood, K., Phillips, J., Xu, Y., Amaral, A., Boyd, A. P., Cehelsky, J. E., McKee, M. D., Schiermeier, A., Harari, O., Murphy, A., … Lebwohl, D. (2021). CRISPR-Cas9 In Vivo Gene Editing for Transthyretin Amyloidosis. *New England Journal of Medicine*, *385*(6), 493–502. https://doi.org/10.1056/NEJMOA2107454

Haapaniemi, E., Botla, S., Persson, J., Schmierer, B., & Taipale, J. (2018). CRISPR–Cas9 genome editing induces a p53-mediated DNA damage response. *Nature Medicine 2018 24:7*, *24*(7), 927–930. https://doi.org/10.1038/s41591-018-0049-z

Hilton, I. B., D'Ippolito, A. M., Vockley, C. M., Thakore, P. I., Crawford, G. E., Reddy, T. E., & Gersbach, C. A. (2015). Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nature Biotechnology*, *33*(5), 510–517. https://doi.org/10.1038/NBT.3199

Hsu, P. D., Lander, E. S., & Zhang, F. (2014). Development and Applications of CRISPR-Cas9 for Genome Engineering. *Cell*, *157*(6), 1262. https://doi.org/10.1016/J.CELL.2014.05.010

Hsu, P. D., Scott, D. A., Weinstein, J. A., Ran, F. A., Konermann, S., Agarwala, V., Li, Y., Fine, E. J., Wu, X., Shalem, O., Cradick, T. J., Marraffini, L. A., Bao, G., & Zhang, F. (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. *Nature Biotechnology*, *31*(9), 827–832. https://doi.org/10.1038/NBT.2647

Huang, X. ;, Yang, D. ;, Zhang, J. ;, Xu, J. ;, Chen, Y. E., Huang, X., Yang, D., Zhang, J., Xu, J., & Chen, Y. E. (2022). Recent Advances in Improving Gene-Editing Specificity through CRISPR–Cas9 Nuclease Engineering. *Cells 2022, Vol. 11, Page 2186*, *11*(14), 2186. https://doi.org/10.3390/CELLS11142186

Hunt, J. M. T., Samson, C. A., Rand, A. du, & Sheppard, H. M. (2023). Unintended CRISPR-Cas9 editing outcomes: a review of the detection and prevalence of structural variants generated by gene-editing in human cells. *Human Genetics*, *142*(6), 705. https://doi.org/10.1007/S00439-023-02561-1

Hyeon, H., Hwang, S., Luo, Y., Shin, E., Yeom, J. H., Kim, H. M., Ryu, M., & Lee, K. (2025). CRISPR-Cas technologies: Emerging tools from research to clinical application. *Journal of Microbiology*, *63*(8), e2504012. https://doi.org/10.71150/JM.2504012

Ihry, R. J., Worringer, K. A., Salick, M. R., Frias, E., Ho, D., Theriault, K., Kommineni, S., Chen, J., Sondey, M., Ye, C., Randhawa, R., Kulkarni, T., Yang, Z., McAllister, G., Russ, C., Reece-Hoyes, J., Forrester, W., Hoffman, G. R., Dolmetsch, R., & Kaykas, A. (2018). p53 inhibits CRISPR–Cas9 engineering in human pluripotent stem cells. *Nature Medicine 2018 24:7*, *24*(7), 939–946. https://doi.org/10.1038/s41591-018-0050-6

Iyer, K. A., Tenchov, R., Lotti Diaz, L. M., Jain, P., Thite, T., Deng, Y., & Zhou, Q. A. (2025). CRISPR Technology: Transforming the Future of Medicine and Diagnostics. *Biochemistry*, *64*(24), 4628. https://doi.org/10.1021/ACS.BIOCHEM.5C00480

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science (New York, N.Y.)*, *337*(6096), 816. https://doi.org/10.1126/SCIENCE.1225829

Kabrah, S. M. (2025). Emerging Gene Therapies in Sickle Cell Disease: A Comparative Review of Efficacy and Safety Against Standard Treatments. *Journal of Blood Medicine*, *16*, 493. https://doi.org/10.2147/JBM.S556513

Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A., & Liu, D. R. (2016). Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature*, *533*(7603), 420–424. https://doi.org/10.1038/NATURE17946

Kosicki, M., Tomberg, K., & Bradley, A. (2018). Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. *Nature Biotechnology*, *36*(8), 765–771. https://doi.org/10.1038/NBT.4192

Lazzarotto, C. R., Malinin, N. L., Li, Y., Zhang, R., Yang, Y., Lee, G. H., Cowley, E., He, Y., Lan, X., Jividen, K., Katta, V., Kolmakova, N. G., Petersen, C. T., Qi, Q., Strelcov, E., Maragh, S., Krenciute, G., Ma, J., Cheng, Y., & Tsai, S. Q.

(2020). CHANGE-seq reveals genetic and epigenetic effects on CRISPR-Cas9 genome-wide activity. *Nature Biotechnology*, *38*(11), 1317–1327. https://doi.org/10.1038/S41587-020-0555-7

Lino, C. A., Harper, J. C., Carney, J. P., & Timlin, J. A. (2018). Delivering CRISPR: a review of the challenges and approaches. *Drug Delivery*, *25*(1), 1234–1257. https://doi.org/10.1080/10717544.2018.1474964

Lopes, R., & Prasad, M. K. (2023). Beyond the promise: evaluating and mitigating off-target effects in CRISPR gene editing for safer therapeutics. *Frontiers in Bioengineering and Biotechnology*, *11*, 1339189. https://doi.org/10.3389/FBIOE.2023.1339189/FULL

Ma, Y., & Qi, L. S. (2024). The realization of CRISPR Gene Therapy. *Nature Chemical Biology*, *20*(7), 791. https://doi.org/10.1038/S41589-024-01645-X

Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., Norville, J. E., & Church, G. M. (2013). RNA-guided human genome engineering via Cas9. *Science (New York, N.Y.)*, *339*(6121), 823–826. https://doi.org/10.1126/SCIENCE.1232033

Massone, S., Ciarlo, E., Vella, S., Nizzari, M., Florio, T., Russo, C., Cancedda, R., & Pagano, A. (2012). NDM29, a RNA polymerase III-dependent non coding RNA, promotes amyloidogenic processing of APP and amyloid β secretion. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1823*(7), 1170–1177. https://doi.org/10.1016/j.bbamcr.2012.05.001

Mehta, A., & Merkel, O. M. (2019). Immunogenicity of Cas9 Protein. *Journal of Pharmaceutical Sciences*, *109*(1), 62. https://doi.org/10.1016/J.XPHS.2019.10.003

Modell, A. E., Lim, D., Nguyen, T. M., Sreekanth, V., & Choudhary, A. (2021). CRISPR-based therapeutics: current challenges and future applications. *Trends in Pharmacological Sciences*, *43*(2), 151. https://doi.org/10.1016/J.TIPS.2021.10.012

Musunuru, K., & Urnov, F. (2025). Moving Therapeutic Genome Editing into Global Clinical Trials and Medicine. *The CRISPR Journal*, *8*(4), 228–231. https://doi.org/10.1089/CRISPR.2025.0049

Navarro, C., Díaz, M. P., Duran, P., Castro, A., Díaz, A., Cano, C., Carbonell-Zabaleta, A. K., Solano-Jimenez, D. S., Rivera-Porras, D., Contreras-Velásquez, J. C., & Bermúdez, V. (2025). CRISPR-Cas Systems: A Functional Perspective and Innovations. *International Journal of Molecular Sciences 2025, Vol. 26, Page 3645*, *26*(8), 3645. https://doi.org/10.3390/IJMS26083645

*News: CRISPR Trial Update: NTLA-2002 For Hereditary Angioedema - CRISPR Medicine*. (n.d.). Retrieved June 17, 2026, from https://crisprmedicinenews.com/news/crispr-trial-update-ntla-2002-for-hereditary-angioedema/

Ortiz-Bueno, M., Zinghirino, F., Serra, P. P., Paschoudi, K., Montoliu, L., Atilla, E., Luo, Y., Cavazza, A., Lederer, C. W., & Benabdellah, K. (2026). From Bench to Bedside: Ethical and Clinical Best Practices for Genome Editing Applications. *International Journal of Molecular Sciences*, *27*(3), 1484. https://doi.org/10.3390/IJMS27031484

Pacesa, M., Pelea, O., & Jinek, M. (2024). Past, present, and future of CRISPR genome editing technologies. *Cell*, *187*(5), 1076–1100. https://doi.org/10.1016/J.CELL.2024.01.042

Qi, L. S., Larson, M. H., Gilbert, L. A., Doudna, J. A., Weissman, J. S., Arkin, A. P., & Lim, W. A. (2013). Repurposing CRISPR as an RNA-γuided platform for sequence-specific control of gene expression. *Cell*, *152*(5), 1173–1183. https://doi.org/10.1016/j.cell.2013.02.022

Rees, H. A., & Liu, D. R. (2018). Base editing: precision chemistry on the genome and transcriptome of living cells. *Nature Reviews. Genetics*, *19*(12), 770–788. https://doi.org/10.1038/S41576-018-0059-1

Rehman, S. U., & Abbas, G. H. (2026). CRISPR/CAS9-based gene editing in cancer therapy: A systematic review and meta-analysis on current status and future directions. *Medicine*, *105*(2), e47114. https://doi.org/10.1097/MD.0000000000047114

Rostami, N., Gomari, M. M., Choupani, E., Abkhiz, S., Fadaie, M., Eslami, S. S., Mahmoudi, Z., Zhang, Y., Puri, M., Monfared, F. N., Demireva, E., Uversky, V. N., Smith, B. R., & Bencherif, S. A. (2024). Exploring Advanced CRISPR Delivery Technologies for Therapeutic Genome Editing. *Small Science*, *4*(10), 2400192. https://doi.org/10.1002/SMSC.202400192;ISSUE:ISSUE:DOI

Saeed, M., Arham, M., Zafar, I., Jamal, A., Hussian, M., Usman, M., Bahwerth, F. S., Noman, M., & Hossain, M. B. (2026). Harnessing Deep Learning Models for Guide RNA Optimization and Off-Target Prediction in CRISPR Systems. *Biotechnology Journal*, *21*(6), e70255. https://doi.org/10.1002/BIOT.70255;WEBSITE:WEBSITE:ANALYTICALSCIENCEJOURNALS;REQUESTEDJOURNAL:JOURNAL:18607314;ISSUE:ISSUE:DOI

Sahel, D. K., Vora, L. K., Saraswat, A., Sharma, S., Monpara, J., D'Souza, A. A., Mishra, D., Tryphena, K. P., Kawakita, S., Khan, S., Azhar, M., Khatri, D. K., Patel, K., & Singh Thakur, R. R. (2023). CRISPR/Cas9 Genome Editing for Tissue-Specific In Vivo Targeting: Nanomaterials and Translational Perspective. *Advanced Science*, *10*(19), 2207512. https://doi.org/10.1002/ADVS.202207512

Scholz, P., Thompson, J., Crosby, K. T., Fauth, T., Krah, N. M., Schlauderaff, G., Back, R., Berkheimer, Z. A., Jolley, A., Sombroek, D., Medert, R., Zurek, C., Dmytrenko, O., Wilson, E., Schut, F. T., Rutter, J., Zhang, X., Krohn, M., Jackson, R. N., … Liu, Y. (2026). RNA-triggered cell killing with CRISPR–Cas12a2. *Nature 2026*, 1–10. https://doi.org/10.1038/s41586-026-10466-y

Seem, K., Kaur, S., Kumar, S., & Mohapatra, T. (2024). Epigenome editing for targeted DNA (de)methylation: a new perspective in modulating gene expression. *Critical Reviews in Biochemistry and Molecular Biology*, *59*(1–2), 69–98. https://doi.org/10.1080/10409238.2024.2320659

Shi, J., Hu, H., & Ung, C. O. L. (2026). Pathing the way from regulatory approval to market access for gene therapy products (GTPs): An integrative review in the US, EU5, Japan and China. *Regenerative Therapy*, *32*, 101102. https://doi.org/10.1016/J.RETH.2026.101102

Singh, A., Irfan, H., Fatima, E., Nazir, Z., Verma, A., & Akilimali, A. (2024). Revolutionary breakthrough: FDA approves CASGEVY, the first CRISPR/Cas9 gene therapy for sickle cell disease. *Annals of Medicine and Surgery*, *86*(8), 4555. https://doi.org/10.1097/MS9.0000000000002146

Slaymaker, I. M., Gao, L., Zetsche, B., Scott, D. A., Yan, W. X., & Zhang, F. (2016). Rationally engineered Cas9 nucleases with improved specificity. *Science (New York, N.Y.)*, *351*(6268), 84–88. https://doi.org/10.1126/SCIENCE.AAD5227

Stadtmauer, E. A., Fraietta, J. A., Davis, M. M., Cohen, A. D., Weber, K. L., Lancaster, E., Mangan, P. A., Kulikovskaya, I., Gupta, M., Chen, F., Tian, L., Gonzalez, V. E., Xu, J., Jung, I. young, Joseph Melenhorst, J., Plesa, G., Shea, J., Matlawski, T., Cervini, A., … June, C. H. (2020). CRISPR-engineered T cells in patients with refractory cancer. *Science (New York, N.Y.)*, *367*(6481). https://doi.org/10.1126/SCIENCE.ABA7365

Strecker, J., Ladha, A., Gardner, Z., Schmid-Burgk, J. L., Makarova, K. S., Koonin, E. V., & Zhang, F. (2019). RNA-guided DNA insertion with CRISPR-associated transposases. *Science (New York, N.Y.)*, *365*(6448), 48–53. https://doi.org/10.1126/SCIENCE.AAX9181

Tsai, S. Q., & Joung, J. K. (2016). Defining and improving the genome-wide specificities of CRISPR-Cas9 nucleases. *Nature Reviews. Genetics*, *17*(5), 300–312. https://doi.org/10.1038/NRG.2016.28

Tsai, S. Q., Nguyen, N. T., Malagon-Lopez, J., Topkar, V. V., Aryee, M. J., & Joung, J. K. (2017). CIRCLE-seq: a highly sensitive in vitro screen for genome-wide CRISPR-Cas9 nuclease off-targets. *Nature Methods*, *14*(6), 607–614. https://doi.org/10.1038/NMETH.4278

Tsai, S. Q., Zheng, Z., Nguyen, N. T., Liebers, M., Topkar, V. V., Thapar, V., Wyvekens, N., Khayter, C., Iafrate, A. J., Le, L. P., Aryee, M. J., & Joung, J. K. (2015). GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nature Biotechnology*, *33*(2), 187–198. https://doi.org/10.1038/NBT.3117

Xu, Y., & Li, Z. (2020). CRISPR-Cas systems: Overview, innovations and applications in human disease research and gene therapy. *Computational and Structural Biotechnology Journal*, *18*, 2401. https://doi.org/10.1016/J.CSBJ.2020.08.031

Xue, C., & Greene, E. C. (2021). DNA repair pathway choices in CRISPR-Cas9 mediated genome editing. *Trends in Genetics : TIG*, *37*(7), 639. https://doi.org/10.1016/J.TIG.2021.02.008

Yin, H., Kauffman, K. J., & Anderson, D. G. (2017). Delivery technologies for genome editing. *Nature Reviews. Drug Discovery*, *16*(6), 387–399. https://doi.org/10.1038/NRD.2016.280

Zetsche, B., Gootenberg, J. S., Abudayyeh, O. O., Slaymaker, I. M., Makarova, K. S., Essletzbichler, P., Volz, S. E., Joung, J., Van Der Oost, J., Regev, A., Koonin, E. V., & Zhang, F. (2015). Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System. *Cell*, *163*(3), 759–771. https://doi.org/10.1016/j.cell.2015.09.038

Zhang, K., Wan, P., Wang, L., Wang, Z., Tan, F., Li, J., Ma, X., Cen, J., Yuan, X., Liu, Y., Sun, Z., Cheng, X., Liu, Y., Liu, X., Hu, J., Zhong, G., Li, D., Xia, Q., & Hui, L. (2024). Efficient expansion and CRISPR-Cas9-mediated gene correction of patient-derived hepatocytes for treatment of inherited liver diseases. *Cell Stem Cell*, *31*(8), 1187-1202.e8. https://doi.org/10.1016/j.stem.2024.04.022

Zhang, X., Ma, D., & Liu, F. (2025). CRISPR Technology and Its Emerging Applications. *Genomics, Proteomics & Bioinformatics*, *23*(3), qzaf034. https://doi.org/10.1093/GPBJNL/QZAF034

Zhao, Q., Shen, S., Shim, W. J., & Palpant, N. J. (2026). The changing landscape of gene expression analysis. *Briefings in Bioinformatics*, *27*(2), bbag185. https://doi.org/10.1093/BIB/BBAG185

Zhao, R., Wang, C., Li, J., Liao, Y., Huang, C., Hu, T., Zhang, H., & Zhang, W. (2026). DNA and RNA editing for the therapy of human diseases: current status, challenges, and future prospects. *Molecular Biomedicine*, *7*(1), 55. https://doi.org/10.1186/S43556-026-00456-X

Chapter 4

# MATERNAL NUTRITION AND EPIGENETICS

*Şule ARSLAN*[1]

*NOOR MANSOOR HASAN ABDULLAH ALSUBARI*[2]

1 Dr. Öğr. Üyesi ŞULE ARSLAN
ORCID no
2 ORCID no 0009-0000-3674-4579

## Introduction

The early stages of life represent a critical and sensitive window, spanning from pregnancy through early childhood, during which nutrition plays a critical role in determining lifelong health outcomes. The concept of the *Developmental Origins of Health and Disease (DOHaD)*, first introduced by David Barker and colleagues, suggests that exposure to environmental factors, particularly nutrition, during early development has lasting effects on health and disease risk later in life (Barker 2007; Ryznar, Phibbs, and Van Winkle 2021). The intrauterine environment acts as a link between maternal physiology and fetal development, influencing metabolic programming, cell differentiation, and organ formation (Schiera et al. 2026). Epigenetic mechanisms, including DNA methylation, histone modifications, and long non-coding RNA (lncRNA) regulation, play a central role in this process by regulating gene expression without altering the underlying DNA sequence. These mechanisms are highly sensitive to intrauterine conditions, and the plasticity of the fetal epigenome makes it particularly responsive to maternal nutritional status during pregnancy period (Andonotopo et al. 2025). Evidence from the Dutch Huger Winter has shown that individuals exposed to prenatal undernutrition have an increased risk of obesity, insulin resistance, and cardiovascular disease in adulthood, providing evidence that adaptive responses to intrauterine undernutrition may increase disease risk later in life (Schiera et al. 2026; Schulz 2010). In addition, maternal diet can influence epigenetic programming through pathways such as one-carbon metabolism, affecting DNA methylation patterns, while maternal metabolic conditions such as gestational diabetes have been associated with altered DNA methylation in offspring, including genes involved in energy regulation such as *LEP* gene (Allard et al. 2015; Anastasopoulou et al. 2026). Recent studies have shown that maternal obesity and a high-fat diet during pregnancy can disrupt fetal development in rats and alter DNA methylation patterns, increasing the risk of metabolic disease in offspring later in life (Tang et al. 2024). In addition, maternal depletion of methyl donors during pregnancy has been associated with adverse metabolic outcomes, including low leptin levels, hypertension, and insulin resistance, through epigenetic modifications in genes involved in energy balance and metabolic regulation (Bokor et al. 2024). These findings highlight the sensitivity of the fetal epigenome to maternal nutritional status during critical developmental periods, and both undernutrition and overnutrition during pregnancy may increase the risk of chronic disease later in life, Most studies investigating fetal and neonatal epigenetic modifications have focused on maternal nutrition, and understanding these mechanisms may contribute to the prevention of metabolic disease and the development of personalized nutritional interventions. Therefore, this review aims to examine the role of maternal nutrition during pregnancy in shaping epigenetic regulation and its impact on offspring health outcomes.

## Nutrition and Epigenetics Mechanisms

### DNA methylation

Epigenetic markers are enzyme-mediated chemical modifications in DNA and associated chromatin proteins. These epigenetic changes play a critical role in regulating genomic function without altering the primary DNA sequence and can be maintained across generations through cellular transmission. Epigenetic mechanisms influence chromatin structure, transcriptional activity, and other functional properties. DNA methylation, histone modifications, and microRNAs are the main epigenetic mechanisms involved in regulating chromatin structure and genomic activity (Lee 2015). The timing and location of specific gene expressions are largely controlled by these mechanisms; therefore, alterations in epigenetic regulation may lead to significant phenotypic differences.

DNA methylation is one of the most extensively studied epigenetic modifications and occurs through specific enzyme-mediated processes catalyzed by DNA methyltransferases (DNAMTs).

DNAMT1 is primarily responsible for maintaining DNA methylation during cell division, whereas DNAMT3A and DNAMT3B establish de novo DNA methylation patterns during early embryonic development (Chango and Pogribny 2015). Alterations in DNA methylation at important genomic regions, such as enhancers and promoters, can affect gene expression and function. DNA methylation is also involved in several biological processes, including gene expression regulation, X-chromosome inactivation, and genomic imprinting.

One-carbon metabolism is the main pathway involved in DNA methylation and depends on several enzymes whose activities require dietary micronutrients. At the cellular level, S-adenosylmethionine (SAM) serves as the primary methyl donor and is synthesized from methionine in an ATP-dependent reaction. Methyltetrahydrofolate (MTHF) participates in the conversion of homocysteine to methionine by donating methyl groups. DNA methyltransferases (DNMTs) covalently attach methyl groups from SAM to the carbon-5 position of cytosine residues, resulting in the formation of 5-methylcytosine (5mC). Nutrients involved in one-carbon metabolism, including folate, vitamin B6 and B12, choline, and methionine, play essential roles in DNA methylation by influencing SAM and S-adenosylhomocysteine (SAH) levels, which acts as methyltransferase inhibitor. In addition, bioactive food compounds such as resveratrol, retinoic acid, curcumin, sulforaphane, and tea polyphenols can regulate DNA methylation and histone modifications, thereby influencing epigenetic mechanisms.

Maternal nutrition can strongly influence fetal epigenetic regulation during early pregnancy, a period in which the fetal epigenome is highly sensitive to environmental signals. (Lee 2015). Animal studies have shown that maternal intake of methyl donors during pregnancy can alter DNA methylation of the Agouti gene, affecting fetal phenotype and susceptibility to disease later in life (Andonotopo et al. 2025).

Human studies have also demonstrated that individuals prenatally exposed to the Dutch Hunger Winter famine had lower DNA methylation levels of the *IGF2* gene compared to individuals of the same sex who were not exposed to the famine (Heijmans et al. 2008).These findings demonstrate that early pregnancy is highly sensitive to epigenetic alterations that may persist throughout life.

A maternal high fat diet has been associated with an increased risk of obesity in offspring through epigenetic alterations in DNA methylation. Studies have shown that maternal high-fat diet may modify the methylation of adipogencic regulators such as *PPARγ* and *C\EBP α*, promoting adipocyte differentiation and fat accumulation (Pant et al. 2021). Altered methylation patterns have also been reported in genes involved in energy balance, appetite regulation, insulin signaling, and blood pressure regulation, including *POMC,LEP, Map2K4, IRS2, Ephb2*, and *Fgf21* (Bokor et al. 2024; Zhang et al. 2019). In animal studies, high maternal intake of sucrose and fructose during early pregnancy reduced DNA methylation of *IGF2* and *IRS2*, contributing to impaired insulin and glucose signaling and increasing susceptibility to obesity and insulin resistance in offspring (Hor et al. 2025). Human cohort studies, including Project Viva have similarly reported that increased maternal consumption of sugar-sweetened beverages is associated with epigenetic alterations in insulin signaling-related genes in umbilical cord blood (Bokor et al. 2024; Sen et al. 2018).

Collectively, these findings suggest that maternal dietary patterns during pregnancy, particularly diets high in fat and sugar, may influence DNA methylation and fetal metabolic programming, thereby increasing susceptibility to obesity and non-communicable disease later in life.

**Histone Modifications**

Histone modifications include several chemical alterations, such as methylation, acetylation, and phosphorylation that occur on histone proteins. Histone methylation is catalyzed by histone methyltransferase enzymes (HMTs or KMTs) and occurs on specific amino acid residues, including lysine and arginine, located on the N-terminal tails of histones. The effect of histone methylation on gene expression depends on the modified amino acid

residue and the number of methyl groups added (Ryznar et al. 2021). These epigenetic modifications influence chromatin structure, accessibility, and the transcriptional activity within the cell. Histone modifications regulate gene expression by altering chromatin structure and function. (Ramírez-Alarcón et al. 2019). Enzymes that modify histones can add or remove epigenetic marks on histone tails, thereby controlling chromatin structure and gene expression (Şanlı and Kabaran 2019).

Several enzyme families are involved in histone modification. Histone acetyltransferases (HATs) add acetyl groups to lysine residues on histone tails, neutralizing their positive charge and relaxing chromatin structure, which enhances transcriptional activity and gene expression. In contrast, histone deacetylases (HDACs) remove acetyl groups, leading to chromatin condensation and transcriptional repression. Maternal nutrition may indirectly influence these mechanisms through macronutrients such as fats and proteins and their metabolic intermediates, including coenzyme A, which is involved in histone acetyltransferase activation (Gujral et al. 2020; Socha, Flis, and Wartęga 2024).

