CURRENT CONCEPTS AND INNOVATIVE RESEARCH IN HEALTH SCIENCES

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CONTENT

Analytical Approaches for Counterfeit Drug Detection Using IR Spectroscopy: A Review of Recent Studies (2015–2025)

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ABSTRACT

Counterfeit and substandard pharmaceuticals pose a significant risk to public health, leading to reduced therapeutic efficacy, drug resistance, toxicity, and economic loss. Infrared (IR) spectroscopy, particularly Fourier Transform Infrared (FTIR) spectroscopy and Attenuated Total Reflection (ATR-FTIR), has emerged as a powerful analytical tool for detecting counterfeit drugs due to its ability to provide rapid, non-destructive, and highly informative chemical fingerprints. This review summarizes studies published between 2015 and 2025 that applied IR spectroscopy for the identification and classification of counterfeit or substandard pharmaceutical products. The review highlights the advantages of IR spectroscopy (minimal sample preparation, rapid analysis, non-destructive nature, and applicability to multiple pharmaceutical forms) while also detailing its limitations, such as structural identification difficulties and spectral overlap. Overall, IR spectroscopy is demonstrated to be a versatile, reliable, and cost-effective method for pharmaceutical quality control and counterfeit drug detection, supporting regulatory enforcement and public health protection.

Keywords – Counterfeit drugs, Substandard pharmaceutical, FTIR spectroscopy, Pharmaceutical quality control, Pharmaceutical quality control

INTRODUCTION

Infrared (IR) spectroscopy is a well-established analytical technique widely employed for the identification, purity assessment, and structural characterization of chemical compounds based on their molecular vibrational transitions. Because IR spectra arise from bond stretching and bending vibrations within molecules, this technique is commonly classified under vibrational spectroscopy.

Among IR-based techniques, Fourier Transform Infrared (FTIR) spectroscopy—especially when combined with Attenuated Total Reflection (ATR)—offers significant advantages such as rapid analysis, minimal or no sample preparation, non-destructive measurement, and applicability to a wide range of pharmaceutical dosage forms. The fingerprint region of the IR spectrum provides unique spectral features for each compound, making IR spectroscopy a powerful tool for distinguishing active pharmaceutical ingredients (APIs), excipients, polymorphic forms, and formulation differences. When supported by chemometric methods, IR spectroscopy allows simultaneous multi-component analysis and detection of subtle chemical variations in complex pharmaceutical matrices.

Counterfeit and substandard medicines represent a growing global public health challenge, often containing incorrect amounts of APIs, harmful contaminants, or entirely different chemical compositions. Such products

compromise therapeutic efficacy, promote drug resistance, and may lead to serious toxicity or fatal outcomes. The increasing sophistication of counterfeiting techniques has made visual inspection insufficient, emphasizing the critical role of rapid and reliable analytical methods in drug authentication. In this context, IR spectroscopy has emerged as a cost-effective and versatile screening tool that provides chemical fingerprinting for the identification and classification of counterfeit pharmaceuticals.

This review focuses on studies published between 2015 and 2025 that utilize IR spectroscopy—including FTIR, ATR-FTIR, and NIR techniques—for the detection of counterfeit or substandard drugs. By summarizing current applications, advantages, and limitations, this work highlights the value of IR-based approaches as supportive tools for pharmaceutical quality control, regulatory enforcement, and public health protection. Articles were evaluated by entering the keywords "Falsified medicines," "Counterfeit medicine," "Falsified drug," "Counterfeit drug," "FTIR", "NIR", "Infrared spectroscopy," and "ATR-FTIR" into various search engines (Google Scholar, PubMed, Web of Science). The inclusion and exclusion criteria for the review are shown in Table 1.

Table 1: Inclusion and Exclusion Criteria for Article Selection

Inclusion criteria
The article must have been published between January 1, 2015, and November 30, 2025.
The article or data must be publicly available.
The article contains data on the prevalence of substandard and counterfeit medical products.
IR spectroscopy was used during the analysis in the article
Exclusion criteria
The article only reports on the physical examination of the product and/or packaging and does not perform content analysis.
The article reports results that have been previously reported elsewhere.
The language of the article is not English.
The article examines the active ingredients in herbal products, not pharmaceuticals.

1. Infrared Spectroscopy

Infrared (IR) spectroscopy is a powerful analytical technique widely used for the structural characterization, purity assessment, and identification of a compound. The results obtained from this technique, known as IR spectra, are based on the vibrational transitions of molecules and are therefore often classified as "vibrational spectroscopy." (Silverstein et al., 2005). The infrared region begins after the visible region (approximately 700 nm) and extends into the microwave region. The energies of the waves used in the infrared spectrum are low. This energy is insufficient to cause electronic transitions and therefore induces vibrational transitions within molecules (bond expansion and contraction) or bending (angle change between three atoms) (Pavia et al., 1998). The infrared region spans approximately from 700 nm to 1 mm, covering NIR, MIR and FIR sub-regions. The infrared region is divided into three sub-regions: Near-IR

(NIR), mid-IR (MIR), and far-IR (FIR) (Griffiths, 1983:297; Workman and Weyer, 2007). Information about these regions is provided in Table 2.