Although studies investigating histone modifications are still limited, increasing evidence suggests that maternal diet can influence histone acetylation and methylation patterns during fetal development. Human studies have reported that prenatal intake of omega-3 fatty acids, particularly docosahexaenoic acid (DHA), is associated with changes in histone H3K9 acetylation at genes involved in inflammation and immune regulation, supporting the role of lipid signaling in chromatin remodeling (Andonotopo et al. 2025). In addition, polyphenolic compounds such as resveratrol have been shown to activate the SIRT1 enzyme and promote histone deacetylation (Khamis, Moselhy, and Rihan 2025). Maternal nutritional status, including lipid-rich diets and glycemic load, can influence fetal histone modifications and increase the risk of chronic diseases (Arslan and Yıldıran 2021). Animal studies on rodents have demonstrated that maternal protein restriction reduces histone H3 and H4 acetylation in the fetal liver and alters glucocorticoid receptor gene expression (Hill and Hill 2024; Tang et al. 2024). Similarly maternal high-fat diets have been associated with altered histone H4 acetylation and changes in gene programming within the fetal liver, placenta, and hippocampus of the offspring (Aslı Akyol Mutlu 2016).

These diets may increase active histone marks such as H3K4me3 and H3K9ac at lipid-metabolism-related gene promoters, contributing to obesity-related phenotypes in offspring (Glendining and Jasoni 2019; Reichetzeder 2021). Human cohort studies, including GENERATION R and Avon Longitudinal Study of Parents and Children (ALSPAC), have also shown

that increased maternal dietary fat intake during pregnancy is associated with epigenetic alterations in obesity-related genes such as *LEP* and *IGF2*, potentially increasing obesity risk in offspring (Alfano et al. 2022; Alum et al. 2025; Sharp et al. 2017). These findings suggest that maternal dietary patterns can influence fetal chromatin remodeling and histone modifications leading to long-term metabolic programming in offspring.

**MicroRNA**

MicroRNAs (MiRNAs) represent another important layer of epigenetic regulation. Although the human genome contains approximately 20,000 protein-coding genes, these genes account for less than 2% of the total genome. A large portion of the genome is transcribed into non-coding RNAs with limited or no protein-coding capacity (Lee 2015). Non-coding RNAs play essential roles in cellular processes such as chromosome organization, mRNA stability, and post-transcriptional regulation of gene expression. Among these, long non-coding RNAs (lncRNAs) are particularly important in germ cell function and embryonic development, and their dysregulation has been associated with various diseases (Cai et al. 2021). MicroRNAs are involved in the regulation of insulin sensitivity, glucose homeostasis, lipid metabolism, and inflammatory pathways, and their dysregulation has been associated with obesity, type 2 diabetes mellitus, and metabolic syndrome (Zhou et al. 2020). Early-life undernutrition and maternal dietary factors may alter miRNA expression profiles and disrupt cellular regulatory mechanisms. Diet-related epigenetic changes associated with maternal nutrition have been shown to affect miRNA expression in offspring, particularly pathways related to insulin resistance, lipid metabolism, and inflammation (Alejandro et al. 2014; Lee 2015). Human studies have reported that maternal vitamin D status during pregnancy is associated with altered expression of miRNAs involved in neurodevelopment and immune function, including miR-21, miR-146a, and miR-155, in umbilical cord blood (Anderson et al. 2018; Chodur and Steinberg 2024). Animal studies have also demonstrated that maternal high-fat diets increase hepatic expression of miR-122 and miR-370 in offspring, contributing to postnatal hepatic fat accumulation (Harmancıoğlu and Kabaran 2023; Nelson and Friedman 2024). In addition, circulating maternal miRNAs such as miR-21 and miR-148a have been associated with maternal obesity and insulin resistance during pregnancy (Dalgaard et al. 2022; Iacomino and Siani 2017). These circulating miRNAs may cross the placenta and influence fetal gene expression (Alum et al. 2025). Together, these findings indicate that maternal nutritional status may affect fetal development through miRNA-mediated epigenetic regulation, thereby influence metabolic pathways in offspring.

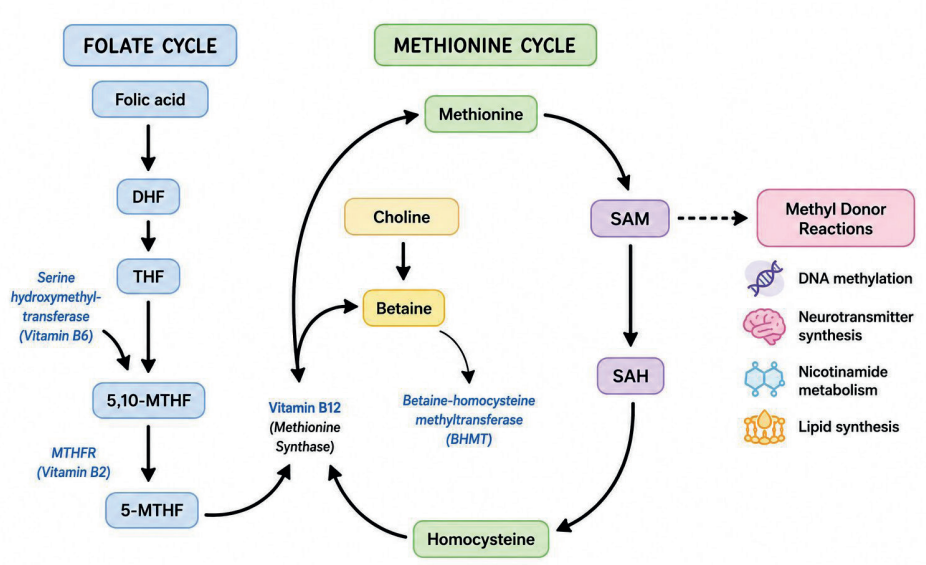



***Figure 1.*** **Overview of one-carbon metabolism and its role in epigenetic regulations.** The folate and methionine cycles interact to generate S-adenosylmethionine (SAM), the primary methyl donor for DNA methylation. Nutrients including folate, choline, betaine, and vitamins B2, B6, and B12 support these metabolic pathways by regulating homocysteine remethylation and methyl group availability. Through the production of SAM, one-carbon metabolism contributes to DNA methylation, neurotransmitter synthesis, nicotinamide metabolism, and lipid synthesis. **Abbreviations:** DHF, dihydrofolate; THF, tetrahydrofolate; 5,10-MTHF, 5,10-methylenetetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; BHMT, betaine-homocysteine methyltransferase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

One-carbon metabolism is a biochemical pathway that maintains cellular homeostasis through methyl donor reactions involved in neurotransmitter synthesis, nicotinamide metabolism, and lipid synthesis. The methionine and folate cycles are central components of this pathway, and nutrients such as folate, choline, methionine, betaine, and vitamins B12, B6, and B2 play essential roles in these reactions (Socha et al. 2024). Choline is oxidized to betaine, and both betaine and folate donate methyl groups for the conversion of homocysteine to methionine, which is subsequently used for the synthesis of S-adenosylmethionine (SAM), the primary methyl donor for DNA methylation. Through this process, one-carbon metabolism contributes to genome stability and epigenetic regulation. Vitamins B2, B6, B12, and folic

acid act as important cofactors in these metabolic reactions and support the maintenance of DNA methylation through the regulation of SAM availability (Yusuf et al. 2016).

Vitamin B6 acts as a coenzyme for serine hydroxymethyltransferase, a vital enzyme that converts tetrahydrofolate (THF) to 5,10-methylenetetrahydrofolate (5,10-MTHF), while riboflavin (B2) is required for the activity of methylenetetrahydrofolate reductase (MTHFR) an enzyme involved in the conversion of 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-methyl-THF. Vitamin B12 is also acts as a cofactor for the methionine synthase, which converts homocysteine to methionine (Yusuf et al. 2016). Because these nutrients directly regulate methyl donor availability, deficiencies in one-carbon metabolism may alter DNA methylation and disrupt epigenetic programming.

Animal studies have demonstrated that maternal supplementation with folic acid and B12 increases DNA methylation of the Agouti gene in offspring, thereby reducing the susceptibility to obesity (Waterland and Jirtle 2003). Studies using viable yellow Agouti (Avy) mice further showed that maternal methyl donor supplementation during pregnancy altered offspring coat color from yellow to brown by suppressing Agouti gene expression through increased DNA methylation. These findings provide strong evidence that early-life exposures such as maternal nutrition during early development can induce long-term effects on gene expression and influence phenotype.

Altered one-carbon metabolism has also been associated with metabolic and cardiovascular disorders. Methyl donor deficiency and elevated homocysteine levels are major risk factors for cardiovascular diseases (Blom and Smulders 2011). Restriction of folate, methionine, and B vitamins during periconceptional period has been linked to altered DNA methylation, insulin resistance, elevated blood pressure, and dysregulation of genes involved in mitochondrial metabolism, renin-angiotensin system, and phospholipid homeostasis (Lee 2015). Similarly, maternal folate and vitamin B12 deficiencies during pregnancy and lactation have been associated with low birth weight, hepatic steatosis, myocardial hypertrophy, and increased adiposity in offspring (Guéant et al. 2013). These effects may result from epigenetic alterations involving hypomethylation and hyperacetylation of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC1-α*) gene, together with decreased expression and activity of sirtuin (*SIRT1*) and protein arginine methyltransferase1( *PRMT1*) (Guéant et al. 2014).

Maternal choline deficiency during pregnancy has also been shown to alter the fetal epigenome. Experimental studies demonstrated that choline supplementation increased the repressive histone marks H3K27me3 and H3K9me2 in the fetal liver and brain, whereas choline deficiency increased

H3K4me2 levels. Choline deficiency may also alter the methylation and expression of imprinted genes such as *IGF2* through changes in DNMT1 activity in the liver (Kovacheva et al. 2007) . Moreover, maternal choline intake may influence genes involved in lipid and methionine metabolism (Medici et al. 2014). These findings suggest that maternal choline intake may influence histone methylation and contribute to developmental programming and long-term health outcomes in offspring.

The maintenance of embryonic epigenetic stability depends on maternal one-carbon metabolism, and may contribute to transgenerational effects (Basak et al. 2024). Adequate maternal folate intake is essential for neural tube development, DNA synthesis, and epigenetic regulation. Epigenetic modifications mediated by folate may exert long-term effects on brain development and neurological function. Methylation processes within the one carbon metabolism rely on the synergistic balance of nutrient-nutrient and nutrient-gene interaction among methyl donor nutrients. Within this network, folate acts as a rate-limiting nutrients within one-carbon metabolism, insufficient folate and related cofactors like vitamins B12, B6, and B2 may impair methylation reactions and epigenetic regulation (Caffrey et al. 2018; Nishigori et al. 2023).

Human studies have demonstrated that folic acid supplementation during the first trimester of pregnancy is associated DNA methylation of genes involved in fetal brain development, including *IGF2*, brain derived neurotrophic factor (*BDNF*), and long interspersed nuclear element-1 (*LINE-1*) , suggesting potential benefits for cognitive development in offspring (Caffrey et al. 2018). In contrast, low maternal vitamin B12 and elevated homocysteine concentration during pregnancy have been associated with low birth weight, preterm birth, intrauterine growth restriction, and increased risk of metabolic disease later in life (Blasetti et al. 2023). Experimental studies have also showed that rats fed a diet restricted in folate, methionine, and choline reduces methylation and expression of *Gria1* gene, leading to hippocampus-related cognitive impairment (Tomizawa et al. 2015). Together, these findings support the important role of one-carbon metabolism in neurodevelopment and cognitive function through epigenetic regulation (Desai and Ross 2020).

In addition, studies have also shown that maternal folate and vitamin B12 deficiencies increase the risk of neural tube defects. Reduced *LINE-1* and genome-wide DNA methylation level in neural tissues of affected fetuses have been associated with low maternal of folic acid and vitamin B12 in maternal plasma (Bokor et al. 2022; Chang et al. 2013). Furthermore, maternal intake of methyl donors such as betaine and folate has been associated with altered DNA methylation of genes related to growth, metabolism, and appetite regulation, including *IGF2*, retinoic acid X receptor (*RXRA*), and *LEP*, Potentially

increasing susceptibility to obesity and non-communicable diseases in later life (Pauwels et al. 2017).

The placenta acts as an important mediator of epigenetic programming. Maternal betaine intake has been inversely associated with placental *IGF2* mRNA expression, suggesting that maternal methyl donor intake may influence fetal growth and phenotype through alterations in gene expression and methylation during critical developmental periods (Kadam et al. 2023). These findings collectively demonstrate that maternal one-carbon metabolism plays a fundamental role in fetal epigenetic programming by regulating DNA methylation and gene expression during critical developmental periods. Disturbances in methyl donor availability during pregnancy may induce persistent epigenetic alterations that influence fetal growth, neurodevelopment, metabolic homeostasis, and susceptibility to chronic disease later in life.

**Transgenerational Risk and Epigenetic Inheritance**

Maternal nutrition is a major determinant of fetal programming and may influence disease susceptibility not only in offspring but also across subsequent generations. The concept of transgenerational epigenetic inheritance suggests that environmental exposures during critical developmental periods can induce epigenetic alterations that persist beyond the directly exposed generation. Both maternal undernutrition and overnutrition have been associated with long-term changes in gene regulation, increasing the risk of obesity, type 2 diabetes, and other metabolic disorders later in life.

Maternal undernutrition represents one of the most extensively studied models of transgenerational programming. Evidence from the Dutch Hunger Winter demonstrated that nutritional deprivation during early pregnancy was associated with persistent epigenetic alterations and adverse metabolic outcomes in offspring. Studies have reported changes in DNA methylation of genes involved in growth, metabolism, and inflammation, including *IGF2, IL10, LEP, ABCA1, GNASAS*, and *MEG3* (Lee 2015). Furthermore, the effects of prenatal famine exposure may extend beyond the directly exposed generation, as a grandchildren of women exposed to famine during early pregnancy have been reported to exhibit altered birth outcomes. These findings suggest that maternal undernutrition may induce epigenetic changes in germ cells, thereby contributing to the transmission of metabolic disorders across generations (Schiera et al. 2026).

Maternal overnutrition and obesity have also been linked to transgenerational metabolic risk. Human studies have shown that exposure to maternal obesity and gestational diabetes is associated with altered DNA methylation of genes involved in appetite regulation, glucose transport,

and energy metabolism, including *LEP, ADIPOQ, SLC2A1, SLC2A3, GLUT1,GLUT3, PPARGC1α, MC4R*, and *LPL* (Franzago et al. 2024; Morais et al. 2025). These epigenetic alterations may affect fetal growth, insulin sensitivity, and energy balance, increasing the risk of obesity and metabolic dysfunction later in life.

Experimental studies further support the transgenerational effects of maternal overnutrition. In mouse models, maternal exposure to a high-fat diet affects the genes encoding adipokines, adiponectin, and leptin, which are responsible for regulating energy balance and insulin sensitivity. First-generation (F0) females were fed a high-fat diet during pregnancy, and second-generation (F1) offspring were exposed to the same diet. When assessing the metabolic outcomes of the third generation (F2), which was not exposed to a high-fat diet, Following cumulative exposure to the previous generations, the F2 offspring exhibited increased body weight, insulin resistance, hypertension, and dyslipidemia, along with impaired metabolism, despite not having been exposed to a high-fat diet (Masuyama et al. 2015). These effects were accompanied by persistent histone modifications in regions regulating leptin and adiponectin expression, including increased H3K9 demethylation and reduced H3K9 acetylation (de Castro Barbosa et al. 2016). Such findings indicate that epigenetic alterations induced by maternal overnutrition can be transmitted across generations and contribute to long-term metabolic dysfunction.

Collectively, current evidence suggests that both maternal undernutrition and overnutrition can induce epigenetic alterations that influence metabolic programming across generations. Through changes in DNA methylation, histone modifications, and gene regulation, maternal nutritional statues may shape susceptibility to obesity, type 2 diabetes, and other chronic diseases not only in offspring but also in subsequent generations. Therefore, transgenerational epigenetic inheritance highlights the importance of maternal metabolic health as a determinant of long-term population health.

**Imprinted Genes**

Imprinted genes play essential roles in fetal growth, metabolism and developmental programming, and their expression is largely regulated through DNA methylation. Because only one parental allele is expressed, these genes are particularly sensitive to environmental influences during fetal development. Studies conducted in umbilical cord blood from infants born to mothers with gestational diabetes have reported altered expression of several imprinted genes, including reduced expression of *PEG3, H19, MEST and MEG3* (Abad et al. 2023). Similarly, increased *IGF2* expression and decreased *H19* expression have been observed in placental and cord

blood samples from pregnancies with gestational diabetes, suggesting that maternal hyperglycemia may alter methylation patterns within the *IGF2/H19* imprinting region (Li, Liu, and Liu 2024; Su et al. 2016). These alterations may contribute to impaired glucose metabolism and increased susceptibility to insulin resistance in offspring .

Imprinted genes are generally protected from extensive epigenetic reprogramming during early embryonic development, making them particularly vulnerable to persistent epigenetic alteration. Maternal obesity has been associated with reduced DNA methylation of *MEG3* and increased methylation of *PLAGL1*, both of which have been linked to dysregulation of energy metabolism and obesity risk in offspring (Alum et al. 2025). In addition, exposure to hyperglycemia in utero has been associated with long-term metabolic disturbances through a process known as metabolic memory, whereby epigenetic changes induced during fetal development remain after birth (Dalfrà et al. 2020). Increase in DNA methylation levels and elevated expression of histone deacetylase 2 (HDAC2) have also been reported in gestational diabetes, further supporting the role of persistent epigenetic modifications in long-term metabolic programming (Sultan and AlMalki 2023).

Beyond imprinted genes, maternal hyperglycemia may influence the epigenetic regulation of genes involved in metabolic processes and pancreatic function, such as the pancreatic and duodenal homeobox 1 (*PDX1*) gene and the peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*PGC1α*) gene. Altered methylation and expression of *PDX1* and *PGC1α* have been associated with glucose metabolism in fetuses exposed to gestational diabetes (Wang et al. 2018). Experimental studies have also demonstrated changes in DNA methylation of cell-cycle regulators such as cyclin-dependent kinase inhibitors CDKN2A/B, suggesting that intrauterine hyperglycemia can induce complex epigenetic alterations that affect metabolic health beyond the fetal period (Li et al. 2024; Nazari et al. 2020).

Collectively, these findings suggest that maternal metabolic conditions, particularly obesity and gestational diabetes, may alter the epigenetic regulation of imprinted genes during fetal development. Because imprinted genes are relatively resistant to epigenetic reprogramming, alterations at these loci may remain throughout life and contribute to long-term metabolic dysfunction, obesity, and increased disease susceptibility in offspring.

**Placental Epigenetics and Epigenetic Clock**

Placental epigenetic regulation plays a key role in placental growth, function, and fetal development. Gestational diabetes can alter placental DNA methylation and the methylation of imprinted genes, leading to placental

dysfunction and impaired fetal growth in utero, which may increase the risk of obesity and metabolic disorders later in life. Studies have shown increased methylation of the *MEG3* gene in women with gestational diabetes, which is associated with maternal blood glucose levels and fetal weight (Chen et al. 2021; Yi et al. 2024). Similarly, decreased methylation of the *MEST* gene in the placenta of women with gestational diabetes, has been linked to fetal macrosomia, while reduced *MEST* methylation has also been observed in obese adults, suggesting a role in obesity programming across the life course (El Hajj et al. 2013). In addition, hypermethylation of the *DLK1* gene was associated with reduced gene expression and was positively correlated with fetal weight and maternal glucose levels following an oral glucose tolerance test. Studies have suggested that *DLK1* methylation may be associated with obesity and metabolic programming disorders in childhood and adulthood (Zhao et al. 2019). These findings indicate that maternal metabolic status can modify placental methylation patterns and influence fetal growth and metabolic programming.

Placental epigenetic modifications also involve non-coding RNAs that regulate fetal growth and nutrient metabolism. Reduced expression of miR-130b-3p, miR-29a-3p, and miR-let-7a-5p have been observed in placentas from women with gestational diabetes and was associated with increased birth weight (Guan et al. 2020). In addition, decreased placental miR-9 and miR-21expression has been linked to increased expression of *PPAR-α, GLUT1* and *HK2*, leading to increased glucose uptake and altered lipid metabolism in trophoblast cells (Song et al. 2021; Yi et al. 2024). Other studies have reported downregulation of *IRS1* and *IRS2*, suggesting that placental miRNAs contribute to changes in insulin signaling and glucose metabolism. Together, these findings support the role of placental miRNAs as important mediators of fetal adaptation to maternal hyperglycemia (Yi et al. 2024).

The concept of the epigenetic clock has provided further evidence that early-life exposures may influence long-term health. Epigenetic clocks are based on DNA methylation patterns and are used to estimate biological age and disease risk. In a 2023 study, gerontologist David reported that epigenetic changes play a key role in mammalian aging and that restoring epigenetic genome integrity reverses signs of aging (Yang et al. 2023). Studies have shown accelerated epigenetic aging in both mothers and children exposed to gestational diabetes, and this acceleration has been associated with fasting insulin levels in children and altered metabolic markers in mothers (Kanney et al. 2022). Similarly, accelerated epigenetic aging in children exposed to gestational diabetes in utero has been associated with an increased risk of cardiovascular and metabolic diseases later in life (Shiau et al. 2021). These findings suggest that maternal metabolic conditions during pregnancy may

have long-lasting effects on biological aging processes through epigenetic mechanisms.

Placental tissue reflects nutritional and metabolic signals exchanged between the mother and the fetus and may serve as an important biomarker of fetal programming (Andonotopo et al. 2026). Studies have shown that maternal nutritional imbalances including deficiencies in micronutrients, protein or fat, can alter placental epigenetic regulation and metabolic programming, thereby increasing disease susceptibility in offspring later in life (Barata et al. 2026). Collectively, current evidence suggests that the placenta acts as a central mediator between maternal nutrition and fetal development. Through alterations in DNA methylation, miRNA expression, and epigenetic aging pathways, placental epigenetic regulation can influence fetal growth, metabolic programming, and long-term disease risk in offspring (Li et al. 2024).

**Conclusion**

Maternal nutrition is one of the most important environmental factors influencing fetal development and long-term health outcomes. This article indicates that nutritional exposure during critical periods of development can modify epigenetic regulation through several mechanisms, including DNA methylation, histone modifications, microRNAs, genomic imprinting, and placental epigenetic adaptations. These mechanisms regulate genes involved in growth, energy metabolism, insulin signaling, neurodevelopment, and inflammatory pathways, thereby influencing susceptibility to obesity, type 2 diabetes, cardiovascular disease, and other non-communicable diseases later in life.

A major finding of this review is that maternal nutrition does not influence fetal programming through a single epigenetic pathway. Instead, nutrients and maternal metabolic status interact with several interconnected mechanisms that collectively shape the fetal epigenome. Evidence from studies on one-carbon metabolism, methyl donor nutrients, imprinted genes, placental epigenetics, and transgenerational inheritance suggests that nutrition during pregnancy may induce persistent changes in gene regulation that extend beyond fetal life. These observations support the concept that fetal epigenome serves as a biological link between the maternal environment and long-term offspring health.

The studies also demonstrated that both maternal undernutrition and overnutrition can affect epigenetic programming. Maternal deficiencies in folate, choline. Vitamin B12, and other nutrients involved in one-carbon metabolism have been associated with altered DNA methylation, impaired

neurodevelopment, and increased metabolic risk. Similarly, maternal obesity, high-fat diets, and gestational diabetes have been linked to epigenetic alterations affecting genes involved in appetite regulation, glucose metabolism, insulin sensitivity, and adipogenesis. Although the exact mechanisms are not fully understood, these findings suggest that maintaining optimal maternal nutritional status during pregnancy may contribute to reducing disease risk in offspring.

Another important observation is the growing evidence for transgenerational effects. Studies in both human and animal models indicate that epigenetic alterations induced by maternal nutritional exposures may persist across generations. However, many of the current studies are from experimental studies, therefore human longitudinal studies are needed to determine the stability and clinical significance of these epigenetics changes throughout life course.

From clinical perspective, epigenetic markers identified in placental tissue, umbilical cord blood, and circulating microRNA may provide beneficial tools for assessing fetal programming and early metabolic risk. Future research should focus on large human studies that combine dietary assessment with epigenomic, transcriptomic, and metabolomic analysis. Further investigations are needed to clarify the effect of specific nutrients, and maternal metabolic conditions on epigenetic regulation during pregnancy.

In conclusion, the available evidence showed that maternal nutrition plays a key role in fetal epigenetic programming and may influence health outcomes throughout life. A better understanding of the interactions between nutrition and epigenetic mechanisms may contribute to the development of more effective nutritional strategies that can improve maternal health and reduce the risk of chronic disease in future generations.