The basic principle is based on measuring the amount of infrared radiation absorbed by or reflected from the sample. In infrared (IR) spectroscopy, analytical measurements are typically performed in the mid-infrared region covering the wavelength range of 400-4000 cm-1 (2500-25000 nm), because most molecules exhibit characteristic spectral bands in this range (Skoog et al., 1998; Stuart, 2004). The IR spectrum consists of the functional group region (4000–1300 cm^{-1}) and the fingerprint region (1300–400 cm^{-1}) (Gupta at al., 2023; Silverstein et al., 2005). The fingerprint region, on the other hand, arises as a result of complex vibration modes unique to the entire molecule. Just like a human fingerprint, this region is unique for each molecule and is extremely useful for the comparative verification of pure substances (Skoog et al., 1998). The position and intensity of the vibration bands observed in an IR spectrum are directly related to factors such as the molecule's dynamic motion, the bonding geometry of the atoms, and the immediate chemical environment (Coates, 2000:10815). Therefore, characteristic absorption bands are created in narrow ranges of wavenumbers and characteristic functional groups. The fundamental principle is that similar atom groups absorb similar energies and therefore form bands at approximately the same frequencies (Silverstein and Bassler, 1962:546). These bands are analyzed using correlation tables (Table 3), which play a key role in interpreting the spectra (Silverstein et al., 2005). Figure 1 schematically summarizes infrared (IR) spectroscopy data, which plays a critical role in determining the structure of organic compounds. The graph displays the absorption bands of characteristic functional groups (O-H, N-H, C=O, C≡N, C≡C, aromatic rings, etc.) as color-coded blocks based on their wavenumber (cm-1). In addition to distinctive signals such as broad hydroxyl (O-H) and sharp carbonyl (C=O) stretching bands, the vibration modes (stretching and bending) of the groups are also indicated.

Table 2: Spectral Characteristics and Application Areas of NIR, MIR, and FIR Regions

Parameters	Near-infrared (NIR)	Mid-infrared (MIR)	Far-infrared (FIR)
Wavenumber Range (cm^{-1})	12,800 - 4,000	4000-400	400-10
Wavelength Range (nm)	700-2,500	2,500-25,000	25,000-1,000,000
Energy (kJ/mol)	171-47.9	4.78-47.9	0.12-4.78
Interaction Type	Harmonic (overtone) and combined vibrations.	Vibrations of stretching and bending chemical bonds	Low-Energy Vibrations
Application Areas	• Moisture, fat, and protein analysis in food • Raw material identification in the pharmaceutical industry • Grain quality determination in agriculture	• Functional group identification (C=O, O-H, N-H, etc.) • Compound identification (organic, inorganic, polymer) • This is the main field of application for classical FTIR spectroscopy	• Characterization of inorganic compounds • Semiconductor analysis • Astronomy (space dust and gas clouds)

Table 3: FTIR Fingerprints of Common Functional Groups with Bond Types, Wavenumbers, and Band Features

Functional Group	Functional Group	Bond Type	Wavenumber (cm^{-1})	Color	Band Feature	Description
Hydrocarbons	Alkane (C–H)	sp^3 C–H stretching	2850–2960	Orange	Medium–weak	Saturated hydrocarbons
	Alkene (C=C)	C=C stretching	1620–1680	Orange	Medium	Unsaturated bonds
	Alkene (C–H)	sp^2 C–H stretching	3010–3100	Orange	Weak	Alkenic hydrogens
	Alkyne (C≡C)	C≡C stretching	2100–2260	Orange	Weak	Terminal/internal alkyne differences
	Alkyne (≡C–H)	sp C–H stretching	3300	Orange	Sharp, strong	Terminal alkynes
	Aromatic (C=C)	Aromatic C=C stretching	1450–1600	Orange	Medium	Terminal alkynes
Oxygen-containing Groups	Alcohol (O–H)	O–H stretching	3200–3600	Blue	Broad, strong	Sensitive to hydrogen bonding
	Phenol (O–H)	O–H stretching	3500–3600	Blue	Medium–strong	Generally similar to alcohol O–H
	Carboxylic acid (O–H)	O–H stretching	2500–3300	Blue	Very broad, strong	Interacts with C=O
	Carboxylic acid (C=O)	C=O stretching	1700–1725	Blue	Strong	Distinct carbonyl band
	Aldehyde (C=O)	C=O stretching	1720–1740	Blue	Strong	Characteristic carbonyl band
	Aldehyde (C–H)	–CHO stretching	2720–2820	Blue	Weak–medium	Specific to aldehydes
	Ketone (C=O)	C=O stretching	1715–1730	Blue	Strong	Typical carbonyl band
	Ester (C=O)	C=O stretching	1735–1750	Blue	Strong	Slightly higher than acids
	Ester (C–O)	C–O stretching	1050–1300	Blue	Strong	Two different C–O stretches possible
	Ether (C–O)	C–O stretching	1050–1150	Blue	Medium	May overlap with alcohol or ester
Nitrogen-containing Groups	Amide (C=O)	C=O stretching	1630–1690	Green	Strong	Varies with N–H in amide
	Amide (N–H)	N–H stretching	3200–3500	Green	Medium	One or two bands possible
	Amine (N–H)	N–H stretching	3300–3500	Green	Medium	Two bands in primary, one in secondary
	Nitro (NO_2)	ν_as, ν_s stretching*	1515 & 1345	Green	Strong	Two characteristic bands
Triple Bonds	Nitrile (C≡N)	C≡N stretching	2210–2260	Red	Medium–strong	Sharp, narrow band
	Alkyne (C≡C)	C≡C stretching	2100–2260	Red	Weak	Alkene difference effects
Halogenated Compounds	(C–Cl)	C–Cl stretching	600–800	Purple	Strong	Heavy atom effect lowers frequency
	(C–Br)	C–Br stretching	500–600	Purple	Strong	Sharp low-frequency band