## References

Abad, Fatemeh Rahimi Mehdi, Mohammad Reza Hajizadeh, Mehdi Mahmoodi, Zahra Jalali, Fatemeh Nazem Kazeruni, Jennifer Swann, Reza Hosseiniara, and Mojgan Noroozi Karimabad. 2023. "Evaluation of H19, Mest, Meg3, and Peg3 Genes Affecting Growth and Metabolism in Umbilical Cord Blood Cells of Infants Born to Mothers with Gestational Diabetes and Healthy Mothers in Rafsanjan City, Iran." *Journal of Developmental Origins of Health and Disease* 14(2):182–89. doi:DOI: 10.1017/S2040174422000393.

Alejandro, Emilyn U., Brigid Gregg, Taylor Wallen, Doga Kumusoglu, Daniel Meister, Angela Chen, Matthew J. Merrins, Leslie S. Satin, Ming Liu, Peter Arvan, and Ernesto Bernal-Mizrachi. 2014. "Maternal Diet-Induced MicroRNAs and MTOR Underlie β Cell Dysfunction in Offspring." *Journal of Clinical Investigation* 124(10):4395–4410. doi:10.1172/JCI74237.

Alfano, Rossella, Oliver Robinson, Evangelos Handakas, Tim S. Nawrot, Paolo Vineis, and Michelle Plusquin. 2022. "Perspectives and Challenges of Epigenetic Determinants of Childhood Obesity: A Systematic Review." *Obesity Reviews* 23(S1):e13389. doi:https://doi.org/10.1111/obr.13389.

Allard, C., V. Desgagné, J. Patenaude, M. Lacroix, L. Guillemette, M. C. Battista, M. Doyon, J. Ménard, J. L. Ardilouze, P. Perron, L. Bouchard, and M. F. Hivert. 2015. "Mendelian Randomization Supports Causality between Maternal Hyperglycemia and Epigenetic Regulation of Leptin Gene in Newborns." *Epigenetics* 10(4):342–51. doi:10.1080/15592294.2015.1029700.

Alum, Esther Ugo, Henry Egi Aloh, David Chukwu Obasi, Prince Nkemakolam Okoroh, Ugonna Cassandra Aniokete, and Akunna Perpetua Emeruwa. 2025. "Maternal Nutrition, Toxicants, and Epigenetic Programming of Obesity Across Generations." *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy* 18(null):4873–4911. doi:10.2147/DMSO.S579409.

Anastasopoulou, Maria, Irene Dereki, Argyro Sgourou, and George Lagoumintzis. 2026. "Interactions between Nutrition and the Epigenome: How Can It Be Harnessed for Public Health?" *Future Science OA* 12(1):2641015. doi:10.1080/20565623.2026.2641015.

Anderson, Cindy M., Shannon L. Gillespie, Doria K. Thiele, Jody L. Ralph, and Joyce E. Ohm. 2018. "Effects of Maternal Vitamin D Supplementation on the Maternal and Infant Epigenome." *Breastfeeding Medicine : The Official Journal of the Academy of Breastfeeding Medicine* 13(5):371–80. doi:10.1089/bfm.2017.0231.

Andonotopo, Wiku, Muhammad Adrianes Bachnas, Julian Dewantiningrum, Mochammad Besari Adi Pramono, Sri Sulistyowati, I. Nyoman Hariyasa Sanjaya, Milan Stanojevic, and Asim Kurjak. 2026. "Nutriepigenomics in Perinatal Medicine: Maternal Nutrition as a Modulator of Fetal Gene Expression and Long-Term Health." 54(2):244–57. doi:doi:10.1515/jpm-2025-0289.

Andonotopo, Wiku, Muhammad Adrianes Bachnas, Julian Dewantiningrum, Mochammad Besari, Adi Pramono, Sri Sulistyowati, I. Nyoman Hariyasa Sanjaya, and Milan Stanojevic. 2025. "Nutriepigenomics in Perinatal Medicine : Mater-

nal Nutrition as a Modulator of Fetal Gene Expression and Long-Term Health." *Journal of Perinatal Medicine* 1–14. doi:10.1515/jpm-2025-0289.

Arslan, Sabriye, and Hilal Yıldıran. 2021. "Maternal Beslenmenin Yavrular Üzerine Etkileri: Fetal Programlama ve Epigenetik Mekanizmalar." *Journal of Nutrition and Dietetics* 1–8. doi:10.33076/2021.bdd.1437.

Aslı Akyol Mutlu. 2016. "Maternal Beslenme, Tip 2 Diyabet ve Epigenetik Görünümü." *Beslenme ve Diyet Dergisi* 44(1):62-67.

Barata, Luisa Annibal, Matheus Naia Fioretto, Natália Magosso, Flávia Alessandra Maciel, Isabelle Tenori Ribeiro, Patrick Vieira de Souza, Ana Luiza Romano Gabriel, Wellerson Rodrigo Scarano, and Luis Antonio Justulin. 2026. "Placental Programming in Maternal Malnutrition as a Crucial Determinant of Offspring Health: A Critical-Interpretative Narrative Review." *Clinical Nutrition ESPEN* 74:103349. doi:https://doi.org/10.1016/j.clnesp.2026.103349.

Barker, D. J. P. 2007. "The Origins of the Developmental Origins Theory." *Journal of Internal Medicine* 261(5):412–17. doi:10.1111/j.1365-2796.2007.01809.x.

Basak, Sanjay, Rahul Mallick, Boga Navya Sree, and Asim K. Duttaroy. 2024. "Placental Epigenome Impacts Fetal Development: Effects of Maternal Nutrients and Gut Microbiota." *Nutrients* 16(12).

Blasetti, Annalisa, Alessia Quarta, Miriana Guarino, Ilenia Cicolini, Daniela Iannucci, Cosimo Giannini, and Francesco Chiarelli. 2023. "Role of Prenatal Nutrition in the Development of Insulin Resistance in Children." *Nutrients* 15(1).

Blom, Henk J., and Yvo Smulders. 2011. "Overview of Homocysteine and Folate Metabolism. With Special References to Cardiovascular Disease and Neural Tube Defects." *Journal of Inherited Metabolic Disease* 34(1):75–81.

Bokor, Szilvia, Ildikó Csölle, Regina Felső, Réka A. Vass, Simone Funke, Tibor Ertl, and Dénes Molnár. 2024. "Dietary Nutrients during Gestation Cause Obesity and Related Metabolic Changes by Altering DNA Methylation in the Offspring." *Frontiers in Endocrinology* Volume 15-2024. doi:10.3389/fendo.2024.1287255.

Bokor, Szilvia, Réka A. Vass, Simone Funke, Tibor Ertl, and Dénes Molnár. 2022. "Epigenetic Effect of Maternal Methyl-Group Donor Intake on Offspring's Health and Disease." *Life* 12(5):609.

Caffrey, Aoife, Rachelle E. Irwin, Helene McNulty, J. J. Strain, Diane J. Lees-Murdock, Breige A. McNulty, Mary Ward, Colum P. Walsh, and Kristina Pentieva. 2018. "Gene-Specific DNA Methylation in Newborns in Response to Folic Acid Supplementation during the Second and Third Trimesters of Pregnancy: Epigenetic Analysis from a Randomized Controlled Trial." *American Journal of Clinical Nutrition* 107(4):566–75. doi:10.1093/ajcn/nqx069.

Cai, Shuang, Shuang Quan, Guangxin Yang, Meixia Chen, Qianhong Ye, Gang Wang, Haitao Yu, Yuming Wang, Shiyan Qiao, and Xiangfang Zeng. 2021. "Nutritional Status Impacts Epigenetic Regulation in Early Embryo Development: A Scoping Review." *Advances in Nutrition* 12(5):1877–92.

de Castro Barbosa, Thais, Lars R. Ingerslev, Petter S. Alm, Soetkin Versteyhe, Julie Massart, Morten Rasmussen, Ida Donkin, Rasmus Sjögren, Jonathan M. Mudry, Laurène Vetterli, Shashank Gupta, Anna Krook, Juleen R. Zierath, and Romain Barrès. 2016. "High-Fat Diet Reprograms the Epigenome of Rat Spermatozoa and Transgenerationally Affects Metabolism of the Offspring." *Molecular Metabolism* 5(3):184–97. doi:10.1016/j.molmet.2015.12.002.

Chang, Shaoyan, Li Wang, Yunqian Guan, Shaofang Shangguan, Qingan Du, Yang Wang, Ting Zhang, and Yu Zhang. 2013. "Cellular Biochemistry." *Journal of Cellular Biochemistry* 1558(45):1549–58. doi:10.1002/jcb.24496.

Chango, Abalo, and Igor P. Pogribny. 2015. "Considering Maternal Dietary Modulators for Epigenetic Regulation and Programming of the Fetal Epigenome." *Nutrients* 7(4):2748–70. doi:10.3390/nu7042748.

Chen, Cheng, Ying Jiang, Ting Yan, Yuan Chen, Mengmeng Yang, Min Lv, Fangfang Xi, Juefei Lu, Baihui Zhao, and Qiong Luo. 2021. "Placental Maternally Expressed Gene 3 Differentially Methylated Region Methylation Profile Is Associated with Maternal Glucose Concentration and Newborn Birthweight." *Journal of Diabetes Investigation* 12(6):1074–82. doi:https://doi.org/10.1111/jdi.13432.

Chodur, Gwen M., and Francene M. Steinberg. 2024. "Human MicroRNAs Modulated by Diet: A Scoping Review." *Advances in Nutrition (Bethesda, Md.)* 15(6):100241. doi:10.1016/j.advnut.2024.100241.

Dalfrà, Maria Grazia, Silvia Burlina, Gloria Giovanna Del Vescovo, and Annunziata Lapolla. 2020. "Genetics and Epigenetics: New Insight on Gestational Diabetes Mellitus." *Frontiers in Endocrinology* Volume 11-2020.

Dalgaard, Louise T., Anja E. Sørensen, Anandwardhan A. Hardikar, and Mugdha V Joglekar. 2022. "The MicroRNA-29 Family: Role in Metabolism and Metabolic Disease." *American Journal of Physiology-Cell Physiology* 323(2):C367–77. doi:10.1152/ajpcell.00051.2022.

Desai, Mina, and Michael G. Ross. 2020. "Maternal-Infant Nutrition and Development Programming of Offspring Appetite and Obesity." *Nutrition Reviews* 78:25–31. doi:10.1093/nutrit/nuaa121.

Franzago, Marica, Paola Borrelli, Marta Di Nicola, Pierluigi Cavallo, Ebe D'Adamo, Luciano Di Tizio, Diego Gazzolo, Liborio Stuppia, and Ester Vitacolonna. 2024. "From Mother to Child: Epigenetic Signatures of Hyperglycemia and Obesity during Pregnancy." *Nutrients* 16(20). doi:10.3390/nu16203502.

Glendining, Kelly A., and Christine L. Jasoni. 2019. "Maternal High Fat Diet-Induced Obesity Modifies Histone Binding and Expression of Oxtr in Offspring Hippocampus in a Sex-Specific Manner." *International Journal of Molecular Sciences* 20(2):329.

Guan, Chun-Yi, Shi Tian, Jing-Li Cao, Xue-Qin Wang, Xu Ma, and Hong-Fei Xia. 2020. "Down-Regulated MiR-21 in Gestational Diabetes Mellitus Placenta Induces PPAR-α to Inhibit Cell Proliferation and Infiltration." *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy* 13(null):3009–34. doi:10.2147/DMSO.S253920.

Guéant, Jean Louis, Rania Elakoum, Olivier Ziegler, David Coelho, Eva Feigerlova, Jean Luc Daval, and Rosa Maria Guéant-Rodriguez. 2014. "Nutritional Models of Foetal Programming and Nutrigenomic and Epigenomic Dysregulations of Fatty Acid Metabolism in the Liver and Heart." *Pflugers Archiv European Journal of Physiology* 466(5):833–50.

Guéant, Jean Louis, Fares Namour, Rosa Maria Guéant-Rodriguez, and Jean Luc Daval. 2013. "Folate and Fetal Programming: A Play in Epigenomics?" *Trends in Endocrinology and Metabolism* 24(6):279–89.

Gujral, Palak, Vishakha Mahajan, Abbey C. Lissaman, and Anna P. Ponnampalam. 2020. "Histone Acetylation and the Role of Histone Deacetylases in Normal Cyclic Endometrium." *Reproductive Biology and Endocrinology* 18(1):84. doi:10.1186/s12958-020-00637-5.

El Hajj, Nady, Galyna Pliushch, Eberhard Schneider, Marcus Dittrich, Tobias Müller, Michael Korenkov, Melanie Aretz, Ulrich Zechner, Harald Lehnen, and Thomas Haaf. 2013. "Metabolic Programming of MEST DNA Methylation by Intrauterine Exposure to Gestational Diabetes Mellitus." *Diabetes* 62(4):1320–28. doi:10.2337/db12-0289.

Harmancıoğlu, Begüm, and Seray Kabaran. 2023. "Maternal High Fat Diets: Impacts on Offspring Obesity and Epigenetic Hypothalamic Programming." *Frontiers in Genetics* 14:1158089. doi:10.3389/fgene.2023.1158089.

Heijmans, Bastiaan T., Elmar W. Tobi, Aryeh D. Stein, Hein Putter, Gerard J. Blauw, Ezra S. Susser, P. Eline Slagboom, and L. H. Lumey. 2008. "Persistent Epigenetic Differences Associated with Prenatal Exposure to Famine in Humans." *Proceedings of the National Academy of Sciences of the United States of America* 105(44):17046–49. doi:10.1073/pnas.0806560105.

Hill, David J., and Thomas G. Hill. 2024. "Maternal Diet during Pregnancy and Adaptive Changes in the Maternal and Fetal Pancreas Have Implications for Future Metabolic Health." *Frontiers in Endocrinology* 15:1456629. doi:10.3389/fendo.2024.1456629.

Hor, Kahyee, Laura Dearden, Emily Herzstein, Susan Ozanne, Giles Hardingham, and Amanda J. Drake. 2025. "Maternal High Fat and High Sugar Diet Impacts on Key DNA Methylation Enzymes in Offspring Brain in a Sex-Specific Manner." *Journal of Neuroendocrinology* 37(8):e70046. doi:https://doi.org/10.1111/jne.70046.

Iacomino, Giuseppe, and Alfonso Siani. 2017. "Role of MicroRNAs in Obesity and Obesity-Related Diseases." *Genes & Nutrition* 12(1):23. doi:10.1186/s12263-017-0577-z.

Kadam, Isma'il, Mudar Dalloul, Jeanette Hausser, Monique Huntley, Lori Hoepner, Lawrence Fordjour, Joan Hittelman, Anjana Saxena, Jia Liu, Itamar D. Futterman, Howard Minkoff, and Xinyin Jiang. 2023. "Associations between Nutrients in One-Carbon Metabolism and Fetal DNA Methylation in Pregnancies with or without Gestational Diabetes Mellitus." *Clinical Epigenetics* 15(1):137. doi:10.1186/s13148-023-01554-1.

Kanney, Nita, Amit Patki, Paula Chandler-Laney, W. T. Garvey, and Bertha A. Hidalgo. 2022. "Epigenetic Age Acceleration in Mothers and Offspring 4–10 Years after a Pregnancy Complicated by Gestational Diabetes and Obesity." *Metabolites* 12(12):1226.

Khamis, Marwa Maher, Said Salama Moselhy, and Shaimaa Rihan. 2025. "Role of Trans-Resveratrol in Ameliorating Biochemical and Molecular Alterations in Obese Rats Induced by a High Fructose/Fat Diet." *Scientific Reports* 15(1):7879. doi:10.1038/s41598-025-91027-7.

Kovacheva, Vesela P., Tiffany J. Mellott, Jessica M. Davison, Nicholas Wagner, Ignacio Lopez-Coviella, Aletta C. Schnitzler, and Jan K. Blusztajn. 2007. "Gestational Choline Deficiency Causes Global and Igf2 Gene DNA Hypermethylation by Up-Regulation of Dnmt1 Expression." *Journal of Biological Chemistry* 282(43):31777–88. doi:10.1074/jbc.M705539200.

Lee, Ho Sun. 2015. "Impact of Maternal Diet on the Epigenome during in Utero Life and the Developmental Programming of Diseases in Childhood and Adulthood." *Nutrients* 7(11):9492–9507.

Li, Nan, Huan-Yu Liu, and Song-Mei Liu. 2024. "Deciphering DNA Methylation in Gestational Diabetes Mellitus: Epigenetic Regulation and Potential Clinical Applications." *International Journal of Molecular Sciences* 25(17):9361.

Masuyama, Hisashi, Takashi Mitsui, Etsuko Nobumoto, and Yuji Hiramatsu. 2015. "The Effects of High-Fat Diet Exposure In Utero on the Obesogenic and Diabetogenic Traits Through Epigenetic Changes in Adiponectin and Leptin Gene Expression for Multiple Generations in Female Mice." *Endocrinology* 156(7):2482–91. doi:10.1210/en.2014-2020.

Medici, Valentina, Noreene M. Shibata, Kusum K. Kharbanda, Mohammad S. Islam, Carl L. Keen, Kyoungmi Kim, Brittany Tillman, Samuel W. French, Charles H. Halsted, and Janine M. LaSalle. 2014. "Maternal Choline Modifies Fetal Liver Copper, Gene Expression, DNA Methylation, and Neonatal Growth in the Tx-j Mouse Model of Wilson Disease." *Epigenetics* 9(2):286–96. doi:10.4161/epi.27110.

Morais, Juliana, Débora Veiga, Ana Filipa Ferreira, Inês Alves, Inês Castela, Carla Ramalho, Joana O. Miranda, Adelino Leite-Moreira, and Inês Falcão-Pires. 2025. "Relationship between Maternal Obesity, Birth Weight, and Fetal Adiponectin/Leptin Ratio: A Potential Early Biomarker of Cardiometabolic Risk." *American Journal of Physiology-Endocrinology and Metabolism* 329(5):E655–62. doi:10.1152/ajpendo.00259.2025.

Nazari, Zahra, Alireza Shahryari, Soraya Ghafari, Mohammad Nabiuni, and Mohammad Jafar Golalipour. 2020. "In Utero Exposure to Gestational Diabetes Alters DNA Methylation and Gene Expression of CDKN2A/B in Langerhans Islets of Rat Offspring." *Cell Journal* 22(2):203–11. doi:10.22074/cellj.2020.6699.

Nelson, Benjamin N., and Jacob E. Friedman. 2024. "Developmental Programming of the Fetal Immune System by Maternal Western-Style Diet: Mechanisms and Implications for Disease Pathways in the Offspring." *International Journal of Molecular Sciences* 25(11):5951.

Nishigori, Hidekazu, Toshie Nishigori, Taku Obara, Taeko Suzuki, Miyuki Mori, Karin Imaizumi, Tsuyoshi Murata, Hyo Kyozuka, Yuka Ogata, Akiko Sato, Kosei Shinoki, Seiji Yasumura, Mitsuaki Hosoya, Koichi Hashimoto, Keiya Fujimori, Michihiro Kamijima, Shin Yamazaki, Yukihiro Ohya, Reiko Kishi, Nobuo Yaegashi, Koichi Hashimoto, Chisato Mori, Shuichi Ito, Zentaro Yamagata, Hidekuni Inadera, Takeo Nakayama, Tomotaka Sofue, Masayuki Shima, Hiroshige Nakamura, Narufumi Suganuma, Koichi Kusuhara, and Takahiko Katoh. 2023. "Prenatal Folic Acid Supplement/Dietary Folate and Cognitive Development in 4-Year-Old Offspring from the Japan Environment and Children's Study." *Scientific Reports* 13(1). doi:10.1038/s41598-023-36484-8.

Pant, Richa, Priyanka Firmal, Vibhuti Kumar Shah, Aftab Alam, and Samit Chattopadhyay. 2021. "Epigenetic Regulation of Adipogenesis in Development of Metabolic Syndrome." *Frontiers in Cell and Developmental Biology* Volume 8-2020.

Pauwels, Sara, Manosij Ghosh, Radu Corneliu Duca, Bram Bekaert, Kathleen Freson, Inge Huybrechts, Sabine A. S. Langie, Gudrun Koppen, Roland Devlieger, and Lode Godderis. 2017. "Maternal Intake of Methyl-Group Donors Affects DNA Methylation of Metabolic Genes in Infants." *Clinical Epigenetics* 9(1):16. doi:10.1186/s13148-017-0321-y.

Ramírez-Alarcón, Karina, Ángela Sánchez-Agurto, Liliana Lamperti, and Miquel Martorell. 2019. "Epigenetics, Maternal Diet and Metabolic Programming." *The Open Biology Journal* 7(1):45–51. doi:10.2174/1874196701907010045.

Reichetzeder, Christoph. 2021. "Overweight and Obesity in Pregnancy: Their Impact on Epigenetics." *European Journal of Clinical Nutrition* 75(12):1710–22. doi:10.1038/s41430-021-00905-6.

Ryznar, Rebecca Jean, Lacie Phibbs, and Lon J. Van Winkle. 2021. "Epigenetic Modifications at the Center of the Barker Hypothesis and Their Transgenerational Implications." *International Journal of Environmental Research and Public Health* 18(23).

Şanlı, Ezgi, and Seray Kabaran. 2019. "Maternal Obesity, Maternal Overnutrition and Fetal Programming: Effects of Epigenetic Mechanisms on the Development of Metabolic Disorders." *Current Genomics* 20(6):419–27. doi:10.2174/1389202920666191030092225.

Schiera, Gabriella, Giulia Macajone, Sara Volpes, Laura Greco, Carlo Maria Di Liegro, Graziella Serio, Fabio Caradonna, and Flores Naselli. 2026. "Maternal Overnutrition and Fetal Programming: Long-Term Metabolic, Cognitive, and Epigenetic Consequences." *Cells* 15(4). doi:10.3390/cells15040366.

Schulz, Laura C. 2010. "The Dutch Hunger Winter and the Developmental Origins of Health and Disease." *Proceedings of the National Academy of Sciences of the United States of America* 107(39):16757–58. doi:10.1073/pnas.1012911107.

Sen, S., S. L. Rifas-Shiman, N. Shivappa, M. D. Wirth, J. R. Hebert, D. R. Gold, M. W. Gillman, and E. Oken. 2018. "Associations of Prenatal and Early Life Dietary Inflammatory Potential with Childhood Adiposity and Cardiometabolic Risk in Project Viva." *Pediatric Obesity* 13(5):292–300. doi:https://doi.org/10.1111/ijpo.12221.

Sharp, Gemma C., Lucas A. Salas, Claire Monnereau, Catherine Allard, Paul Yousefi, Todd M. Everson, Jon Bohlin, Zongli Xu, Rae-Chi Huang, Sarah E. Reese, Cheng-Jian Xu, Nour Baïz, Cathrine Hoyo, Golareh Agha, Ritu Roy, John W. Holloway, Akram Ghantous, Simon K. Merid, Kelly M. Bakulski, Leanne K. Küpers, Hongmei Zhang, Rebecca C. Richmond, Christian M. Page, Liesbeth Duijts, Rolv T. Lie, Phillip E. Melton, Judith M. Vonk, Ellen A. Nohr, ClarLynda Williams-DeVane, Karen Huen, Sheryl L. Rifas-Shiman, Carlos Ruiz-Arenas, Semira Gonseth, Faisal I. Rezwan, Zdenko Herceg, Sandra Ekström, Lisa Croen, Fahimeh Falahi, Patrice Perron, Margaret R. Karagas, Bilal M. Quraishi, Matthew Suderman, Maria C. Magnus, Vincent W. V Jaddoe, Jack A. Taylor, Denise Anderson, Shanshan Zhao, Henriette A. Smit, Michele J. Josey, Asa Bradman, Andrea A. Baccarelli, Mariona Bustamante, Siri E. Håberg, Göran Pershagen, Irva Hertz-Picciotto, Craig Newschaffer, Eva Corpeleijn, Luigi Bouchard, Debbie A. Lawlor, Rachel L. Maguire, Lisa F. Barcellos, George Davey Smith, Brenda Eskenazi, Wilfried Karmaus, Carmen J. Marsit, Marie-France Hivert, Harold Snieder, M. Daniele Fallin, Erik Melén, Monica C. Munthe-Kaas, Hasan Arshad, Joseph L. Wiemels, Isabella Annesi-Maesano, Martine Vrijheid, Emily Oken, Nina Holland, Susan K. Murphy, Thorkild I. A. Sørensen, Gerard H. Koppelman, John P. Newnham, Allen J. Wilcox, Wenche Nystad, Stephanie J. London, Janine F. Felix, and Caroline L. Relton. 2017. "Maternal BMI at the Start of Pregnancy and Offspring Epigenome-Wide DNA Methylation: Findings from the Pregnancy and Childhood Epigenetics (PACE) Consortium." *Human Molecular Genetics* 26(20):4067–85. doi:10.1093/hmg/ddx290.

Shiau, Stephanie, Leishen Wang, Huikun Liu, Yinan Zheng, Alex Drong, Brian T. Joyce, Jun Wang, Weiqin Li, Junhong Leng, Yun Shen, Ru Gao, Gang Hu, Lifang Hou, and Andrea A. Baccarelli. 2021. "Prenatal Gestational Diabetes Mellitus Exposure and Accelerated Offspring DNA Methylation Age in Early Childhood." *Epigenetics* 16(2):186–95. doi:10.1080/15592294.2020.1790924.

Socha, Maciej W., Wojciech Flis, and Mateusz Wartęga. 2024. "Epigenetic Genome Modifications during Pregnancy: The Impact of Essential Nutritional Supplements on DNA Methylation." *Nutrients* 16(5):678.

Song, Tian-Rong, Gui-Dong Su, Ya-Li Chi, Ting Wu, Yue Xu, and Chun-Chun Chen. 2021. "Dysregulated MiRNAs Contribute to Altered Placental Glucose Metabolism in Patients with Gestational Diabetes via Targeting GLUT1 and HK2." *Placenta* 105:14–22. doi:https://doi.org/10.1016/j.placenta.2021.01.015.

Su, Rina, Chen Wang, Hui Feng, Li Lin, Xinyue Liu, Yumei Wei, and Huixia Yang. 2016. "Alteration in Expression and Methylation of IGF2/H19 in Placenta and Umbilical Cord Blood Are Associated with Macrosomia Exposed to Intrauterine Hyperglycemia." *PLOS ONE* 11(2):e0148399.

Sultan, Samar, and Sultanh AlMalki. 2023. "Analysis of Global DNA Methylation and Epigenetic Modifiers (DNMTs and HDACs) in Human Foetal Endothelium Exposed to Gestational and Type 2 Diabetes." *Epigenetics* 18(1):2201714. doi:10.1080/15592294.2023.2201714.

Tang, Shoubin, Huihua Wu, Qiuzhen Chen, Tao Tang, Jiashuo Li, Huiqing An, Shuai Zhu, Longsen Han, Hongzheng Sun, Juan Ge, Xu Qian, Xi Wang, and Qiang Wang. 2024. "Maternal Obesity Induces the Meiotic Defects and Epigenetic Alterations During Fetal Oocyte Development." *Advanced Science* 11(30):2309184. doi:https://doi.org/10.1002/advs.202309184.

Tomizawa, H., D. Matsuzawa, D. Ishii, S. Matsuda, K. Kawai, Y. Mashimo, C. Sutoh, and E. Shimizu. 2015. "Methyl-Donor Deficiency in Adolescence Affects Memory and Epigenetic Status in the Mouse Hippocampus." *Genes, Brain and Behavior* 14(3):301–9. doi:10.1111/gbb.12207.

Wang, Lizhen, Hailing Fan, Ludan Zhou, Yanjun Wu, Hongping Lu, and Jing Luo. 2018. "Altered Expression of PGC-1α and PDX1 and Their Methylation Status Are Associated with Fetal Glucose Metabolism in Gestational Diabetes Mellitus." *Biochemical and Biophysical Research Communications* 501(1):300–306. doi:https://doi.org/10.1016/j.bbrc.2018.05.010.

Waterland, Robert A., and Randy L. Jirtle. 2003. "Transposable Elements: Targets for Early Nutritional Effects on Epigenetic Gene Regulation." *Molecular and Cellular Biology* 23(15):5293–5300. doi:10.1128/mcb.23.15.5293-5300.2003.

Yang, Jae-Hyun, Motoshi Hayano, Patrick T. Griffin, João A. Amorim, Michael S. Bonkowski, John K. Apostolides, Elias L. Salfati, Marco Blanchette, Elizabeth M. Munding, Mital Bhakta, Yap Ching Chew, Wei Guo, Xiaojing Yang, Sun Maybury-Lewis, Xiao Tian, Jaime M. Ross, Giuseppe Coppotelli, Margarita V Meer, Ryan Rogers-Hammond, Daniel L. Vera, Yuancheng Ryan Lu, Jeffrey W. Pippin, Michael L. Creswell, Zhixun Dou, Caiyue Xu, Sarah J. Mitchell, Abhirup Das, Brendan L. O'Connell, Sachin Thakur, Alice E. Kane, Qiao Su, Yasuaki Mohri, Emi K. Nishimura, Laura Schaevitz, Neha Garg, Ana-Maria Balta, Meghan A. Rego, Meredith Gregory-Ksander, Tatjana C. Jakobs, Lei Zhong, Hiroko Wakimoto, Jihad El Andari, Dirk Grimm, Raul Mostoslavsky, Amy J. Wagers, Kazuo Tsubota, Stephen J. Bonasera, Carlos M. Palmeira, Jonathan G. Seidman, Christine E. Seidman, Norman S. Wolf, Jill A. Kreiling, John M. Sedivy, George F. Murphy, Richard E. Green, Benjamin A. Garcia, Shelley L. Berger, Philipp Oberdoerffer, Stuart J. Shankland, Vadim N. Gladyshev, Bruce R. Ksander, Andreas R. Pfenning, Luis A. Rajman, and David A. Sinclair. 2023. "Loss of Epigenetic Information as a Cause of Mammalian Aging." *Cell* 186(2):305-326.e27. doi:10.1016/j.cell.2022.12.027.

Yi, Yan, Tao Wang, Wei Xu, and San-Hong Zhang. 2024. "Epigenetic Modifications of Placenta in Women with Gestational Diabetes Mellitus and Their Offspring." *World Journal of Diabetes* 15(3):378–91. doi:10.4239/wjd.v15.i3.378.

Yusuf, Mohamed S., Ahmed Kamel El-sayed, A. Hashish, and Zhen Sun. 2016. "Tasks of Some Dietary Essential Nutrients on Epigenetics and One Carbon Metabolism in Livestock during Production." *Journal of Agriculture and Veterinary Science (IOSR-JAVS)* 9(8):86–90. doi:10.9790/2380-0908028690.

Zhang, Qian, Xinhua Xiao, Jia Zheng, Ming Li, Miao Yu, Fan Ping, Tong Wang, and Xiaojing Wang. 2019. "A Maternal High-Fat Diet Induces DNA Methylation Changes That Contribute to Glucose Intolerance in Offspring." *Frontiers in Endocrinology* Volume 10-2019.

Zhao, Bai-Hui, Ying Jiang, Hong Zhu, Fang-Fang Xi, Yuan Chen, Ye-Tao Xu, Fang Liu, Ya-Yun Wang, Wen-Sheng Hu, Wei-Guo Lv, and Qiong Luo. 2019. "Placental Delta-Like 1 Gene DNA Methylation Levels Are Related to Mothers' Blood Glucose Concentration." *Journal of Diabetes Research* 2019(1):9521510. doi:https://doi.org/10.1155/2019/9521510.

Zhou, Li Yuan, Ming Qun Deng, Qian Zhang, and Xin Hua Xiao. 2020. "Early-Life Nutrition and Metabolic Disorders in Later Life: A New Perspective on Energy Metabolism." *Chinese Medical Journal* 133(16):1961–70.

Chapter 5

# MICROFLUIDICS, HIGH-SPEED IMAGING AND ARTIFICIAL INTELLIGENCE IN DIGITAL HEMORHEOLOGY

*Ahmet Alperen SUBAŞI*[1]
*Nurten BAHTİYAR*[2]
*Birsen AYDEMİR*[3]

1 Ahmet Alperen SUBAŞI, PhD Student, Sakarya University, Institute of Health Sciences, Faculty of Medicine, Department of Physiology, Sakarya, Turkey, ORCID ID: https:// orcid.org/0009-0005-8984-9426
2 Nurten BAHTİYAR, Assoc. Prof, Istanbul University-Cerrahpasa, Cerrahpaşa Medical Faculty, Department of Biophysics, Istanbul, Turkey, ORCID ID: https:// orcid.org/0000-0003-2420-8415
3 Birsen AYDEMİR, Prof, Sakarya University, Medical Faculty, Department of Biophysics, Sakarya, Turkey, ORCID ID: https://orcid.org/0000-0003-1406-864X

## 1. Introduction

Hemorheology is defined as a discipline concerned with the assessment of the biophysical and rheological properties that determine the flow behavior of blood within the circulatory system. In this context, plasma viscosity, hematocrit level, and the deformability and aggregation characteristics of erythrocytes constitute the primary parameters of investigation (Baskurt & Meiselman, 2024). Blood behaves as a non-Newtonian fluid, exhibiting variable viscosity depending on the applied shear stress due to the deformability of erythrocytes (Nader et al., 2019). Accordingly, the flow properties of blood are not merely physical characteristics but are also directly related to biological function. Indeed, fundamental physiological processes such as tissue perfusion, oxygen transport, and vascular resistance are significantly influenced by these rheological parameters. This highlights the critical role of hemorheology both in understanding normal physiological conditions and in evaluating various pathophysiological states (Popel & Johnson, 2005).

According to classical hemorheological approaches, erythrocyte deformability and aggregation are considered two fundamental determinants of microvascular flow behavior (Baskurt et al., 2011). Erythrocyte deformability enables cells to traverse capillaries narrower than their resting diameter, thereby playing a vital role in maintaining adequate tissue oxygenation (Mokken et al., 1992). In contrast, erythrocyte aggregation leads to the reversible formation of rouleaux structures under low shear conditions, increasing the apparent viscosity of blood and thereby affecting flow resistance (Nader et al., 2019). This dynamic interaction between deformability and aggregation underlies the shear-thinning behavior of blood; that is, viscosity decreases with increasing shear rate as aggregates are disrupted and erythrocytes align in the direction of flow (Lanotte et al., 2016).

However, the classical hemorheological approach has important limitations. In this framework, measurements are predominantly based on bulk parameters that represent averaged values across cell populations (Tomaiuolo, 2014).

Such measurements largely neglect the heterogeneity in morphological characteristics, membrane composition, and mechanical behavior of circulating erythrocytes (Pretorius et al., 2015). The reduction of erythrocyte variability to a single mean value is not only a methodological limitation but also carries significant physiological implications. Indeed, subpopulations of mechanically impaired erythrocytes may induce pronounced alterations in the microcirculation, thereby contributing to the development of pathological conditions such as diabetes, cardiovascular diseases, and sickle cell anemia (Connes et al., 2016).

In response to these limitations, the field of blood rheology has undergone a significant transformation over time. Researchers have developed the concept of "mechanical phenotyping," which enables the evaluation of cellular mechanical behavior at the single-cell level (Gossett et al., 2012). In this approach, each cell is analyzed independently based on its deformability, viscoelastic properties, and dynamic behavior under flow, rather than being represented through population averages (Otto et al., 2015). This development reflects a growing recognition that microcirculatory function is influenced not only by average rheological properties but also by the distribution and temporal variability of distinct cellular subpopulations (Gossett et al., 2012; Otto et al., 2015).

Today, hemorheology has evolved beyond a standalone discipline into an interdisciplinary research field situated at the intersection of biomechanics, microfluidic technologies, and computational science (Tomaiuolo, 2014). Through the integration of microfluidic systems, high-throughput imaging techniques, and advanced data analytics, cellular behavior can now be investigated with a level of precision and resolution previously unattainable (Otto et al., 2015). In this context, hemorheology has shifted from a field focused solely on macroscopic blood flow properties to one that enables detailed analysis of each cell's response to its mechanical environment (Otto et al., 2015; Tomaiuolo, 2014).

This study aims to present a comprehensive overview of the field of hemorheology by examining its developmental trajectory from classical methodologies to modern approaches, including mechanical phenotyping, microfluidic technologies, and artificial intelligence–assisted analyses. In doing so, it seeks to provide an integrated perspective on both the biophysical foundations of hemorheology and its contemporary methodological transformation.

## 2. Classical Measurement Techniques and Their Limitations

Classical hemorheology relies on three main methodological approaches—viscometry, ektacytometry, and aggregometry—to evaluate both the bulk flow characteristics of blood and the collective properties of blood cells (Baskurt & Meiselman, 2024). Through these techniques, blood has been established as a non-Newtonian fluid, and the relationship between its physical properties and physiological processes has been substantially elucidated (Nader et al., 2019). However, these methods predominantly provide measurements that reflect average behaviors of cell populations rather than resolving single-cell dynamics.

Viscometry is one of the primary techniques used to assess the viscosity of whole blood under varying shear rates. The interactions among erythrocytes, their aggregation behavior, and their alignment in the direction of flow during

shear-thinning collectively lead to significant changes in blood viscosity (Chien, 1970). In this respect, viscometric measurements provide essential information about the overall flow behavior of the system. Nevertheless, since this approach reflects only global rheological performance, it does not allow for the discrimination of contributions from distinct erythrocyte subpopulations (Tomaiuolo, 2014).

Ektacytometry, typically based on laser diffraction methods, is used to evaluate erythrocyte deformability. In this technique, the elongation index of red blood cells exposed to defined shear stresses is measured to quantify their deformability (Baskurt et al., 2009; Mannini et al., 2007). The elongation index is a key parameter as it reflects the ability of erythrocytes to traverse narrow capillaries. However, similar to viscometry, ektacytometry yields results that represent the averaged behavior of large cell populations and therefore fails to capture cellular heterogeneity at the single-cell level (Mokken et al., 1992). This limitation restricts the detection of mechanically distinct subpopulations that may be clinically relevant (Clark et al., 1983).

Aggregometry is another fundamental method used to investigate the reversible formation of erythrocyte rouleaux structures under low shear conditions and their disaggregation under increasing mechanical stress (Baskurt et al., 2011). Aggregation plays a particularly significant role in determining apparent blood viscosity at low shear rates, making it a key determinant of flow behavior (Nader et al., 2019). However, compared to advanced imaging-based techniques, aggregometry has limited capacity to resolve the structural organization and heterogeneity of erythrocyte aggregates in detail (Menichini et al., 2015).

A common limitation of these methods is that they rely on population-averaged properties when evaluating blood as a highly heterogeneous system. In contrast, erythrocytes exhibit substantial variability in terms of age, morphology, and mechanical properties. Moreover, small subpopulations of mechanically impaired or poorly deformable erythrocytes can exert disproportionately large effects on microcirculatory flow. However, such effects cannot be adequately captured by measurements based on averaged rheological values (Connes et al., 2016).

In addition, conventional experimental techniques fail to sufficiently represent the complex geometry and dynamic flow characteristics of the microcirculation. In microvascular networks, where vessel diameters may be comparable to or even smaller than those of erythrocytes, cellular deformability and interactions with the vessel wall become critically important (Popel & Johnson, 2005). Under in vivo conditions, blood flow within branched and constricted vascular networks is governed by cell–wall interactions and

localized flow heterogeneities. However, these complex processes cannot be effectively reproduced using classical rheological assays (Pries et al., 1990).

### 3. Microfluidic Systems: Biomimetic Approaches in Hemorheology

Microfluidic technologies have profoundly transformed the methodological framework of hemorheological research. Owing to their ability to accurately reproduce the physical microenvironment and mechanical forces encountered in the microcirculation, these systems enable detailed investigation of blood flow behavior under a wide range of shear conditions (Whitesides, 2006). In contrast to conventional rheological techniques, which typically assume a homogeneous shear stress distribution across the sample, microfluidic platforms can be engineered to generate spatially heterogeneous shear fields at the micrometer scale. This allows for the observation of critical phenomena such as cell crowding, altered cell–cell interactions, and geometry-dependent flow behavior (Sackmann et al., 2014). Consequently, hemorheology can be approached from a more mechanistic perspective, enabling the detailed characterization of individual cellular responses within physiologically relevant environments (Sackmann et al., 2014; Tomaiuolo, 2014).

One of the key advantages of microfluidic systems is their ability to recreate biomimetic geometries that closely resemble real vascular structures. Within this context, artificial stenoses, bifurcations, and capillary-scale constrictions can be precisely designed and implemented (Guo et al., 2014; Liu et al., 2025). Such geometric constraints influence not only the flow field but also the mechanical behavior of erythrocytes. In order to traverse narrow regions, erythrocytes must undergo deformation or alter their trajectory, even in the absence of pronounced shear stress (Tomaiuolo, 2014). In microfluidic channels, erythrocyte dynamics are commonly described by two principal behaviors: “tank-treading” and “tumbling.” In tank-treading motion, erythrocytes rotate around their membrane while maintaining a stable orientation aligned with the flow direction, whereas in tumbling motion, cells exhibit a flipping or rolling behavior along curved trajectories. These dynamic states depend on flow velocity, wall shear stress, and the mechanical properties of the cell membrane (Abkarian et al., 2007).

Microfluidic systems also enable precise control of critical parameters such as shear stress, pressure gradients, and residence time. This capability allows researchers to systematically investigate hemorheological responses under well-defined mechanical forces and stress conditions (Sackmann et al., 2014). In particular, it is well established that the mechanical properties of erythrocytes are altered in pathological conditions such as diabetes mellitus and sickle cell anemia, often resulting in increased cellular rigidity (Connes et al., 2016). Microfluidic platforms provide significant advantages

in capturing such pathological alterations by enabling the detection of subtle differences in erythrocyte deformation and dynamic transit behavior within microchannels—differences that are often not detectable using bulk measurement techniques (Gossett et al., 2012).

To further enhance physiological relevance, advanced microfluidic platforms such as "organ-on-a-chip" systems have emerged as a key development. In these systems, endothelial cells and various biochemical cues are integrated into microfluidic environments to recreate a more realistic microphysiological setting (Bhatia & Ingber, 2014; Huh et al., 2010). Organ-on-a-chip technologies enable the direct investigation of critical interactions such as blood cell–endothelium adhesion, margination, and inflammatory responses, all of which play central roles in the pathogenesis of vascular diseases. Accordingly, these systems significantly bridge the gap between in vivo conditions and conventional in vitro experimental models (Bhatia & Ingber, 2014).

Another major advantage of microfluidic technologies is their ability to perform high-throughput analysis using extremely small sample volumes. These systems can operate with microliter-scale samples while simultaneously analyzing thousands of cells per second. This makes microfluidic platforms highly suitable for potential clinical applications (Sia & Kricka, 2008). Furthermore, their seamless integration with high-speed imaging techniques and computer-assisted data analysis pipelines enables the generation of large-scale datasets and the execution of advanced quantitative analyses on cellular behavior (Sackmann et al., 2014).

Finally, microfluidic systems provide a powerful platform for single-cell mechanical phenotyping (Otto et al., 2015). Within these systems, parameters such as deformation, transit time, and mechanical recovery are measured individually for each cell. This approach fundamentally differs from conventional methods that rely on population-averaged values. Single-cell analyses have demonstrated that even small subpopulations of mechanically abnormal erythrocytes can exert a disproportionate influence on overall flow behavior. Therefore, single-cell–based approaches are critical for achieving a more comprehensive understanding of disease mechanisms and their biophysical underpinnings (Gossett et al., 2012).

## 4. High-Speed Imaging and Data Acquisition in Hemorheology

High-speed imaging techniques have become a fundamental component of modern hemorheology. These methods go beyond indirect, bulk-parameter-based approaches by enabling direct observation of blood cell morphology, deformation, and interaction dynamics under flow conditions (Otto et al., 2015). This capability is particularly important, as erythrocytes, leukocytes,

platelets, and cellular aggregates undergo rapid shape transformations within milliseconds while traversing microchannels or capillary-like constrictions (Toepfner et al., 2018).

One of the key technologies in this field is Real-Time Deformability Cytometry (RT-DC), which integrates microfluidic deformation systems with high-speed imaging (Otto et al., 2015). RT-DC allows for the analysis of cellular deformation under controlled hydrodynamic forces while simultaneously capturing high-frame-rate images. This enables quantitative, single-cell–level measurement of deformation, projected area, and other morphometric parameters in real time (Rosendahl et al., 2018).

Recent advances in deformability cytometry have extended beyond static deformation measurements to include the viscoelastic properties of cells (Asghari et al., 2024). Real-time viscoelastic deformability cytometry enables the analysis of both deformation and recovery dynamics, providing a more comprehensive characterization of cellular mechanical phenotypes. This is particularly significant because erythrocytes exhibit viscoelastic rather than purely elastic behavior under physiological flow conditions. Accordingly, capillary passage performance depends not only on the magnitude of deformation but also on the rate at which cells recover their original shape after deformation (Fedosov et al., 2010; Kubánková et al., 2021).

In addition to staining or fluorescence-based approaches, label-free optical imaging techniques have gained increasing importance in hemorheological analysis. In this context, methods such as Digital Holographic Microscopy (DHM) and Quantitative Phase Imaging (QPI) provide high-precision measurements by detecting optical phase shifts induced by cells (Park et al., 2018). These techniques enable the extraction of multiple biophysical parameters, including cell volume, refractive index, and membrane fluctuations (Masters, 2012). Furthermore, their integration with microfluidic platforms allows for the dynamic investigation of erythrocyte morphological properties and aggregation behavior under flow conditions (Kim et al., 2016).

Image-based approaches also offer significant advantages in evaluating the three-dimensional structure and morphological characteristics of erythrocyte aggregates. This enables researchers to move beyond conventional aggregometry and obtain detailed information regarding the structural organization of aggregates (Lee et al., 2013; Menichini et al., 2015). Such analyses are particularly critical in the study of diseases in which abnormal aggregation plays a central role in pathophysiology (Kim et al., 2016; Lee et al., 2013; Park et al., 2018).

In order to convert the large volumes of data generated by high-speed imaging systems into meaningful hemorheological parameters, advanced image processing and data analytics methods are required. In this regard, automated segmentation techniques enable the detection and quantification of cells in flow. Notably, DHM-based approaches have made it possible to determine parameters such as erythrocyte count, size distribution, concentration, and dry mass without the use of labeling agents (Xiong et al., 2024). These automated processing pipelines have become essential not only due to the sheer volume of data but also because of their critical role in ensuring reproducibility and scalability (Zhou et al., 2025).

High-speed imaging technologies also offer important advantages in clinical applications. Cellular morphological and mechanical features extracted from imaging data can reveal disease-specific phenotypes that are not detectable using standard hematological tests (Doan et al., 2020). Moreover, QPI-based approaches have been used to identify platelet aggregates associated with disease severity, demonstrating that label-free imaging techniques can serve as effective tools for evaluating clinically relevant biomarkers (Klenk et al., 2023).

In conclusion, high-speed imaging and associated data processing technologies have fundamentally transformed the field of hemorheology. The discipline has increasingly evolved into a "big data" problem, in which each cell is characterized through high-dimensional datasets describing morphological, mechanical, and dynamic properties. Current research efforts are therefore focused on translating these large-scale imaging datasets into biologically and clinically meaningful parameters (Xiong et al., 2024; Zhou et al., 2025).

## 5.Artificial Intelligence and Machine Learning Integration in Hemorheology

The development of next-generation imaging and data acquisition techniques has enabled the generation of large-scale, high-dimensional datasets in hemorheology. However, the transformation of this data richness into meaningful biological and clinical insights requires advanced computational approaches. In this context, Artificial Intelligence (AI), Machine Learning (ML), and Deep Learning (DL) methods have come to play a central role in the analysis of hemorheological data (Caicedo et al., 2017; Wang, 2024).

One of the most common applications of AI in hemorheology is the automated segmentation and classification of blood cell images. In particular, Convolutional Neural Networks (CNNs) have demonstrated high accuracy in identifying erythrocytes, leukocytes, and other cellular components, as well as in detecting morphological abnormalities. These models are capable of performing effectively even in complex imaging conditions where cells are

densely clustered or overlapping (Khan et al., 2024). By learning hierarchical and multi-layered feature representations directly from raw image data, CNN-based approaches reduce the need for manual feature engineering and outperform traditional image processing methods (Litjens et al., 2017).

Beyond classification tasks, AI techniques also enable the extraction of biomechanical parameters from imaging data. Recent deep learning models have been trained to estimate mechanical properties such as erythrocyte deformability directly from microscopic images. The strong agreement between model predictions and conventional microfluidic measurements supports the reliability of these approaches (Lamoureux et al., 2024; Liang et al., 2024). This development represents a significant paradigm shift, as parameters previously accessible only through specialized experimental setups can now be derived from image-based analysis (Ravì et al., 2017).

AI methods are not limited to descriptive analysis but are also widely used in predictive modeling. Deep learning-based approaches have been shown to estimate clinical parameters such as hemoglobin concentration, hematocrit levels, and erythrocyte counts from peripheral blood smear images. This indicates that such models are capable of capturing complex relationships between cellular morphological patterns and physiological states (Doan et al., 2020; Yang et al., 2026).

Recently, physics-guided machine learning approaches have gained increasing attention. In particular, Physics-Informed Neural Networks (PINNs) integrate fundamental physical laws, such as those governing fluid mechanics and cell mechanics, directly into the training process. This enables the development of models that are not only data-driven but also more interpretable and generalizable (Raissi et al., 2019). Accordingly, this framework allows hemorheological phenomena to be modeled in a manner consistent with underlying physical principles, rather than relying solely on empirical data-driven correlations.

Anomaly detection and disease classification also constitute important application areas of artificial intelligence in hemorheology. Through the integration of computer vision and machine learning, fully automated systems have been developed that can accurately identify pathological cell morphologies and disease-associated phenotypes (El Othmani et al., 2025). These systems reduce observer-dependent variability, enabling more objective and reproducible assessments while enhancing the reliability of diagnostic processes (Eulenberg et al., 2017).

However, several important limitations currently hinder the full integration of AI methods into hemorheology. One of the primary challenges

is the limited availability of high-quality, well-annotated datasets required for robust model training (Dalvi & Gawas, 2025). In addition, the "black box" nature of many deep learning models restricts interpretability, thereby limiting their acceptance in clinical applications. Consequently, the development of explainable artificial intelligence (XAI) approaches and the standardization of model validation procedures are critical for the future advancement and clinical translation of these technologies (Wang, 2024).

## 6. Predictive Analysis and Clinical Applications in Hemorheology

The integration of high-throughput imaging systems, microfluidic technologies, and machine learning methods has transformed hemorheology from a purely descriptive discipline into a predictive research field with strong clinical potential (Caicedo et al., 2017; Otto et al., 2015). By combining quantitative cellular measurements with data-driven modeling approaches, individualized profiles that capture the mechanical and morphological properties of circulating blood cells at high resolution can be generated. These profiles are often referred to as a "hemorheological fingerprint" (Toepfner et al., 2018).

One of the most promising applications of this approach is the development of predictive models for early disease detection. It is well established that pathological conditions such as diabetes mellitus, cardiovascular diseases, and sepsis are closely associated with alterations in erythrocyte deformability, aggregation tendency, and viscoelastic properties (Connes et al., 2016; Kubánková et al., 2021). Through machine learning–based analysis of high-dimensional imaging-derived features, disease-associated phenotypes can be identified even before the onset of clinical symptoms. These findings strongly support the potential of hemorheological parameters as early-stage biomarkers (Doan et al., 2020; Eulenberg et al., 2017).

Predictive hemorheology is not limited to early diagnosis but also offers important advantages in disease monitoring and progression assessment. Longitudinal analysis of the mechanical properties of blood cells enables the tracking of functional changes at the microcirculatory level over time. This allows for objective evaluation of disease progression as well as response to therapeutic interventions (Kubánková et al., 2021). In particular, given the established relationship between changes in erythrocyte deformability and aggregation behavior and vascular complications, these parameters may serve as important biophysical indicators for assessing disease severity in chronic conditions (Connes et al., 2016).

Personalized medicine applications constitute another key component of predictive hemorheology. Through the concept of "individualized hemorheological profiling," it becomes possible to optimize therapeutic

strategies based on patients' cellular and mechanical characteristics (Toepfner et al., 2018). This approach is particularly important in diseases with heterogeneous pathophysiology such as sickle cell disease, where inter-individual differences in erythrocyte mechanical properties can directly influence clinical outcomes (Connes et al., 2016).

Hemorheological assessments also provide significant contributions beyond clinical medicine, extending into exercise physiology and rehabilitation. It is well established that both acute and chronic exercise induce measurable changes in blood viscosity, erythrocyte deformability, and aggregation tendency, thereby affecting oxygen transport at the capillary level (Connes et al., 2013). AI-assisted modeling approaches enable the quantitative analysis of these physiological responses and contribute to the identification of optimal exercise protocols aimed at preventing cardiovascular and metabolic diseases (Hua & Chen, 2026).

However, the full integration of predictive hemorheology into clinical practice is still limited by several methodological and technical challenges. One of the primary limitations is the lack of standardized protocols for data acquisition, analysis, and interpretation. This reduces comparability across studies and leads to issues related to reproducibility (Tomaiuolo, 2014). In addition, for AI-based models to be reliably implemented in clinical settings, they must undergo rigorous validation procedures, obtain regulatory approval, and be developed with a strong emphasis on interpretability and transparency (Litjens et al., 2017).

## 7. Standardization and Challenges

Despite significant technological advances in microfluidic hemorheology, high-speed imaging, and artificial intelligence, the clinical translation of these approaches remains limited due to methodological variability, insufficient data quality, and constraints in validation frameworks. Hemorheological measurements are highly sensitive to a wide range of pre-analytical and post-analytical factors, which complicates their use as reliable clinical tools. These variables include the type of anticoagulant used, the duration and conditions of sample storage prior to analysis, temperature and environmental conditions during measurements, whether hematocrit levels are adjusted during sample preparation, and the technical specifications of the instruments employed (Valério de Arruda et al., 2021). This variability is particularly critical for deformability measurements; for instance, ektacytometry results are directly influenced by device settings and the conditions under which diffraction patterns are acquired. Indeed, methodological differences have been shown to introduce substantial variability in elongation index values, thereby directly affecting the clinical interpretability of results (Renoux et al., 2016).

In addition to methodological limitations, the development of AI-based hemorheological analysis is also hindered by the lack of large-scale, diverse, and well-annotated datasets. Medical imaging datasets frequently suffer from class imbalance, inter-device variability, and site-specific artifacts, which introduce systematic biases. As a result, models trained on such datasets may demonstrate strong performance during internal validation but experience significant performance degradation when applied to external datasets (Varoquaux & Cheplygina, 2022). This highlights the necessity of external validation as a prerequisite for the clinical reliability of AI-based hemorheological models. Similarly, studies have shown that model performance often decreases when tested across different institutions and populations, indicating that internal validation alone is insufficient to establish clinical generalizability (Yu et al., 2022).

A further critical limitation of artificial intelligence applications is the lack of model interpretability. Deep learning models typically operate as "black boxes," making it difficult to clearly explain which biological or mechanical features underlie their predictions (Wang, 2024). This uncertainty complicates the distinction between whether model outputs reflect true cellular mechanical properties or are driven by non-biological technical artifacts. In clinical decision-making contexts, it is essential to explicitly and interpretably link image-derived features with erythrocyte mechanics, microvascular flow behavior, and disease pathophysiology. Accordingly, the development of explainable artificial intelligence (XAI) approaches is crucial both for improving model reliability and for enabling clinicians to better interpret underlying biological processes (Holzinger et al., 2019).

Recent studies have further demonstrated that AI models can inadvertently learn demographic and site-specific information even when such variables are not explicitly provided as inputs, thereby influencing subgroup performance (Yang et al., 2024). Variables such as age, sex, ethnicity, hematocrit levels, comorbidities, medication use, and disease stage can all contribute to significant differences in erythrocyte morphology and mechanical behavior.

In addition, the integration of next-generation hemorheological analyses into existing clinical guidelines remains an important open question. Reporting frameworks such as TRIPOD+AI and STARD-AI define standards for clinical prediction and diagnostic accuracy studies involving AI and statistical modeling, including data description, model development, validation strategies, and performance evaluation (Collins et al., 2024; Sounderajah et al., 2025). For clinical decision-support systems, the DECIDE-AI framework provides structured guidance for safe and effective implementation (Vasey et al., 2022). Furthermore, the CLAIM checklist serves as an important

methodological reference for assessing the validity and transparency of AI applications in optical imaging–based analyses (Tejani et al., 2024).

In conclusion, the primary challenge in modern hemorheology is no longer solely technological capability, but rather reproducibility, standardization, validation, interpretability, and clinical reliability. Without standardized protocols, heterogeneous multi-center datasets, robust external validation, and transparent reporting practices, next-generation hemorheological technologies are likely to remain powerful research tools, yet face significant barriers to becoming fully reliable clinical systems.

**8. Future Perspectives: Toward Digital Hemorheology**

The integration of artificial intelligence, high-speed imaging, and microfluidic technologies has recently advanced hemorheology into a new paradigm referred to as "digital hemorheology." This concept describes data-driven systems that enable continuous modeling and interpretation of blood flow properties both in laboratory settings and in clinical applications (Sackmann et al., 2014; Varoquaux & Cheplygina, 2022). This transformation aligns with the broader trend in biomedical research toward large-scale data generation and computational analysis of complex biological systems (Rajpurkar et al., 2022; Topol, 2019).

One of the key developments enabling this transition is the emergence of point-of-care (POC) hemorheology systems. Through microfluidic technologies and lab-on-a-chip platforms, traditional large-scale laboratory setups have been miniaturized into portable devices capable of analyzing microliter-scale blood samples (Sia & Kricka, 2008; Whitesides, 2006). These systems can rapidly measure fundamental hemorheological parameters such as erythrocyte deformability, aggregation, and flow behavior immediately after sample collection (Guo et al., 2014). As a result, POC technologies enable rapid assessment not only in specialized laboratory environments but also in routine clinical practice and community-based healthcare settings (Sia & Kricka, 2008).

Real-time analytical capability is another essential component of digital hemorheology. The integration of high-speed imaging systems with automated data-processing pipelines allows continuous monitoring of blood cell behavior under physiological flow conditions (Xiong et al., 2024; Zhou et al., 2025). This approach extends beyond static measurements by enabling time-series analysis of microcirculatory dynamics and capturing transient mechanical changes. Such capabilities are particularly valuable in critical care settings, where rapid physiological changes must be monitored in real time (Xiong et al., 2024; Zhou et al., 2025).

Artificial intelligence plays a central role in translating these next-generation systems into clinical practice. AI-based algorithms can automatically segment, classify, and extract features from imaging data, transforming large volumes of raw images into clinically meaningful biological indicators (Caicedo et al., 2017; Esteva et al., 2019; Litjens et al., 2017). In the future, integration of these models directly into data acquisition devices may enable real-time decision-support systems and adaptive hemorheological assessment tools (Rajpurkar et al., 2022; Vasey et al., 2022). Such integration is expected to be a key factor in making hemorheological analysis faster, more reliable, and clinically actionable (Varoquaux & Cheplygina, 2022).

One of the most promising applications of digital hemorheology lies in personalized medicine. High-resolution, individual-level analysis of the mechanical and morphological properties of blood cells enables the creation of patient-specific "hemorheological signatures." These signatures can be considered digital biomarkers reflecting the state of microvascular health (Toepfner et al., 2018). This approach allows for more precise monitoring of disease progression and treatment response while contributing to the development of novel biomarkers that support clinical decision-making (Coravos et al., 2019; Topol, 2019). Ultimately, hemorheology has the potential to evolve from a descriptive discipline into a predictive, personalized, and patient-centered clinical assessment tool (Rajpurkar et al., 2022; Toepfner et al., 2018).

A key future objective is the development of fully automated hemorheological diagnostic systems. Integrating sample preparation, measurement, data processing, analysis, and clinical interpretation into a single unified workflow would significantly reduce user-dependent variability (Esteva et al., 2019; Vasey et al., 2022). Such automated systems are expected to play a major role in clinical translation by enabling higher reproducibility and standardized decision-making processes (Collins et al., 2024). In this context, digital hemorheology holds strong potential as a core component of next-generation diagnostic technologies capable of reliably assessing changes in blood properties with minimal human intervention (Rajpurkar et al., 2022; Tejani et al., 2024).

However, several structural and methodological barriers must be overcome to achieve these goals. Interoperability, standardization, and clinical validation of digital systems remain among the primary limiting factors preventing the full realization of digital hemorheology's potential (Collins et al., 2024; Coravos et al., 2019; Valério de Arruda et al., 2021). In addition, the development of explainable artificial intelligence systems is essential to enable clinicians to understand the biological basis of model outputs and to safely integrate them into clinical decision-making processes (Holzinger et al., 2019). Addressing these challenges will enhance clinical adoptability

and support the safe and sustainable integration of these technologies into healthcare systems (Varoquaux & Cheplygina, 2022).

## 9. Conclusion

Hemorheology, originally defined as a field focused on the bulk flow properties of blood, has evolved into a multidisciplinary research area encompassing single-cell biophysical analyses and data-driven approaches. Although classical methods such as viscometry, ektacytometry, and aggregometry have provided valuable insights into blood rheology, they remain limited in their ability to capture cellular heterogeneity and biomechanical differences at the single-cell level. In contrast, mechanical phenotyping techniques have enabled the analysis of individual erythrocyte properties, revealing substantial biomechanical variability among blood cells.

Microfluidic systems and high-speed imaging technologies provide experimental platforms that closely mimic physiological microcirculation, enabling real-time investigation of cellular flow behavior. These integrated approaches have transformed hemorheology into a more quantitative, high-resolution, and multidimensional field of study.

In recent years, artificial intelligence–based methods have facilitated the extraction of meaningful biophysical features from large-scale imaging datasets, leading to significant advances in classification, prediction, and anomaly detection. However, challenges related to data standardization, model generalizability, and interpretability remain key barriers to clinical translation.

Looking ahead, "digital hemorheology" is expected to emerge as an integrated framework combining microfluidic technologies, advanced imaging systems, and artificial intelligence–based analytics. This approach is anticipated to bring hemorheology closer to clinical practice by enabling personalized diagnostics and real-time analysis capabilities.

## References

Abkarian, M., Faivre, M., & Viallat, A. (2007). Swinging of Red Blood Cells under Shear Flow. *Physical Review Letters*, *98*(18), 188302. https://doi.org/10.1103/PhysRevLett.98.188302

Asghari, M., Ivetich, S. D., Aslan, M. K., Aramesh, M., Melkonyan, O., Meng, Y., Xu, R., Colombo, M., Weiss, T., Balabanov, S., Stavrakis, S., & deMello, A. J. (2024). Real-time viscoelastic deformability cytometry: High-throughput mechanical phenotyping of liquid and solid biopsies. *Science Advances*, *10*(49). https://doi.org/10.1126/sciadv.abj1133

Baskurt, O. K., Hardeman, M. R., Uyuklu, M., Ulker, P., Cengiz, M., Nemeth, N., Shin, S., Alexy, T., & Meiselman, H. J. (2009). Parameterization of red blood cell elongation index – shear stress curves obtained by ektacytometry. *Scandinavian Journal of Clinical and Laboratory Investigation*, *69*(7), 777–788. https://doi.org/10.3109/00365510903266069

Baskurt, O. K., & Meiselman, H. J. (2024). Blood Rheology and Hemodynamics. *Seminars in Thrombosis and Hemostasis*, *50*(06), 902–915. https://doi.org/10.1055/s-0043-1777802

Baskurt, O., Neu, B., & Meiselman, H. J. (2011). *Red Blood Cell Aggregation*. CRC Press. https://doi.org/10.1201/b11221

Bhatia, S. N., & Ingber, D. E. (2014). Microfluidic organs-on-chips. *Nature Biotechnology*, *32*(8), 760–772. https://doi.org/10.1038/nbt.2989

Caicedo, J. C., Cooper, S., Heigwer, F., Warchal, S., Qiu, P., Molnar, C., Vasilevich, A. S., Barry, J. D., Bansal, H. S., Kraus, O., Wawer, M., Paavolainen, L., Herrmann, M. D., Rohban, M., Hung, J., Hennig, H., Concannon, J., Smith, I., Clemons, P. A., … Carpenter, A. E. (2017). Data-analysis strategies for image-based cell profiling. *Nature Methods*, *14*(9), 849–863. https://doi.org/10.1038/nmeth.4397

Chien, S. (1970). Shear Dependence of Effective Cell Volume as a Determinant of Blood Viscosity. *Science*, *168*(3934), 977–979. https://doi.org/10.1126/science.168.3934.977

Clark, M., Mohandas, N., & Shohet, S. (1983). Osmotic gradient ektacytometry: comprehensive characterization of red cell volume and surface maintenance. *Blood*, *61*(5), 899–910. https://doi.org/10.1182/blood.V61.5.899.899

Collins, G. S., Moons, K. G. M., Dhiman, P., Riley, R. D., Beam, A. L., Van Calster, B., Ghassemi, M., Liu, X., Reitsma, J. B., van Smeden, M., Boulesteix, A.-L., Camaradou, J. C., Celi, L. A., Denaxas, S., Denniston, A. K., Glocker, B., Golub, R. M., Harvey, H., Heinze, G., Logullo, P. (2024). TRIPOD+AI statement: updated guidance for reporting clinical prediction models that use regression or machine learning methods. *BMJ*, *385*, e078378. https://doi.org/10.1136/bmj-2023-078378

Connes, P., Alexy, T., Detterich, J., Romana, M., Hardy-Dessources, M.-D., & Ballas, S. K. (2016). The role of blood rheology in sickle cell disease. *Blood Reviews*, *30*(2), 111–118. https://doi.org/10.1016/j.blre.2015.08.005

Connes, P., Simmonds, M. J., Brun, J.-F., & Baskurt, O. K. (2013). Exercise hemorheology: Classical data, recent findings and unresolved issues. *Clinical Hemorheology and Microcirculation*, *53*(1–2), 187–199. https://doi.org/10.3233/CH-2012-1643

Coravos, A., Khozin, S., & Mandl, K. D. (2019). Developing and adopting safe and effective digital biomarkers to improve patient outcomes. *Npj Digital Medicine*, *2*(1), 14. https://doi.org/10.1038/s41746-019-0090-4

Dalvi, P. T., & Gawas, M. A. (2025). A comprehensive review of machine learning approaches for detecting anemia and abnormal red blood cells using RBC indices and medical imaging. *Discover Artificial Intelligence*, *6*(1), 66. https://doi.org/10.1007/s44163-025-00698-8

Doan, M., Sebastian, J. A., Caicedo, J. C., Siegert, S., Roch, A., Turner, T. R., Mykhailova, O., Pinto, R. N., McQuin, C., Goodman, A., Parsons, M. J., Wolkenhauer, O., Hennig, H., Singh, S., Wilson, A., Acker, J. P., Rees, P., Kolios, M. C., & Carpenter, A. E. (2020). Objective assessment of stored blood quality by deep learning. *Proceedings of the National Academy of Sciences*, *117*(35), 21381–21390. https://doi.org/10.1073/pnas.2001227117

El Othmani, O., Mosbah, A., Yahyaoui, A., Bouatay, A., & Dhaouadi, R. (2025). AI-driven Automated Blood Cell Anomaly Detection: Enhancing Diagnostics and Telehealth in Hematology. *Journal of Imaging*, *11*(5), 157. https://doi.org/10.3390/jimaging11050157

Esteva, A., Robicquet, A., Ramsundar, B., Kuleshov, V., DePristo, M., Chou, K., Cui, C., Corrado, G., Thrun, S., & Dean, J. (2019). A guide to deep learning in healthcare. *Nature Medicine*, *25*(1), 24–29. https://doi.org/10.1038/s41591-018-0316-z

Eulenberg, P., Köhler, N., Blasi, T., Filby, A., Carpenter, A. E., Rees, P., Theis, F. J., & Wolf, F. A. (2017). Reconstructing cell cycle and disease progression using deep learning. *Nature Communications*, *8*(1), 463. https://doi.org/10.1038/s41467-017-00623-3

Fedosov, D. A., Caswell, B., & Karniadakis, G. E. (2010). Systematic coarse-graining of spectrin-level red blood cell models. *Computer Methods in Applied Mechanics and Engineering*, *199*(29–32), 1937–1948. https://doi.org/10.1016/j.cma.2010.02.001

Gossett, D. R., Tse, H. T. K., Lee, S. A., Ying, Y., Lindgren, A. G., Yang, O. O., Rao, J., Clark, A. T., & Di Carlo, D. (2012). Hydrodynamic stretching of single cells for large population mechanical phenotyping. *Proceedings of the National Academy of Sciences*, *109*(20), 7630–7635. https://doi.org/10.1073/pnas.1200107109

Guo, Q., Duffy, S. P., Matthews, K., Santoso, A. T., Scott, M. D., & Ma, H. (2014). Microfluidic analysis of red blood cell deformability. *Journal of Biomechanics*, *47*(8), 1767–1776. https://doi.org/10.1016/j.jbiomech.2014.03.038

Holzinger, A., Langs, G., Denk, H., Zatloukal, K., & Müller, H. (2019). Causability and explainability of artificial intelligence in medicine. *WIREs Data Mining and Knowledge Discovery*, *9*(4). https://doi.org/10.1002/widm.1312

Hua, Y. H., & Chen, Y. S. (2026). [Artificial intelligence empowering sports medicine]. *Zhonghua Yi Xue Za Zhi*, *106*(1), 6–12. https://doi.org/10.3760/cma.j.cn112137-20250725-01852

Huh, D., Matthews, B. D., Mammoto, A., Montoya-Zavala, M., Hsin, H. Y., & Ingber, D. E. (2010). Reconstituting Organ-Level Lung Functions on a Chip. *Science*, *328*(5986), 1662–1668. https://doi.org/10.1126/science.1188302

Khan, R. U., Almakdi, S., Alshehri, M., Haq, A. U., Ullah, A., & Kumar, R. (2024). An intelligent neural network model to detect red blood cells for various blood structure classification in microscopic medical images. *Heliyon*, *10*(4), e26149. https://doi.org/10.1016/j.heliyon.2024.e26149

Kim, K., Yoon, J., Shin, S., Lee, S., Yang, S.-A., & Park, Y. (2016). Optical diffraction tomography techniques for the study of cell pathophysiology. *Journal of Biomedical Photonics & Engineering*, 020201-1-020201–020216. https://doi.org/10.18287/JBPE16.02.020201

Klenk, C., Erber, J., Fresacher, D., Röhrl, S., Lengl, M., Heim, D., Irl, H., Schlegel, M., Haller, B., Lahmer, T., Diepold, K., Rasch, S., & Hayden, O. (2023). Platelet aggregates detected using quantitative phase imaging associate with COVID-19 severity. *Communications Medicine*, *3*(1), 161. https://doi.org/10.1038/s43856-023-00395-6

Kubánková, M., Hohberger, B., Hoffmanns, J., Fürst, J., Herrmann, M., Guck, J., & Kräter, M. (2021). Physical phenotype of blood cells is altered in COVID-19. *Biophysical Journal*, *120*(14), 2838–2847. https://doi.org/10.1016/j.bpj.2021.05.025

Lamoureux, E. S., Cheng, Y., Islamzada, E., Matthews, K., Duffy, S. P., & Ma, H. (2024). Biophysical profiling of red blood cells from thin-film blood smears using deep learning. *Heliyon*, *10*(15), e35276. https://doi.org/10.1016/j.heliyon.2024.e35276

Lanotte, L., Mauer, J., Mendez, S., Fedosov, D. A., Fromental, J.-M., Claveria, V., Nicoud, F., Gompper, G., & Abkarian, M. (2016). Red cells' dynamic morphologies govern blood shear thinning under microcirculatory flow conditions. *Proceedings of the National Academy of Sciences*, *113*(47), 13289–13294. https://doi.org/10.1073/pnas.1608074113

Lee, K., Kim, K., Jung, J., Heo, J., Cho, S., Lee, S., Chang, G., Jo, Y., Park, H., & Park, Y. (2013). Quantitative Phase Imaging Techniques for the Study of Cell Pathophysiology: From Principles to Applications. *Sensors*, *13*(4), 4170–4191. https://doi.org/10.3390/s130404170

Liang, M., Zhong, J., Shannon, C. S., Agrawal, R., & Ai, Y. (2024). Intelligent image-based deformability assessment of red blood cells via dynamic shape classification. *Sensors and Actuators B: Chemical*, *401*, 135056. https://doi.org/10.1016/j.snb.2023.135056

Litjens, G., Kooi, T., Bejnordi, B. E., Setio, A. A. A., Ciompi, F., Ghafoorian, M., van der Laak, J. A. W. M., van Ginneken, B., & Sánchez, C. I. (2017). A survey on deep learning in medical image analysis. *Medical Image Analysis*, *42*, 60–88. https://doi.org/10.1016/j.media.2017.07.005

Liu, W., Xie, L., Yang, J., Gong, X., Sun, D., & Zhang, C. (2025). A Microfluidic Device for Detecting the Deformability of Red Blood Cells. *Biosensors*, *15*(11). https://doi.org/10.3390/bios15110758

Mannini, L., Cecchi, E., Fatini, C., Marcucci, R., Alessandriello Liotta, A., Matucci-Cerinic, M., Abbate, R., & Gensini, G. F. (2007). Clinical haemorheology and microcirculation. *Annali Dell'Istituto Superiore Di Sanita*, *43*(2), 144–155.

Masters, B. R. (2012). Quantitative Phase Imaging of Cells and Tissues. *Journal of Biomedical Optics*, *17*(2), 029901. https://doi.org/10.1117/1.JBO.17.2.029901

Menichini, P. A., Larese, M. G., & Riquelme, B. D. (2015). *Automatic analysis of microscopic images of red blood cell aggregates* (C. Kurachi, K. Svanberg, B. J. Tromberg, & V. S. Bagnato, Eds.; p. 953132). https://doi.org/10.1117/12.2181110

Mokken, F. Ch., Kedaria, M., Henny, Ch. P., Hardeman, M. R., & Gelb, A. W. (1992). The clinical importance of erythrocyte deformability, a hemorrheological parameter. *Annals of Hematology*, *64*(3), 113–122. https://doi.org/10.1007/BF01697397

Nader, E., Skinner, S., Romana, M., Fort, R., Lemonne, N., Guillot, N., Gauthier, A., Antoine-Jonville, S., Renoux, C., Hardy-Dessources, M.-D., Stauffer, E., Joly, P., Bertrand, Y., & Connes, P. (2019). Blood Rheology: Key Parameters, Impact on Blood Flow, Role in Sickle Cell Disease and Effects of Exercise. *Frontiers in Physiology*, *10*. https://doi.org/10.3389/fphys.2019.01329

Otto, O., Rosendahl, P., Mietke, A., Golfier, S., Herold, C., Klaue, D., Girardo, S., Pagliara, S., Ekpenyong, A., Jacobi, A., Wobus, M., Töpfner, N., Keyser, U. F., Mansfeld, J., Fischer-Friedrich, E., & Guck, J. (2015). Real-time deformability cytometry: on-the-fly cell mechanical phenotyping. *Nature Methods*, *12*(3), 199–202. https://doi.org/10.1038/nmeth.3281

Park, Y., Depeursinge, C., & Popescu, G. (2018). Quantitative phase imaging in biomedicine. *Nature Photonics*, *12*(10), 578–589. https://doi.org/10.1038/s41566-018-0253-x

Popel, A. S., & Johnson, P. C. (2005). Microcirculation and Hemorheology. *Annual Review of Fluid Mechanics*, *37*(1), 43–69. https://doi.org/10.1146/annurev.fluid.37.042604.133933

Pretorius, E., Bester, J., Vermeulen, N., Alummoottil, S., Soma, P., Buys, A. V, & Kell, D. B. (2015). Poorly controlled type 2 diabetes is accompanied by significant morphological and ultrastructural changes in both erythrocytes and in thrombin-generated fibrin: implications for diagnostics. *Cardiovascular Diabetology*, *14*(1), 30. https://doi.org/10.1186/s12933-015-0192-5

Pries, A. R., Secomb, T. W., Gaehtgens, P., & Gross, J. F. (1990). Blood flow in microvascular networks. Experiments and simulation. *Circulation Research*, *67*(4), 826–834. https://doi.org/10.1161/01.RES.67.4.826

Raissi, M., Perdikaris, P., & Karniadakis, G. E. (2019). Physics-informed neural networks: A deep learning framework for solving forward and inverse problems involving nonlinear partial differential equations. *Journal of Computational Physics*, *378*, 686–707. https://doi.org/10.1016/j.jcp.2018.10.045

Rajpurkar, P., Chen, E., Banerjee, O., & Topol, E. J. (2022). AI in health and medicine. *Nature Medicine*, *28*(1), 31–38. https://doi.org/10.1038/s41591-021-01614-0

Ravì, D., Wong, C., Deligianni, F., Berthelot, M., Andreu-Perez, J., Lo, B., & Yang, G.-Z. (2017). Deep Learning for Health Informatics. *IEEE Journal of Biomedical and Health Informatics*, *21*(1), 4–21. https://doi.org/10.1109/JBHI.2016.2636665

Renoux, C., Parrow, N., Faes, C., Joly, P., Hardeman, M., Tisdale, J., Levine, M., Garnier, N., Bertrand, Y., Kebaili, K., Cuzzubbo, D., Cannas, G., Martin, C., & Connes, P. (2016). Importance of methodological standardization for the ektacytometric measures of red blood cell deformability in sickle cell anemia. *Clinical Hemorheology and Microcirculation*, *62*(2), 173–179. https://doi.org/10.3233/CH-151979

Rosendahl, P., Plak, K., Jacobi, A., Kraeter, M., Toepfner, N., Otto, O., Herold, C., Winzi, M., Herbig, M., Ge, Y., Girardo, S., Wagner, K., Baum, B., & Guck, J. (2018). Real-time fluorescence and deformability cytometry. *Nature Methods*, *15*(5), 355–358. https://doi.org/10.1038/nmeth.4639

Sackmann, E. K., Fulton, A. L., & Beebe, D. J. (2014). The present and future role of microfluidics in biomedical research. *Nature*, *507*(7491), 181–189. https://doi.org/10.1038/nature13118

Sia, S. K., & Kricka, L. J. (2008). Microfluidics and point-of-care testing. *Lab on a Chip*, *8*(12), 1982. https://doi.org/10.1039/b817915h

Sounderajah, V., Guni, A., Liu, X., Collins, G. S., Karthikesalingam, A., Markar, S. R., Golub, R. M., Denniston, A. K., Shetty, S., Moher, D., Bossuyt, P. M., Darzi, A., Ashrafian, H., Acharya, A., Mateen, B. A., Kelly, C., Ting, D., Treanor, D., King, D., Saria, S. (2025). The STARD-AI reporting guideline for diagnostic accuracy studies using artificial intelligence. *Nature Medicine*, *31*(10), 3283–3289. https://doi.org/10.1038/s41591-025-03953-8

Tejani, A. S., Klontzas, M. E., Gatti, A. A., Mongan, J. T., Moy, L., Park, S. H., Kahn, C. E., Abbara, S., Afat, S., Anazodo, U. C., Andreychenko, A., Asselbergs, F. W., Badano, A., Baessler, B., Bold, B., Bisdas, S., Brismar, T. B., Cacciamani, G. E., Carrino, J. A., Zins, M. (2024). Checklist for Artificial Intelligence in Medical Imaging (CLAIM): 2024 Update. *Radiology: Artificial Intelligence*, *6*(4). https://doi.org/10.1148/ryai.240300

Toepfner, N., Herold, C., Otto, O., Rosendahl, P., Jacobi, A., Kräter, M., Stächele, J., Menschner, L., Herbig, M., Ciuffreda, L., Ranford-Cartwright, L., Grzybek, M., Coskun, Ü., Reithuber, E., Garriss, G., Mellroth, P., Henriques-Normark, B., Tregay, N., Suttorp, M., Guck, J. (2018). Detection of human disease conditions by single-cell morpho-rheological phenotyping of blood. *ELife*, *7*. https://doi.org/10.7554/eLife.29213

Tomaiuolo, G. (2014). Biomechanical properties of red blood cells in health and disease towards microfluidics. *Biomicrofluidics*, *8*(5). https://doi.org/10.1063/1.4895755

Topol, E. J. (2019). High-performance medicine: the convergence of human and artificial intelligence. *Nature Medicine*, *25*(1), 44–56. https://doi.org/10.1038/s41591-018-0300-7

Valério de Arruda, M., Cruz Silva, A., Fernandes Galduróz, J. C., & Ferreira Galduróz, R. (2021). Standardization for obtaining blood viscosity: A systematic review. *European Journal of Haematology*, *106*(5), 597–605. https://doi.org/10.1111/ejh.13594

Varoquaux, G., & Cheplygina, V. (2022). Machine learning for medical imaging: methodological failures and recommendations for the future. *Npj Digital Medicine*, *5*(1), 48. https://doi.org/10.1038/s41746-022-00592-y

Vasey, B., Nagendran, M., Campbell, B., Clifton, D. A., Collins, G. S., Denaxas, S., Denniston, A. K., Faes, L., Geerts, B., Ibrahim, M., Liu, X., Mateen, B. A., Mathur, P., McCradden, M. D., Morgan, L., Ordish, J., Rogers, C., Saria, S., Ting, D. S. W., Perkins, Z. B. (2022). Reporting guideline for the early-stage clinical evaluation of decision support systems driven by artificial intelligence: DECIDE-AI. *Nature Medicine*, *28*(5), 924–933. https://doi.org/10.1038/s41591-022-01772-9

Wang, J. (2024). Deep Learning in Hematology: From Molecules to Patients. *Clinical Hematology International*, *6*(4). https://doi.org/10.46989/001c.124131

Whitesides, G. M. (2006). The origins and the future of microfluidics. *Nature*, *442*(7101), 368–373. https://doi.org/10.1038/nature05058

Xiong, Z., Yu, L., An, S., Zheng, J., Ma, Y., Micó, V., & Gao, P. (2024). Automatic identification and analysis of cells using digital holographic microscopy and Sobel segmentation. *Frontiers in Photonics*, *5*. https://doi.org/10.3389/fphot.2024.1359595

Yang, C., Li, D., Zhao, C., Zhang, X., Ouyang, N., Guo, Y., Feng, L., He, Y., Situ, B., Sun, D., & Zheng, L. (2026). Prediction of hemoglobin levels and red blood cell parameters using deep learning on blood smear images. *Intelligent Medicine*, *6*(1), 47–55. https://doi.org/10.1016/j.imed.2025.05.011

Yang, Y., Zhang, H., Gichoya, J. W., Katabi, D., & Ghassemi, M. (2024). The limits of fair medical imaging AI in real-world generalization. *Nature Medicine*, *30*(10), 2838–2848. https://doi.org/10.1038/s41591-024-03113-4

Yu, A. C., Mohajer, B., & Eng, J. (2022). External Validation of Deep Learning Algorithms for Radiologic Diagnosis: A Systematic Review. *Radiology: Artificial Intelligence*, *4*(3). https://doi.org/10.1148/ryai.210064

Zhou, J., Mei, L., Yu, M., Ma, X., Hou, D., Yin, Z., Liu, X., Ding, Y., Yang, K., Xiao, R., Yuan, X., Weng, Y., Long, M., Hu, T., Hou, J., Xu, Y., Tao, L., Mei, S., Shen, H., … Lei, C. (2025). Imaging flow cytometry with a real-time throughput beyond 1,000,000 events per second. *Light: Science & Applications*, *14*(1), 76. https://doi.org/10.1038/s41377-025-01754-9

Chapter 6

# AN OVERVIEW OF ANALYTICAL METHODS USED IN CELL CULTURE

*SALİH ERAT*[1]

1 Izmir Bakırçay University, Menemen Vocational School, Department of Veterinary Medicine, salih.erat@bakircay.edu.tr, ORCID: 0000-0003-4413-6041.

## 1. INTRODUCTION

Cell culture constitutes one of the cornerstones of biomedical research and serves as a critical tool across a broad range of applications, from elucidating disease mechanisms to drug development processes, from toxicological testing to biotechnological production practices. The effective use of this experimental platform necessitates the development and standardization of analytical methods capable of reliably measuring cellular behavior, function, and responses (Freshney, 2010).

Analytical approaches in cell culture have undergone a historical evolution. Beginning with the earliest cell counting methods performed using light microscopy and hemocytometers, this development has progressed to today's high-content imaging platforms and flow cytometry, thereby enabling researchers to investigate biological processes rapidly, accurately, and in a multidimensional manner (Pampaloni et al., 2007; Freshney, 2010). In particular, with the implementation of three-dimensional (3D) cell culture models and organ-on-chip platforms, the necessity of adapting existing analytical methods to these novel biological systems has become increasingly evident (Bhise et al., 2014).

Thanks to the various analytical methods developed over the years, cell culture research is now able to provide far more detailed and reliable data. These methods, ranging from the assessment of cell viability and proliferation rates to the investigation of programmed cell death, from molecular-level analyses of proteins and nucleic acids to visual imaging techniques, offer researchers the opportunity to examine cell biology from multiple perspectives. In addition, approaches aimed at understanding how cells generate energy and how they move have attracted increasing attention. Naturally, no single method is perfect on its own; each possesses not only distinct strengths but also limitations that should not be overlooked. Throughout all these processes, quality control and standardization play a critical role in ensuring that results are meaningful and reproducible. Looking toward the future of the field, it is evident that next-generation technologies are opening the door to resolving many questions that still await answers.

## 2. CELL VIABILITY AND PROLIFERATION ANALYSES

### 2.1. Dye Exclusion Methods

The most fundamental approach for assessing cell viability is the trypan blue exclusion test. In this method, dead cells with compromised membrane integrity take up the dye, whereas viable cells exclude it and are subsequently counted using a hemocytometer or automated cell counters. Although the method is inexpensive and rapid, it has been criticized for its susceptibility to

subjective interpretation and for relying solely on membrane permeability as an indicator of cell death, which may lead to the misclassification of some early apoptotic cells (Strober, 2015).

Propidium iodide (PI) and 7-aminoactinomycin D (7-AAD) are also fluorescent dyes based on membrane integrity; when used in conjunction with flow cytometry, these agents enable high-throughput and quantitative assessment of cell viability (Freshney, 2010; Vermes et al., 1995). Calcein-AM, by contrast, is a fluorescent esterase substrate that is hydrolyzed by cytosolic esterases only in metabolically active viable cells, thereby producing green fluorescence. This property functionally distinguishes it from membrane-impermeant dyes used to assess viability and cell death (Wu et al., 2007; Freshney, 2010).

### 2.2. Metabolic Activity–Based Assays

The MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is based on the conversion of the yellow tetrazolium salt into purple formazan crystals through mitochondrial dehydrogenase activity, and this color change is measured spectrophotometrically, with metabolic activity serving as an indirect indicator of cell viability. This assay has become one of the most widely used methods for evaluating cytotoxicity, antiproliferative effects, and cell proliferation. However, the results should be interpreted with caution, as many chemical compounds and experimental conditions may directly interfere with the tetrazolium reduction process (Mosmann, 1983).

Compared with MTT, WST-1 and WST-8 assays, which generate water-soluble formazan products, offer a more convenient protocol, whereas XTT and MTS assays represent alternative methods based on similar mechanisms (Berridge et al., 2005). The resazurin (Alamar Blue) assay, on the other hand, relies on the conversion of resazurin, a reversible redox indicator, into resorufin by metabolically active cells and can be quantified fluorometrically (O'Brien et al., 2000).

### 2.3. ATP Bioluminescence Assays

Because ATP is a universal indicator of metabolic activity, this method has found increasingly widespread application (Crouch et al., 1993). Requiring substantially fewer cells than the standard MTT assay, this approach offers a particularly advantageous option for experiments involving limited amounts of patient-derived material or primary cell cultures.

### 2.4. Methods for Monitoring Proliferation

BrdU (5-bromo-2′-deoxyuridine) and EdU (5-ethynyl-2′-deoxyuridine) are nucleoside analogs incorporated into DNA during the S phase and can be used to directly quantify the proliferating cell fraction (Dolbeare, 1995). Ki-67

immunofluorescence staining provides a frequently used indirect method for the assessment of proliferation (Freshney, 2010).

*Table 1. Comparison of Cell Viability and Proliferation Assays*

| Method | Principle | Advantage | Limitation | Key Reference(s) |
|---|---|---|---|---|
| Trypan Blue Exclusion | Membrane integrity (dye uptake by dead cells) | Simple, rapid, inexpensive | Subjective; misclassifies early apoptotic cells | Strober, 2015 |
| MTT / WST / MTS | Mitochondrial dehydrogenase reduces tetrazolium salt | Well-established; high-throughput | Chemical interference possible; endpoint only | Mosmann, 1983; Berridge et al., 2005 |
| Resazurin (Alamar Blue) | Cellular redox reduction of resazurin | Fluorometric; non-lytic; continuous monitoring | MDR transporter efflux possible | O'Brien et al., 2000 |
| ATP Bioluminescence | Luciferase-based ATP quantification | Very sensitive; requires few cells | Requires cell lysis; reagent cost | Crouch et al., 1993 |
| BrdU / EdU Incorporation | Nucleoside analog incorporated during S-phase | Direct measure of DNA synthesis | Mild cytotoxicity at high doses | Dolbeare, 1995 |
| Ki-67 Immunostaining | Nuclear antigen in proliferating cells | Applicable to fixed samples | No cell-cycle phase specificity | Freshney, 2010 |

*Note: MDR = multidrug resistance; BrdU = bromodeoxyuridine; EdU = 5-ethynyl-2′-deoxyuridine.*

## 3. CELL DEATH AND APOPTOSIS ANALYSES

### 3.1. Annexin V / PI Double Staining

Phosphatidylserine (PS) is normally located on the inner leaflet of the plasma membrane; however, when apoptotic signaling pathways are activated, it is translocated to the outer leaflet. During this process, Annexin V, which binds to PS with high affinity, reveals through fluorescent labeling that the cell has entered the early apoptotic stage. When combined with propidium iodide (PI), this method allows a clear distinction between Annexin V-negative/PI-negative viable cells, Annexin V-positive/PI-negative early apoptotic cells, and Annexin V-positive/PI-positive late apoptotic or necrotic cells (Vermes et al., 1995).

### 3.2. Caspase Activity Assays

Caspases are proteases that play a central role in the execution of apoptosis. Caspase-9 is generally classified among the initiator caspases associated with the intrinsic (mitochondrial) apoptotic pathway, whereas caspase-8 is associated with the extrinsic (death receptor-mediated) pathway (Thornberry & Lazebnik, 1998). The activity of these enzymes can be quantitatively assessed using colorimetric or fluorometric assays based on synthetic tetrapeptide

substrates such as DEVD (particularly for caspase-3/7-like activity) or IETD (for caspase-8-like activity), which are conjugated to chromogenic or fluorogenic groups such as p-nitroanilide (pNA) or 7-amino-4-trifluoromethyl coumarin (AFC). Although these assays are useful for demonstrating the extent of caspase activation associated with the apoptotic process, they do not directly reflect the proportion of apoptotic cells; rather, they indicate the level of caspase-like proteolytic activity in the sample (Nicholson et al., 1995).

### 3.3. DNA Fragmentation Analyses

The TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assay is used to detect double-stranded DNA breaks, one of the characteristic features of apoptosis. Based on the enzymatic addition of modified nucleotides to free 3′-OH ends, this method can be applied using both flow cytometry and fluorescence microscopy and provides high sensitivity for the detection of apoptosis (Gavrieli et al., 1992). The DNA ladder observed on agarose gel electrophoresis is regarded as semiquantitative evidence of internucleosomal DNA cleavage occurring at nucleosomal intervals (Freshney, 2010).

### 3.4. Analysis of Mitochondrial Membrane Potential

Loss of mitochondrial membrane potential (ΔΨm) is one of the early indicators of the intrinsic apoptotic pathway. JC-1 [5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide] accumulates in the mitochondria of healthy cells and forms red-fluorescent J-aggregates; when membrane potential decreases, it remains in its monomeric form and emits green fluorescence. The ratio between these two signals is used as an indicator of mitochondrial integrity and function (Smiley et al., 1991).

*Table 2. Overview of Apoptosis Detection Methods*

| Method | Molecular Target / Principle | Key Advantage | Key Reference(s) |
|---|---|---|---|
| Annexin V / PI Double Staining | Phosphatidylserine (PS) externalization + membrane integrity | Distinguishes viable, early apoptotic, and late apoptotic/ necrotic populations | Vermes et al., 1995 |
| Caspase Activity Assays (DEVD/ IETD substrates) | Caspase-3/7 or caspase-8 proteolytic cleavage activity | Quantitative; distinguishes intrinsic vs. extrinsic apoptotic pathway | Nicholson et al., 1995; Thornberry & Lazebnik, 1998 |
| TUNEL Assay | Enzymatic labeling of free 3′-OH DNA ends | High sensitivity; compatible with flow cytometry and fluorescence microscopy | Gavrieli et al., 1992 |
| JC-1 ΔΨm Assay | Mitochondrial J-aggregate (red) vs. monomer (green) fluorescence ratio | Early apoptosis indicator; ratiometric signal; live cells | Smiley et al., 1991 |

*Note: ΔΨm = mitochondrial membrane potential; PI = propidium iodide; TUNEL = terminal deoxynucleotidyl transferase dUTP nick-end labeling.*

## 4. CELL MORPHOLOGY AND IMAGING METHODS

### 4.1. Light Microscopy Techniques

Phase-contrast microscopy is the principal imaging method preferred for routine morphological evaluation in cell culture studies; its ability to enable live-cell imaging without the need for fixation or staining makes this technique particularly valuable (Zernike, 1955). Differential interference contrast (DIC) microscopy provides a higher-resolution, three-dimensional-like appearance, whereas Hoffman modulation contrast microscopy has been specifically adapted for studies performed in plastic culture vessels. All of these methods can also be combined with time-lapse live-cell imaging, thereby allowing cell behavior to be monitored continuously and in real time (Freshney, 2010; Boutros et al., 2015).

### 4.2. Fluorescence Microscopy

Fluorescence microscopy enables the labeling of specific cellular structures and molecules through the use of antibodies, fluorescent proteins (GFP and its derivatives), or organelle-specific dyes such as Hoechst, MitoTracker, and LysoTracker. Confocal laser scanning microscopy reduces background fluorescence by means of optical sectioning, thereby permitting the acquisition of optical slices and enabling the three-dimensional reconstruction of intracellular structures (Pawley, 2006).

### 4.3. High-Content Screening

High-content imaging (HCI) and high-content analysis (HCA) platforms combine automated image acquisition with computer-assisted image analysis, making it possible to measure hundreds of cellular parameters simultaneously (Boutros et al., 2015).

### 4.4. Electron Microscopy

Transmission electron microscopy (TEM) is the principal method used to visualize cellular ultrastructure on ultrathin fixed sections and allows detailed examination of fine structures such as mitochondrial cristae, the endoplasmic reticulum, and autophagosomes (Lam et al., 2021). Scanning electron microscopy (SEM), by contrast, is an ideal technique for the three-dimensional evaluation of the organization of surface projections such as microvilli and membrane folds (Passey et al., 2007). Although both techniques have limited applicability for live-cell imaging, they provide unique information in terms of the depth and resolution of morphological data (Varsano & Wolf, 2022).

## 5. PROTEIN ANALYSIS METHODS

### 5.1. Western Blot

Western blotting (immunoblotting) is one of the most fundamental methods for evaluating protein expression levels and post-translational

modifications in cell lysates. After proteins are separated according to molecular weight by SDS-PAGE, they are transferred onto a nitrocellulose or PVDF membrane and subsequently detected using antibodies specific to the target antigen (Towbin et al., 1979).

### 5.2. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a quantitative analytical method widely used for the detection of cytokines, growth factors, and other secreted proteins in cell culture supernatants or lysates. In the sandwich ELISA format, the target antigen is captured between two distinct antibodies (capture and detection antibodies), thereby providing high specificity, whereas competitive ELISA offers a more suitable alternative for low-molecular-weight analytes (Leng et al., 2008).

### 5.3. Flow Cytometry

Flow cytometry is a powerful analytical platform that enables the simultaneous measurement of multiple parameters at the single-cell level. Cell surface receptor expression, intracellular signaling molecules, and transcription factors can be evaluated by labeling fixed and permeabilized cells with fluorochrome-conjugated antibodies (Shapiro, 2003).

**Table 3. Protein and Gene Expression Analysis Methods at a Glance**

| Method | Target | Strength | Limitation | Key Reference(s) |
|---|---|---|---|---|
| Western Blot | Protein levels / post-translational modifications | Resolves protein by molecular weight; widely validated | Low throughput; semi-quantitative | Towbin et al., 1979 |
| ELISA | Secreted or lysate proteins (cytokines, growth factors) | Highly quantitative; 96-well compatible | Limited to known antigens | Leng et al., 2008 |
| Flow Cytometry | Surface & intracellular markers at single-cell level | Multiparameter; single-cell resolution | Requires large cell numbers | Shapiro, 2003 |
| RT-qPCR | mRNA expression of defined target genes | High sensitivity; broad dynamic range | Requires validated reference genes | Bustin et al., 2009 |
| RNA-Seq | Whole-transcriptome expression | Unbiased; detects novel transcripts | High cost; complex bioinformatics | Wang et al., 2009 |
| Microarray | Expression of predefined gene panels | Cost-effective; established pipelines | Probe saturation; no novel transcripts | Schena et al., 1995; Zhao et al., 2014 |
| CRISPR-Cas9 Screens | Genome-wide gene function | Higher specificity than RNAi screens | Requires lentiviral delivery + NGS | Doudna & Charpentier, 2014; Joung et al., 2017 |

*Note: RT-qPCR = real-time quantitative PCR; RNA-Seq = RNA sequencing; NGS = next-generation sequencing; RNAi = RNA interference.*

## 6. GENE EXPRESSION AND NUCLEIC ACID ANALYSES

### 6.1. Real-Time Quantitative PCR

Real-time quantitative PCR (RT-qPCR) enables the amplification and simultaneous quantitative measurement of target genes through the use of gene-specific primers following the conversion of messenger RNA into complementary DNA (cDNA) by reverse transcriptase. Owing to its high sensitivity, broad linear dynamic range, and relatively low RNA requirement, this technique has become the routine standard for gene expression analyses (Bustin et al., 2009). To ensure the reliability of the results, normalization against appropriate reference genes such as GAPDH, β-actin, or 18S rRNA is essential; however, the selection of these reference genes requires careful evaluation depending on the experimental conditions.

### 6.2. RNA Sequencing

RNA sequencing (RNA-seq), based on next-generation sequencing technologies, provides a comprehensive profile of the cellular transcriptome. It offers a wide range of information, from differential gene expression analysis to the discovery of novel transcripts, from the identification of alternative splicing patterns to the characterization of long non-coding RNAs (Wang et al., 2009). Single-cell RNA sequencing (scRNA-seq) has further transformed the field by enabling the resolution of transcriptomic profiles at the level of individual cells within heterogeneous cell populations (Macosko et al., 2015).

### 6.3. Microarray Analysis

Microarray technology is a high-throughput platform that enables the simultaneous evaluation of the expression levels of thousands of genes through predefined probes (Schena et al., 1995). However, technical limitations such as background hybridization and probe saturation may affect the accurate detection and quantitative assessment of genes expressed at either very low or very high levels. For this reason, RNA-seq is regarded as an important alternative to microarray technology in comprehensive transcriptomic analyses, as it offers a broader dynamic range and allows a more extensive evaluation of the transcriptome without dependence on predefined probes (Zhao et al., 2014).

### 6.4. CRISPR and Gene Editing Tools

CRISPR-Cas9 technology is a powerful gene-editing platform that enables targeted investigation of gene function in cell culture systems and facilitates genome-wide functional screening (Doudna & Charpentier, 2014). Genome-wide CRISPR screens, which are based on the delivery of pooled sgRNA

libraries targeting protein-coding genes into cell lines via lentiviral systems, are among the methods widely used in functional genomics research because they can provide higher target specificity and more reliable loss-of-function outputs compared with RNA interference–based approaches (Joung et al., 2017).

## 7. METABOLIC AND BIOCHEMICAL ANALYSES

### 7.1. Metabolic Profiling Using the Seahorse Cell Analyzer

The Seahorse XF Analyzer is a platform that measures the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in real time, thereby enabling the simultaneous assessment of mitochondrial respiration and glycolytic activity (Wu et al., 2007). Nevertheless, because intact-cell models may be insufficient for detailed mechanistic analyses in the evaluation of certain respiratory parameters, the resulting data should be interpreted carefully within the specific experimental context and, when necessary, supported by analyses performed in plasma membrane–permeabilized cells (Divakaruni et al., 2014).

### 7.2. Glucose and Lactate Analysis

The measurement of glucose consumption and lactate production in cell culture media remains a classical biochemical indicator for the assessment of glycolytic metabolism (Freshney, 2010). Periodic monitoring of these parameters in cell culture supernatants by enzymatic methods represents one of the principal approaches used to evaluate the Warburg effect at the cellular level, particularly in tumor cells, where increased glucose consumption and lactate production occur even in the presence of oxygen (Koppenol et al., 2011).

### 7.3. Reactive Oxygen Species (ROS) Analysis

Intracellular reactive oxygen species (ROS) can be measured using ROS-sensitive fluorescent probes such as DCFH-DA (2′,7′-dichlorofluorescein diacetate). This compound is hydrolyzed by intracellular esterases and, in the presence of ROS, is oxidized to the fluorescent DCF form (Eruslanov & Kusmartsev, 2009). However, because the ROS specificity of DCFH-DA is limited, the use of mitochondria-targeted fluorescent probes such as MitoSOX Red, in combination with flow cytometry, is recommended for the more selective detection of mitochondrial superoxide (De Biasi et al., 2016).

### 7.4. Analysis of Calcium Signaling

Intracellular calcium dynamics can be monitored using ratiometric calcium indicators such as Fura-2/AM. These dyes are hydrolyzed by intracellular esterases, retained within the cell, and undergo changes in their

fluorescence properties upon binding calcium (Grynkiewicz et al., 1985). The principal advantage of this method is that the fluorescence ratio obtained from excitation wavelengths of 340 nm and 380 nm allows the quantitative estimation of intracellular calcium levels in a manner that is independent of variables such as dye concentration, light intensity, and optical path length (Barreto-Chang & Dolmetsch, 2009).

**Table 4. Metabolic and Biochemical Analysis Methods**

| Method | Measured Parameter | Key Feature | Key Reference(s) |
|---|---|---|---|
| Seahorse XF Analyzer | OCR (mitochondrial respiration) & ECAR (glycolysis) in real time | Live-cell; simultaneous mitochondrial and glycolytic readout | Wu et al., 2007; Divakaruni et al., 2014 |
| Glucose / Lactate Enzymatic Assay | Glucose consumption & lactate production in conditioned media | Reflects Warburg effect; simple and inexpensive | Freshney, 2010; Koppenol et al., 2011 |
| DCFH-DA / MitoSOX (ROS Detection) | Total intracellular ROS or mitochondrial superoxide | Flow cytometry-compatible; MitoSOX is mitochondria-targeted | Eruslanov & Kusmartsev, 2009; De Biasi et al., 2016 |
| Fura-2/AM Calcium Imaging | Intracellular free $[Ca^{2+}]$ via 340/380 nm excitation ratio | Quantitative; signal independent of dye concentration variability | Grynkiewicz et al., 1985; Barreto-Chang & Dolmetsch, 2009 |

*Note: OCR = oxygen consumption rate; ECAR = extracellular acidification rate; ROS = reactive oxygen species; DCFH-DA = 2′,7′-dichlorofluorescein diacetate.*

## 8. CELL MIGRATION AND INVASION ANALYSES

### 8.1. Wound Healing (Scratch) Assay

The wound healing assay is based on creating a uniform wound area in a monolayer cell culture using a sterile pipette tip and monitoring the rate at which cells close this gap through time-series imaging. Although this method is technically simple and low-cost, it also has several limitations, including non-standardized variations in wound generation, the risk of cell damage during assay performance, and the inability to clearly distinguish the respective contributions of proliferation and migration to wound closure (Liang et al., 2007).

### 8.2. Boyden Chamber and Transwell Migration Assay

The Boyden chamber assay is based on quantifying the number of cells that migrate through a porous membrane to its lower surface in a two-compartment system designed to assess migratory capacity (Boyden, 1962). For the evaluation of invasive capacity, the upper surface of the porous membrane is generally coated with extracellular matrix components such as Matrigel; in this way, not only the ability of cells to migrate across the membrane but also their capacity to traverse an extracellular matrix–like barrier can be assessed.

By comparing data obtained from Matrigel-coated and uncoated transwell systems, this approach allows invasive behavior to be distinguished from simple cell migration (Krämer et al., 2013).

### 8.3. Single-Cell Tracking

Single-cell tracking analyses combined with live-cell imaging enable high-dimensional characterization of migration parameters such as the movement speed, displacement, and migratory directionality of individual cells. Software tools such as ImageJ/Fiji facilitate these analyses and make it possible to use complex cell-tracking algorithms that also account for changes in cell density, cell division, and cell death (Meijering et al., 2012).

**Table 5. Comparison of Cell Migration and Invasion Assays**

| Method | What Is Measured | Advantage | Limitation | Key Reference(s) |
|---|---|---|---|---|
| Wound Healing (Scratch) Assay | Rate of 2D gap closure over time | Simple; low cost; time-lapse imaging compatible | Cannot separate migration from proliferation; not 3D | Liang et al., 2007 |
| Boyden Chamber Migration | Cells crossing a porous membrane toward chemoattractant | Quantitative; chemotaxis gradient applicable | Endpoint only; no real-time data | Boyden, 1962 |
| Transwell Invasion (Matrigel-coated) | Cells penetrating an ECM-like barrier | Distinguishes invasion from simple migration | Matrigel batch variability | Krämer et al., 2013 |
| Single-Cell Tracking (Live Imaging) | Speed, displacement, and directionality per individual cell | High-dimensional kinetic data; per-cell resolution | Computationally intensive; requires time-lapse setup | Meijering et al., 2012 |

*Note: ECM = extracellular matrix; 2D = two-dimensional.*

## 9. QUALITY CONTROL AND STANDARDIZATION

### 9.1. Cell Line Authentication

Contamination and misidentification of cell lines represent major problems that seriously threaten reproducibility in cell biology research. Short tandem repeat (STR) profiling generates a genetic fingerprint unique to each cell line and enables comparison with reference repositories such as the ATCC. STR profiling should be adopted as a standard practice to be performed at the initiation of culture, at regular intervals, and prior to publication (Lorsch et al., 2014).

### 9.2. Mycoplasma Testing

Mycoplasma contamination is a serious problem that can silently affect numerous cellular parameters, ranging from morphology to gene expression,

while remaining undetectable by visual inspection (Drexler & Uphoff, 2002). PCR-based mycoplasma assays, nucleic acid staining with fluorescent dyes, or commercial detection kits should be applied at regular intervals, and both newly acquired cultures and cultures thawed from liquid nitrogen should be tested routinely. Cultures found to be contaminated with mycoplasma should either be decontaminated using appropriate antibiotic regimens or removed from use.

### 9.3. Passage Control and Cell Bank Management

Continuous passaging may lead to genetic drift, senescence, and cumulative alterations in phenotypic characteristics. To minimize this risk, early-passage stocks should be established, and experiments should be conducted under consistent conditions using cells within the lowest feasible passage range. A suitably sized cell bank maintained in liquid nitrogen serves as an essential safeguard for consistency in long-term research projects. Passage number should be recorded as an experimental variable and must be reported in publications (Freshney, 2010).

### 9.4. Experimental Reproducibility

Cell culture analyses, which are directly related to the broader reproducibility crisis in biomedical research, require substantial improvements in experimental design, statistical power analysis, and data reporting practices. It is particularly important to clearly distinguish independent biological replicates from technical replicates within the same experiment. Blinded experiments, the use of the same cell batch throughout the study whenever possible, and appropriate positive and negative control groups are among the fundamental requirements for obtaining reliable results (Begley & Ellis, 2012).

## 10. CONCLUSION AND FUTURE PERSPECTIVES

Analytical methods used in cell culture have made remarkable progress in advancing our understanding of the fundamental mechanisms underlying human diseases. This technological diversity, ranging from simple biochemical assays to high-content phenotypic screening, from mass cytometry to single-cell genomic analyses, provides researchers with the opportunity to investigate biological systems at an unprecedented level of detail and scale. Nevertheless, the major challenges in the field remain issues of reproducibility, standardization, and physiological relevance (Baker, 2016).

From the perspective of future developments, it is evident that the emergence of organoid and organ-on-chip technologies, with their potential to provide more physiologically realistic models, will inevitably be accompanied by profound transformations in the analytical platforms designed to support these systems. The integration of cell culture experiments with molecular profiling

techniques—that is, multi-omics approaches—enables a multidimensional and holistic understanding of cellular states (Bhise et al., 2014). In addition, the application of artificial intelligence and machine learning algorithms to high-content imaging datasets is expected to substantially expand both the depth and breadth of biological information obtained from phenotypic screens.

In conclusion, the careful selection of appropriate analytical methods, the implementation of rigorous quality control protocols, and the complementary use of multifaceted methodologies are indispensable prerequisites for generating reliable and biologically meaningful cell culture data. For researchers working in this field, achieving a thorough understanding of both the strengths and the limitations of current methods is essential for conducting robust, independently reproducible studies that ultimately contribute to the advancement of biomedical knowledge.

## REFERENCES

Baker, M. 1,500 scientists lift the lid on reproducibility. *Nature* **533**, 452–454 (2016). https://doi.org/10.1038/533452a

Barreto-Chang, O. L., & Dolmetsch, R. E. (2009). Calcium imaging of cortical neurons using Fura-2 AM. *Journal of Visualized Experiments*, (23), e1067. https://doi.org/10.3791/1067

Begley, C. G., & Ellis, L. M. (2012). Raise standards for preclinical cancer research. *Nature*, *483*(7391), 531-533. https://doi.org/10.1038/483531a

Berridge, M. V., Herst, P. M., & Tan, A. S. (2005). Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnology annual review*, *11*, 127-152.

Bhise, N. S., Ribas, J., Manoharan, V., Zhang, Y. S., Polini, A., Massa, S., Dokmeci, M. R., & Khademhosseini, A. (2014). Organ-on-a-chip platforms for studying drug delivery systems. *Journal of Controlled Release*, 190, 82-93. https://doi.org/10.1016/j.jconrel.2014.05.004

Boutros, M., Heigwer, F., & Laufer, C. (2015). Microscopy-based high-content screening. *Cell*, *163*(6), 1314-1325. https://doi.org/10.1016/j.cell.2015.11.007

Boyden, S. (1962). The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *The Journal of Experimental Medicine*, *115*(3), 453-466. https://doi.org/10.1084/jem.115.3.453

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, 55(4), 611-622. https://doi.org/10.1373/clinchem.2008.112797

Crouch, S. P., Kozlowski, R., Slater, K. J., & Fletcher, J. (1993). The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *Journal of Immunological Methods*, 160(1), 81-88. https://doi.org/10.1016/0022-1759(93)90011-U

De Biasi, S., Gibellini, L., Bianchini, E., Nasi, M., Pinti, M., Salvioli, S., & Cossarizza, A. (2016). Quantification of mitochondrial reactive oxygen species in living cells by using multi-laser polychromatic flow cytometry. *Cytometry Part A*, *89*(10), 1106-1110. https://doi.org/10.1002/cyto.a.22936

Divakaruni, A. S., Paradyse, A., Ferrick, D. A., Murphy, A. N., & Jastroch, M. (2014). Analysis and interpretation of microplate-based oxygen consumption and pH data. *Methods in Enzymology*, *547*, 309-354. https://doi.org/10.1016/B978-0-12-801415-8.00016-3

Dolbeare, F. (1995). Bromodeoxyuridine: a diagnostic tool in biology and medicine, Part I: Historical perspectives, histochemical methods and cell kinetics. *The Histochemical Journal*, *27*(5), 339-369. https://doi.org/10.1007/BF02389022

Doudna, J. A., & Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. *Science*, *346*(6213), 1258096. https://doi.org/10.1126/science.1258096

Drexler, H. G., & Uphoff, C. C. (2002). Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention. *Cytotechnology*, *39*(2), 75-90. https://doi.org/10.1023/A:1022913015916

Eruslanov, E., & Kusmartsev, S. (2009). Identification of ROS using oxidized DCFDA and flow-cytometry. In *Advanced protocols in oxidative stress II* (pp. 57-72). Totowa, NJ: Humana Press. https://doi.org/10.1007/978-1-60761-411-1_4

Freshney, R. I. (2010). Culture of animal cells: A manual of basic technique and specialized applications (6th ed.). Wiley-Blackwell.

Gavrieli, Y., Sherman, Y., & Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *The Journal of cell biology*, *119*(3), 493-501. https://doi.org/10.1083/jcb.119.3.493

Grynkiewicz, G., Poenie, M., & Tsien, R. Y. (1985). A new generation of Ca2+ indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry*, *260*(6), 3440-3450. https://doi.org/10.1016/S0021-9258(19)83641-4

Joung, J., Konermann, S., Gootenberg, J. S., Abudayyeh, O. O., Platt, R. J., Brigham, M. D., Sanjana, N. E., & Zhang, F. (2017). Genome-scale CRISPR-Cas9 knockout and transcriptional activation screening. *Nature Protocols*, *12*(4), 828-863. https://doi.org/10.1038/nprot.2017.016

Koppenol, W. H., Bounds, P. L., & Dang, C. V. (2011). Otto Warburg's contributions to current concepts of cancer metabolism. *Nature Reviews Cancer*, *11*(5), 325-337. https://doi.org/10.1038/nrc3038

Krämer, N., Walzl, A., Unger, C., Rosner, M., Krupitza, G., Hengstschläger, M., & Dolznig, H. (2013). In vitro cell migration and invasion assays. *Mutation Research/Reviews in Mutation Research*, *752*(1), 10-24. https://doi.org/10.1016/j.mrrev.2012.08.001

Lam, J., Katti, P., Biete, M., Mungai, M., AshShareef, S., Neikirk, K., Garza Lopez, E., Vue, Z., Christensen, T. A., Beasley, H. K., Rodman, T. A., Murray, S. A., Salisbury, J. L., Glancy, B., Shao, J., Pereira, R. O., Abel, E. D., & Hinton, A., Jr. (2021). A Universal Approach to Analyzing Transmission Electron Microscopy with ImageJ. *Cells*, *10*(9), 2177. https://doi.org/10.3390/cells10092177

Leng, S. X., McElhaney, J. E., Walston, J. D., Xie, D., Fedarko, N. S., & Kuchel, G. A. (2008). ELISA and multiplex technologies for cytokine measurement in inflammation and aging research. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *63*(8), 879-884. https://doi.org/10.1093/gerona/63.8.879

Liang, C. C., Park, A. Y., & Guan, J. L. (2007). In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nature protocols*, *2*(2), 329-333. https://doi.org/10.1038/nprot.2007.30

Lorsch, J. R., Collins, F. S., & Lippincott-Schwartz, J. (2014). Fixing problems with cell lines. *Science*, *346*(6216), 1452-1453. https://doi.org/10.1126/science.1259110

Macosko, E. Z., Basu, A., Satija, R., Nemesh, J., Shekhar, K., Goldman, M., Tirosh, I., Bialas, A. R., Kamitaki, N., Martersteck, E. M., Trombetta, J. J., Weitz, D. A.,

Sanes, J. R., Shalek, A. K., Regev, A., & McCarroll, S. A. (2015). Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. *Cell*, *161*(5), 1202-1214. https://doi.org/10.1016/j.cell.2015.05.002

Meijering, E., Dzyubachyk, O., & Smal, I. (2012). Methods for cell and particle tracking. *Methods in enzymology*, 504, 183-200. https://doi.org/10.1016/B978-0-12-391857-4.00009-4

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods*, *65*(1-2), 55-63. https://doi.org/10.1016/0022-1759(83)90303-4

Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T. T., Yu, V. L., & Miller, D. K. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature*, *376*(6535), 37-43. https://doi.org/10.1038/376037a0

O'Brien, J., Wilson, I., Orton, T., & Pognan, F. (2000). Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *European journal of biochemistry*, *267*(17), 5421-5426. https://doi.org/10.1046/j.1432-1327.2000.01606.x

Pampaloni, F., Reynaud, E. G., & Stelzer, E. H. (2007). The third dimension bridges the gap between cell culture and live tissue. *Nature reviews Molecular cell biology*, *8*(10), 839-845. https://doi.org/10.1038/nrm2236

Passey, S., Pellegrin, S., & Mellor, H. (2007). Scanning electron microscopy of cell surface morphology. *Current Protocols in Cell Biology*, *37*(1), 4-17. https://doi.org/10.1002/0471143030.cb0417s37

Pawley, J. (Ed.). (2006). *Handbook of biological confocal microscopy* (Vol. 236). Springer Science & Business Media.

Riccardi, C., & Nicoletti, I. (2006). Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nature protocols*, *1*(3), 1458-1461. https://doi.org/10.1038/nprot.2006.238

Schena, M., Shalon, D., Davis, R. W., & Brown, P. O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science*, *270*(5235), 467-470. https://doi.org/10.1126/science.270.5235.467

Shapiro, H. M. (2005). *Practical flow cytometry*. John Wiley & Sons.

Smiley, S. T., Reers, M., Mottola-Hartshorn, C., Lin, M., Chen, A., Smith, T. W., Steele, G. D., Jr., & Chen, L. B. (1991). Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proceedings of the National Academy of Sciences*, *88*(9), 3671-3675. https://doi.org/10.1073/pnas.88.9.3671

Strober, W. (2015). Trypan blue exclusion test of cell viability. *Current Protocols in Immunology, 111*(1), A3.B.1–A3.B.3. https://doi.org/10.1002/0471142735.ima03bs111

Thornberry, N. A., & Lazebnik, Y. (1998). Caspases: enemies within. *Science*, *281*(5381), 1312-1316. https://doi.org/10.1126/science.281.5381.1312

Towbin, H., Staehelin, T., & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the national academy of sciences*, *76*(9), 4350-4354. https://doi.org/10.1073/pnas.76.9.4350

Varsano, N., & Wolf, S. G. (2022). Electron microscopy of cellular ultrastructure in three dimensions. *Current opinion in structural biology*, *76*, 102444. https://doi.org/10.1016/j.sbi.2022.102444

Vermes, I., Haanen, C., Steffens-Nakken, H., & Reutellingsperger, C. (1995). A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. *Journal of immunological methods*, *184*(1), 39-51. https://doi.org/10.1016/0022-1759(95)00072-I

Wang, Z., Gerstein, M., & Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nature reviews genetics*, *10*(1), 57-63. https://doi.org/10.1038/nrg2484

Wu, M., Neilson, A., Swift, A. L., Moran, R., Tamagnine, J., Parslow, D., Armistead, S., Lemire, K., Orrell, J., Teich, J., Chomicz, S., & Ferrick, D. A. (2007). Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. *American Journal of Physiology-Cell Physiology*, *292*(1), C125-C136. https://doi.org/10.1152/ajpcell.00247.2006

Zernike, F. (1955). How I discovered phase contrast. *Science*, *121*(3141), 345-349. https://doi.org/10.1126/science.121.3141.345

Zhao, S., Fung-Leung, W. P., Bittner, A., Ngo, K., & Liu, X. (2014). Comparison of RNA-Seq and microarray in transcriptome profiling of activated T cells. *PLOS ONE*, *9*(1), e78644. https://doi.org/10.1371/journal.pone.0078644

Chapter 7

# THERAPEUTİC POTENTIAL OF ALKALOID-CONTAINING PLANTS IN NEURODEGENERATİVE DİSEASES

*Nouralyakin MANAFİKHİ*[1]
*Beyza Nur YILMAZ*[2]
*Duygu TAŞKIN*[3]
*Turgut TAŞKIN*[4]

1 Öğrenci: Nouralyakin MANAFİKHİ, Marmara University, Faculty of Pharmacy, Department of Pharmacognosy, Istanbul, Türkiye
2 Arş.Gör. Beyza Nur YILMAZ, Marmara University, Faculty of Pharmacy, Department of Pharmacognosy, Istanbul, Türkiye 0000-0002-4418-3182
3 Doç.Dr. Duygu TAŞKIN, Department of Analytical Chemistry, Faculty of Pharmacy, University of Health Sciences, Istanbul, Turkiye. 0000-0002-5279-0900
4 Doç.Dr. Turgut TAŞKIN, Marmara University, Faculty of Pharmacy, Department of Pharmacognosy, Istanbul, Türkiye 0000-0001-8475-6478

Neuronal degeneration can arise from genetic or environmental factors and is observed in many neurological disorders. At the cellular level, apoptosis (programmed cell death) mechanisms play a crucial role in this process. These mechanisms, driven by genetically encoded programs, lead to the selective elimination of specific cells over time. In diseases such as Alzheimer's, Parkinson's, Huntington's chorea, and ALS, abnormal protein production, increased oxidative stress, and elevated intracellular calcium levels have all been shown to contribute to apoptosis (Bredesen, D.E. et al., 2006). In the long term, structural changes in neurons can result in cell death. Unlike apoptosis, this process progresses without leaving distinct traces in glial tissue. Recent studies have demonstrated that the intracellular accumulation of proteins such as amyloid, synuclein, ubiquitin, and huntingtin is associated with neurodegenerative diseases. These proteins may be overproduced due to genetic mutations or accumulate as a result of impaired cellular waste clearance mechanisms. In particular, in Alzheimer's disease, the abnormal processing of amyloid precursor proteins leads to toxic accumulations. Scientists continue to investigate the effects of these proteins on cells and the mechanisms by which they contribute to neuronal death (Du, J. et al., 2009; Rogaeva, E. et al., 2007).

**Neurodegenerative Diseases**

**1. Alzheimer's Disease**

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative disorders and is primarily characterized by memory impairments resulting from the progressive loss of brain cells. Although the exact causes of the disease remain unclear, genetic predisposition, social and psychological factors, dietary habits, and lifestyle choices are believed to play a role. The disease was first described in 1907 by German physician Alois Alzheimer, based on specific clinical findings. Today, it is estimated that approximately five million new cases occur worldwide each year (Ferri et al., 2005).

The incidence of Alzheimer's increases significantly with age. For instance, while the prevalence is approximately 0.1% at age 60, it rises to 47% among individuals aged 85 and above. By 2050, it is projected that more than a quarter of the global population will be over the age of 65, suggesting a significant increase in Alzheimer's prevalence in the coming decades (Katzman, 1991; Taneli, et al., 2010).

Although the pathological processes of Alzheimer's disease are not fully understood, it is characterized by senile amyloid plaques, neurofibrillary tangles (NFTs), neuronal loss, and marked brain atrophy. The main component of NFTs is hyperphosphorylated tau protein, which, when unable to bind to

microtubules, leads to structural cellular disruption. Over time, this results in the formation of paired helical filaments that develop into intracellular NFTs, ultimately leading to neuronal death. Another key feature of the disease is the formation of amyloid plaques. One hypothesis explaining the onset of Alzheimer's is the oxidative stress theory. An increase in free oxygen radicals, coupled with a decline in antioxidant defense mechanisms, causes neuronal damage that can progress to cell death. Elevated levels of iron and mercury, lipid peroxidation, and DNA/protein oxidation abnormalities are known to contribute to oxidative stress. These physiological changes become more pronounced with aging (Demir et al., 2011).

Currently, the most commonly used class of medications in Alzheimer's treatment is acetylcholinesterase inhibitors. Research indicates that acetylcholine levels in the brains of Alzheimer's patients are reduced by up to 50%. Acetylcholine is a critical neurotransmitter involved in neural communication. The enzyme acetylcholinesterase (AChE) breaks down acetylcholine, leading to impaired neurotransmission. Thus, AChE inhibitors work by preventing this breakdown, thereby maintaining communication between neurons. The primary cholinesterase inhibitors approved by the FDA for Alzheimer's treatment include tacrine, galantamine, donepezil, and rivastigmine. Although these drugs do not halt disease progression, they aim to alleviate symptoms and improve patients' quality of life (Winblad & Jelic, 2004).

**2. Parkinson's Disease**

Parkinson's disease (PD) is one of the most common movement disorders and ranks second only to essential tremor in prevalence. It typically emerges in middle to late adulthood, most often between the ages of 40 and 70. Given that both incidence and prevalence increase with age, PD is considered an age-related disorder. Originally classified solely as a motor disorder, Parkinson's disease is now recognized as a complex condition affecting the autonomic nervous system, cognitive functions, sensory perception, and behavioral processes. The primary pathological feature of the disease is the loss of dopaminergic neurons in the substantia nigra region of the brain, along with the accumulation of eosinophilic cytoplasmic inclusions known as Lewy bodies. This neurodegeneration leads to decreased dopamine production, which underlies the core symptoms of the disease. Clinically, symptoms often begin unilaterally and the most characteristic early sign is resting tremor. Additional symptoms include bradykinesia (slowness of movement), muscle rigidity, and postural instability (impaired balance). Diagnosis is primarily based on medical history and neurological examination, as no definitive laboratory test currently exists. The disease generally follows a slow, progressive course, becoming more pronounced over time. Dopaminergic therapies are

particularly effective in the early stages of the disease in alleviating symptoms and improving quality of life. However, responsiveness to these treatments may diminish in the later stages, and symptoms may worsen. In advanced stages, additional complications such as freezing of gait, postural instability, and cognitive impairments can arise (Apaydın, et al., 2013).

### 3. Huntington's Disease

Huntington's disease (HD) is a hereditary neurodegenerative disorder that causes damage to specific regions of the nervous system, resulting in behavioral and cognitive impairments. In its advanced stages, the disease leads to severe cognitive decline and frequent emotional disturbances. First described in 1872 by Dr. George Huntington, HD is inherited in an autosomal dominant manner, meaning it is passed from generation to generation via a single mutated copy of the responsible gene. The disorder is also known as Huntington's chorea. The gene associated with HD normally regulates essential brain functions, but a mutation impairs neuronal function and leads to gradual degeneration. Every individual carries two copies of the Huntington gene; however, only one mutated copy is sufficient to cause the disease. Children of affected individuals have a 50% chance of inheriting the mutated gene. The initial symptoms typically appear after age 30 and include involuntary movements (chorea), loss of balance, clumsiness, mood disturbances, and short-term memory loss. As the disease progresses, patients may experience worsening motor function, speech difficulties, cognitive decline, reflex impairments, and weight loss. A critical marker in HD is the number of CAG trinucleotide repeats in the gene. Healthy individuals have fewer than 35 repeats, whereas affected individuals usually have 40 or more. Those with repeat counts between 36 and 39 are in a borderline zone with uncertain disease expression. Currently, there is no definitive cure for Huntington's disease. However, symptomatic treatments—including medications for involuntary movements and mood disorders, along with physical and speech therapy—can help improve quality of life. Ongoing research into gene therapy and targeted pharmacological interventions offers hope for slowing disease progression in the future (Vega et al., 2025; Gatto et al., 2020; Gaba, 2025 2014).

### 4. Dementia with Lewy Bodies

Dementia with Lewy Bodies (DLB) is a type of dementia characterized by cognitive impairments accompanied by motor and behavioral disturbances. This disease is marked by abnormal protein accumulations within brain cells and is sometimes confused with Parkinson's disease dementia. However, the onset phases of these two conditions differ: in Parkinson's disease dementia, motor impairments typically appear first, whereas in DLB, cognitive and behavioral changes precede motor symptoms. In the early stages, most

patients experience only depression, but as the disease progresses, symptoms such as memory decline, confusion, and Parkinson-like motor abnormalities become apparent. Over time, cognitive impairments such as memory loss and disorientation become more prominent. One of the distinguishing features of the disease is the fluctuating nature of these symptoms throughout the day. Motor impairments typically include muscle rigidity, decreased reflexes, and a mask-like facial expression. At present, there is no definitive cure for DLB. However, due to the involvement of cholinergic and dopaminergic pathways, certain dopaminergic and cholinergic medications used in Parkinson's disease may also be beneficial in treating this condition (King et al., 2019; Chin et al., 2019; Brenowitz et al., 2017).

**5. Multiple Sclerosis**

Multiple Sclerosis (MS) is an autoimmune disease that affects the central nervous system, characterized by demyelination, inflammation, and damage to nerve fibers. In MS, not only neurons but also the myelin sheath and oligodendrocytes are affected. The disease typically emerges in young adulthood, with a prevalence ranging from 2 to 200 cases per 100,000 individuals. First described by Jean-Martin Charcot in 1868, MS is a chronic condition. Common symptoms include muscle weakness, bladder control issues, fatigue, visual disturbances, and attention deficits. Less commonly, patients may also experience epileptic seizures, headaches, and hearing loss (Dobson and Giovannoni, 2019).

Various therapeutic approaches are employed to control relapses and slow disease progression. Corticosteroids are widely used to treat acute exacerbations, although it remains unclear whether all relapses require intervention. Steroid therapy is considered when an attack results in functional impairment or when the disease shows signs of progression. Since the 1990s, disease-modifying treatments aimed at preventing relapses and delaying progression have also been implemented (Ünal, (Akyıldız & Sav, 2004).

**6. Amyotrophic Lateral Sclerosis**

Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disease that affects motor neurons in the cerebral cortex, brainstem, and spinal cord (BrooksMD, 1994). It was first described by Jean-Martin Charcot in 1874. The name of the disease derives from the terms "amyotrophy" (referring to muscle wasting due to grey matter degeneration) and "lateral sclerosis" (referring to the involvement of the spinal cord's lateral white matter) (Goetz, 2000).

According to research conducted in 2016, the annual incidence of ALS is 1–2 cases per 100,000 people, while its prevalence is approximately 6 cases per 100,000. On average, disease onset occurs around the age of 60, and the average survival time following diagnosis is estimated at 3–4 years (Talbott et

al., 2016). Approximately 5–10% of ALS cases are inherited, and in 10–20% of these familial cases, mutations in the Cu/Zn superoxide dismutase-1 (SOD1) gene are responsible. Laboratory findings in familial and sporadic ALS are largely similar (Pizzasegola et al, 2009).

**Alkaloid-Containing Plants**

Alkaloids compounds, noted for their "anti-Alzheimer" effects, possess central nervous system-stimulating, anti-inflammatory properties and enhance the functionality of the cholinergic system. One of the most well-known examples of alkaloids is galantamine, which was first isolated from species of *Galanthus* L. and later produced synthetically. It exerts its effect by inhibiting the enzyme acetylcholinesterase. Currently, research on various alkaloid-containing plants is ongoing, and new potential therapeutic applications are being explored (Ng, Or, & Ip, 2015). The aim of this study is to reveal the potential of alkaloid-containing plants in the prevention and treatment of neurodegenerative diseases.

***Clausena lansium* (Lour.) Skeels**

*Clausena lansium* (Lour.) Skeels, a plant species belonging to the Rutaceae family, is a medicinally valuable species that grows predominantly in India, tropical Asia, and South Africa. This plant, which can grow up to about six meters tall, is commonly known as "wampee" due to its white or yellow fruits and is cultivated as a fruit tree in various tropical regions, especially Southern China. Different parts of the plant—including the leaves, stem, fruit, flowers, branches, and roots—have long been used in traditional medicine for the treatment of various diseases. For instance, in traditional Chinese medicine, leaf extracts have been used to treat edema, while the fruits have been consumed both as human food and animal feed (Arbab et al., 2012). Among the main compounds that determine the pharmacological significance of *Clausena lansium* are carbazole alkaloids, coumarins, and essential oils. These compounds have been isolated from the plant's stem, fruit, and branches. Particularly, carbazole alkaloids are believed to have therapeutic potential in neurological disorders due to their effects on the nervous system (Arbab et al., 2012). One of the important compounds isolated from *C. lansium* is (-)-clausenamide [(3S,4R,5R,6S)-diastereomer], which is one of 16 possible enantiomers due to the presence of four asymmetric carbon atoms. Pharmacological studies have shown that this compound may improve cognitive functions in various neurodegenerative diseases, especially Alzheimer's disease. It has been reported that (-)-clausenamide alleviates memory impairments, inhibits beta-amyloid (Aβ) toxicity, and prevents neurofibrillary tangle formation

by blocking tau protein phosphorylation (Chu & Zhang, 2014). The main underlying mechanisms of these effects include enhancement of synaptic plasticity, modulation of the cholinergic system, maintenance of intracellular calcium balance, and strengthening of antioxidative responses. In this context, it is suggested that the compound may have memory-enhancing potential not only in diseased individuals but also in healthy individuals (Chu et al., 2016). In a study conducted by Chang et al., sixteen different carbazole alkaloids isolated from *C. lansium* fruits were tested on SH-SY5Y neuronal cells damaged by 6-hydroxydopamine (6-OHDA). A significant number of these compounds increased neuronal viability and reduced apoptotic cell death. Their $EC_{50}$ values ranged between 0.36 ± 0.02 μM and 10.69 ± 0.15 μM, indicating the strong neuroprotective properties of these alkaloids (Liu et al., 2019). In a more recent study, a novel alkaloid derived from *C. lansium* called CZK (CZK = alkaloid derived from *C. lansium*) was shown to reduce brain damage by suppressing oxidative stress following ischemic stroke. This effect was particularly mediated through the activation of Nrf2-dependent antioxidant mechanisms. CZK increased the levels of glutathione (GSH) and superoxide dismutase (SOD), while reducing malondialdehyde (MDA) levels, thus contributing to the protection of brain cells against oxidative stress (Chen, H. et al., 2023). *C. lansium* stands out as a natural plant with therapeutic potential in neurodegenerative diseases such as Alzheimer's and Parkinson's, particularly due to its alkaloid compounds. Specific compounds like (-)-clausenamide, with their effects on enhancing synaptic plasticity, reducing oxidative stress, and preventing amyloid accumulation, make this plant a strong candidate among next-generation neuroprotective agents. However, these findings need to be validated in human clinical studies.

*Clausena lansium*'s neuroprotective capacity is closely linked to its carbazole alkaloid content. Therefore, accurate phytochemical characterization is essential for establishing structure–activity relationships. High-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS) have been widely employed for the identification and profiling of carbazole alkaloids in this species (Huang et al., 2023). Beyond the characterization of individual alkaloids, recent metabolomic studies have highlighted the importance of evaluating the overall phytochemical profile of *C. lansium*. Variations in metabolic fingerprints among plant samples have been associated with differences in biological activities, suggesting that neuroprotective effects may depend on the collective metabolite composition rather than on a single bioactive constituent. Consequently, metabolomics-based fingerprinting represents a promising analytical approach for extract standardization and quality control (Deng et al., 2025).

***Huperzia serrata* Thunb:**

*Huperzia serrata* is a plant mainly cultivated in China and is known for its alkaloid compound called "Huperzine A." Huperzine A acts as a selective and reversible acetylcholinesterase inhibitor and has notable effects on brain functions. It also functions as an N-methyl-D-aspartate (NMDA) receptor antagonist, preventing glutamate-induced neuronal damage. Due to its good solubility in lipids, it can cross the blood-brain barrier easily when administered orally and reach brain tissues, increasing its potential in neurological therapies (Zhang & Tang, 2006; Mao & Cao, 2014).

The positive effects of Huperzine A on memory and cognitive functions have been investigated in various animal models and human studies. A study by Zhang and Tang (2006) demonstrated that Huperzine A improved memory performance in rats. Additionally, research has confirmed the potential of Huperzine A to support cognitive functions (Mao & Cao, 2014). In a study conducted by Ma et al. (2013), the potential therapeutic effects of Huperzine A in the treatment of neurological diseases were examined. Notably, Huperzine A was found to have significant effects on hippocampal neurogenesis. The study observed that Huperzine A promoted the proliferation of hippocampal neural stem cells derived from embryonic mice and increased the formation of new neurons in the adult mouse hippocampus. In vitro studies showed that low doses of Huperzine A stimulated the proliferation of neural stem cells, whereas higher doses diminished this effect. These findings highlight the role of Huperzine A in the neurogenesis process and its potential benefits in the treatment of neurological disorders.

The efficacy of Huperzine A in clinical studies positions it as a strong option for the treatment of cognitive impairments. A randomized, double-blind clinical trial conducted in China observed a significant improvement in cognitive performance in students with memory problems who were administered Huperzine A at a dose of 50 micrograms twice daily (Sun, Xu & Pan, 1999). Furthermore, another study on Alzheimer's disease examined the effectiveness of Huperzine A. A meta-analysis revealed that daily doses of 300–500 micrograms administered over a period of 8 to 24 weeks significantly improved cognitive functions and daily living activities in Alzheimer's patients. These studies indicate that Huperzine A is an important therapeutic option in the treatment of Alzheimer's disease and is well-tolerated (Wang et al., 2009).

Huperzine A is considered one of the most prominent marker compounds in *Huperzia serrata* and is widely used for quality control of plant extracts and herbal preparations. Various analytical techniques, particularly HPLC-UV and LC-MS/MS, have been employed for its quantitative determination (Yang et al., 2003; Wu et al., 2006). Reported variations in huperzine A content

among plant materials highlight the importance of analytical standardization and quality control. Accurate quantification of this alkaloid is essential for ensuring consistency among samples and supporting the reproducibility of pharmacological studies.

***Securinega suffruticosa* (Linnaeus)**

*Securinega suffruticosa* (family Euphorbiaceae) grows wild in the tropical regions of Southeast Asia, particularly from the south of Korea extending to the northwestern Caucasus. This plant is notably rich in indolizidine-type alkaloids, especially the alkaloid securinine. Securinega suffruticosa has been reported to exhibit stimulating effects on the central nervous system and has been used for this purpose in regions such as Russia and Poland (Raj & Luczkiewicz, 2008). A study conducted in 2017 investigated the relationship between oxidative stress and β-amyloid accumulation, two pathological hallmarks of Alzheimer's disease. The findings indicated that oxidative stress could stimulate the production and aggregation of β-amyloid and lead to the accumulation of pathogenic forms. The study revealed that securinine alkaloid possesses antioxidant and metal-chelating properties and is effective in inhibiting β-amyloid aggregation. These results suggest that securinine could be a promising compound in the development of new drugs for the treatment of neurodegenerative diseases (Neganova, Klochkov, Petrova, & Shevtsova, 2017). In a 2004 study, the neuroprotective effects of D-securinine against β-amyloid-induced neurotoxicity were explored. Rats received β-amyloid injections into the intracerebral ventricle, and the effects of securinine on cognitive functions were evaluated. The study demonstrated that D-securinine significantly reduced acetylcholinesterase (AChE) activity, without affecting choline acetyltransferase. These results suggest that D-securinine may alleviate cognitive deficits and neurodegeneration in disorders such as Alzheimer's disease (Lin & Jun-tian, 2004). Another study in 2008 found that securinine improved quality of life in patients with amyotrophic lateral sclerosis (ALS), facilitated learning processes, strengthened memory, and prevented alcohol-induced memory loss. Moreover, in mice, securinine exhibited stimulatory effects on the central nervous system and acted as a GABA receptor antagonist (Raj & Luczkiewicz, 2008).

From an analytical perspective, recent phytochemical investigations have considerably expanded the known alkaloid diversity of *Securinega suffruticosa*. Advanced analytical approaches have enabled the isolation and characterization of several previously undescribed Securinega alkaloids, including securingines and episecurinol derivatives. Structural elucidation has primarily relied on spectroscopic techniques, particularly one- and two-dimensional NMR spectroscopy, together with mass spectrometric analyses.

More recent studies have further incorporated computational approaches such as electronic circular dichroism (ECD) calculations and DP4+ probability analysis to support structural assignments. These studies demonstrate the importance of modern analytical methodologies in revealing the chemical complexity of *S. suffruticosa* and in supporting subsequent investigations of its biological activities. Furthermore, these analytical studies provide valuable insight into the structural diversity of Securinega alkaloids and support future efforts to establish relationships between alkaloid composition and biological activity (Park et al., 2019; Guo et al., 2009).

## REFERENCES

Akyıldız, E., & Sav, A. M. (2004). Myelin hastalıkları. *Türkiye Ekopatoloji Dergisi, 10*(1–2), 43–48.

Apaydın, H., Özemekçi, S., Oğuz, S., & Zileli, İ. (2013). *Parkinson hastalığı: Hasta ve yakınları için el kitabı*. İstanbul, Türkiye: Bayçınar Tıbbi Yayıncılık.

Arbab, I., Abdul, A., Aspollah, M., Abdullah, R., Abdelwahab, S., Ibrahim, M., ... (2012). A review of traditional uses, phytochemical and pharmacological aspects of selected members of Clausena genus (Rutaceae). Journal of Medicinal Plants Research, 6, 5107–5118.

Bredesen, D. E., Rammohan, V. R., & Mehlen, P. (2006). Cell death in the nervous system. Nature, 443, 796–802.

Brenowitz, W. D., Keene, C. D., Hawes, S. E., Hubbard, R. A., Longstreth, W. T., Woltjer, R. L., Crane, P. K., Larson, E. B., & Kukull, W. A. (2017). Alzheimer's disease neuropathologic change, Lewy body disease, and vascular brain injury in clinic- and community-based samples. *Neurobiology of Aging, 53*, 83–92.

Brooks, B. R. (1994). El Escorial World Federation of Neurology criteria for the diagnosis of amyotrophic lateral sclerosis. *Journal of the Neurological Sciences, 124*(Suppl.), 96–107.

Chen, W., et al. (2023). Neuroprotective effect of total flavonoids in stems and leaves of Glycyrrhiza uralensis Fisch. on oxidative stress in HT-22 cells and Caenorhabditis elegans. Aging, 15(12), 5290–5303.

Chin, K. S., Teodorczuk, A., & Watson, R. (2019). Dementia with Lewy bodies: Challenges in the diagnosis and management. *Australian & New Zealand Journal of Psychiatry, 53*(4), 291–303.

Chu, S., Liu, S., Duan, W., Cheng, Y., Jiang, X., Zhu, C., ... (2016). The anti-dementia drug candidate, (−)-clausenamide, improves memory impairment through its multi-target effect. Pharmacology & Therapeutics, 162, 179–187.

Chu, S.-F., & Zhang, J.-T. (2014). Recent advances in the study of (−)-clausenamide: Chemistry, biological activities and mechanism of action. Acta Pharmaceutica Sinica B, 4(6), 417–423.

Demir Özkay, Ü., Öztürk, Y., & Can, Ö. D. (2011). Yaşlanan dünyanın hastalığı: Alzheimer hastalığı. *Süleyman Demirel Üniversitesi Tıp Fakültesi Dergisi, 18*(2), 35–42.

Deng, W.-H., Zheng, Y.-Y., Li, Y., Zhang, H., Lin, K.-X., Jiang, H.-Z., ... Lin, H.-B. (2025). Phytochemical profile influences the e-tongue responses, antioxidant, anti-inflammation, and hypoglycemic effects of wampee fruits from different regions. Food Chemistry: X, 29, 102860.

Dobson, R., & Giovannoni, G. (2019). Multiple sclerosis—A review. *European Journal of Neurology, 26*(1), 27–40. https://doi.org/10.1111/ene.13819

Du, J., Sun, B., Chen, K., Zhang, L., Liu, S., & Gu, Q. (2009). Metabolites of cerebellar neurons and hippocampal neurons play opposite roles in pathogenesis of Alzheimer's disease. PLoS ONE, 4(5), e5530.

Ferri, C. P., Prince, M., Brayne, C., Brodaty, H., Fratiglioni, L., Ganguli, M., ... Scazufca, M. (2005). Global prevalence of dementia: A Delphi consensus study. *The Lancet, 366*(9503), 2112–2117. https://doi.org/10.1016/S0140-6736(05)67889-0

Gaba, A. (2025). Nutrition and Huntington's disease: A review of current practice and theory. *Current Nutrition Reports, 14*, 1–8. https://doi.org/10.1007/s13668-025-00610-x

Gatto, E. M., González Rojas, N., Persi, G., Etcheverry, J. L., Cesarini, M. E., & Perandones, C. (2020). Huntington disease: Advances in the understanding of its mechanisms. *Clinical Parkinsonism & Related Disorders, 3*, 100056. https://doi.org/10.1016/j.prdoa.2020.100056

Goetz, C. G. (2000). Amyotrophic lateral sclerosis: Early contributions of Jean-Martin Charcot. *Muscle & Nerve, 23*(3), 336–343.

Guo, Y.-W. (2009). Two new securinega alkaloids from Securinega suffruticosa. Helvetica Chimica Acta.

Huang, X., Wang, M., Zhong, S., & Xu, B. (2023). Comprehensive review of phytochemical profiles and health-promoting effects of different portions of wampee (Clausena lansium). ACS Omega, 8(30), 26699–26714.

Katzman, R., & Saitoh, T. (1991). Advances in Alzheimer's disease. *The FASEB Journal, 5*(3), 278–286.

King, E., O'Brien, J. T., Donaghy, P., Morris, C., Barnett, N., Olsen, K., Martin-Ruiz, C., Taylor, J. P., & Thomas, A. J. (2019). Peripheral inflammation in mild cognitive impairment with possible and probable Lewy body disease and Alzheimer's disease. *International Psychogeriatrics, 31*(4), 551–560.

Liu, Y. P., Guo, J. M., Liu, Y. Y., Hu, S., Yan, G., Qiang, L., & Fu, Y. H. (2019). Carbazole alkaloids with potential neuroprotective activities from the fruits of Clausena lansium. Journal of Agricultural and Food Chemistry, 67(20), 5764–5771.

Ma, T., Gong, K., Yan, Y., Zhang, L., & Tang, P. (2013). Huperzine A promotes hippocampal neurogenesis in vitro and in vivo. Brain Research, 1506, 35–43.

Mao, X.-Y., & Cao, D.-F. (2014). Huperzine A ameliorates cognitive deficits in streptozotocin-induced diabetic rats. International Journal of Molecular Sciences, 15, 7667–7683.

Ng, Y., Or, T., & Ip, N. (2015). Plant alkaloids as drug leads for Alzheimer's disease. Neurochemistry International, 89, 260–270.

Park, K. J., Kim, C. S., Khan, Z., Oh, J., Kim, S. Y., Choi, S. U., & Lee, K. R. (2019). Securinega alkaloids from the twigs of Securinega suffruticosa and their biological activities. Journal of Natural Products, 82(5), 1345–1353.

Pizzasegola, C., Caron, I., Daleno, C., Ronchi, A., Minoia, C., Carri, M. T., & Bendotti, C. (2009). Treatment with lithium carbonate does not improve disease progression in two different strains of SOD1 mutant mice. *Amyotrophic Lateral Sclerosis, 10*(1), 1–8.

Raj, R., & Luczkiewicz, M. (2008). Neuroprotective effects of Securinega suffruticosa in central nervous system disorders. Journal of Ethnopharmacology, 115(1), 7–13.

Rogaeva, E., Meng, Y., Lee, J. H., Gu, Y., Kawarai, T., Zou, F., ... Mayeux, R. (2007). The neuronal sortilin-related receptor SORL1 is genetically associated with Alzheimer disease. Nature Genetics, 39(2), 168–177.

Sun, Q.-Q., Xu, S., & Pan, J.-L. (1999). Huperzine-A capsules enhance memory and learning performance in 34 pairs of matched adolescent students. Acta Pharmaceutica Sinica, 34(8), 601–603.

Talbott, E. O., Malek, A. M., & Lacomis, D. (2016). The epidemiology of amyotrophic lateral sclerosis. In *Handbook of Clinical Neurology* (Vol. 138, pp. 225–238). https://doi.org/10.1016/B978-0-12-802973-2.00013-6

Taneli, B., Sivrioğlu, Y., & Taneli, T. (2010). Alzheimer disease. *Gerofam Tip Dergisi, 1*, 35–40.

Vega, A. J., Hernandez, G. V., O'Malley, P. A., Robin, C. J., Parra, A. N., Varrassi, G., Shekoohi, S., & Kaye, A. D. (2025). Overview of Huntington's disease and emerging treatment strategies: A narrative review. *Cureus, 17*(12), e98243. https://doi.org/10.7759/cureus.98243

Wang, B.-S., Wang, H., Wei, Z.-H., Song, Y.-Y., & Zhang, L. (2009). Efficacy and safety of natural acetylcholinesterase inhibitor huperzine A in the treatment of Alzheimer's disease: An updated meta-analysis. Journal of Neural Transmission, 116, 457–465.

Winblad, B., & Jelic, V. (2004). Long-term treatment of Alzheimer disease: Efficacy and safety of acetylcholinesterase inhibitors. *Alzheimer Disease & Associated Disorders, 18*(Suppl. 1), S2–S8.

Wu, Q., & Gu, Y. (2006). Quantification of huperzine A in Huperzia serrata by HPLC-UV and identification of the major constituents in its alkaloid extracts by HPLC-DAD-MS-MS. Journal of Pharmaceutical and Biomedical Analysis, 40(4), 993–998.

Yang, Q.-P., Kou, X.-L., Fugal, K. B., & McLaughlin, J. L. (2003). Determination of huperzine A in formulated products by reversed-phase liquid chromatography using diode array and electrospray ionization mass spectrometric detection. Phytomedicine, 10(2–3), 200–205.

Zhang, H. Y., & Tang, X. C. (2006). Neuroprotective effects of huperzine A: New therapeutic targets for neurodegenerative diseases. Trends in Pharmacological Sciences, 27(12), 619–625.