*ν_as = asymmetric stretching vibration; ν_s = symmetric stretching vibration

Source: Silverstein et al., 2005

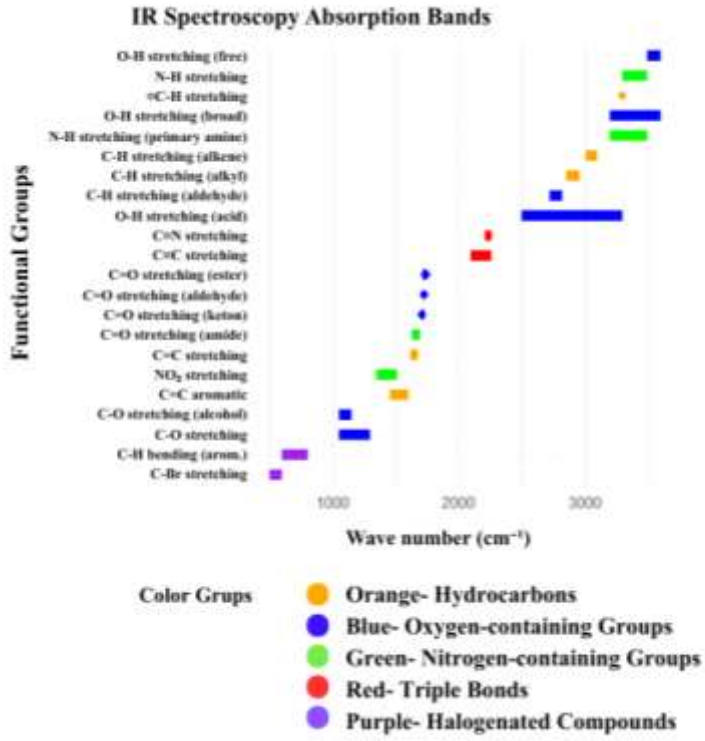


Figure 1: Schematic representation of characteristic infrared spectroscopy absorption bands for major organic functional groups. The chart displays the characteristic absorption regions of major functional groups as color-coded blocks, serving as a qualitative guide for spectrum interpretation

In IR spectroscopy, wave number (cm^{-1}) is generally used instead of wavelength (nm) to avoid large numbers and make spectra easier to interpret. In IR spectra, the x-axis usually shows wavenumbers cm^{-1} while wavelength (nm or μm), Electron Volts (eV), kilo-Electron Volts (keV), and Terahertz (THz) can be given. The y-axis can show absorbance, intensity, transmittance (0-1 or %), and reflectance (0-1 or %) (Larkin, 2017). However, the most preferred parameters in the obtained spectra are expressed as wave number (cm^{-1}) on the x-axis and % transmittance on the y-axis.

There are two types of imaging methods in infrared spectroscopy. One of these is the transmission mode. With this method, the sample is prepared in a very thin form, illuminated with IR light from end to end, and the absorption of light passing through the sample is measured. This method, which generally uses KBr disks, has the drawbacks of difficult disk preparation and moisture absorption. In ATR mode, IR light undergoes total

internal reflection inside the crystal, creating an evanescent wave that interacts with the sample surface. Combined with an ATR head, this method uses a high refractive index crystal to achieve total internal reflection and measures “evanescent wave” waves that penetrate the sample by a few micrometers. The advantages of this method include no sample preparation and suitability for drug forms such as thick tablets (Smith, 2011). The methods are shown in Figure 2. Among these, FTIR imaging in ATR mode is particularly advantageous because it requires minimal sample preparation and is independent of sample thickness.

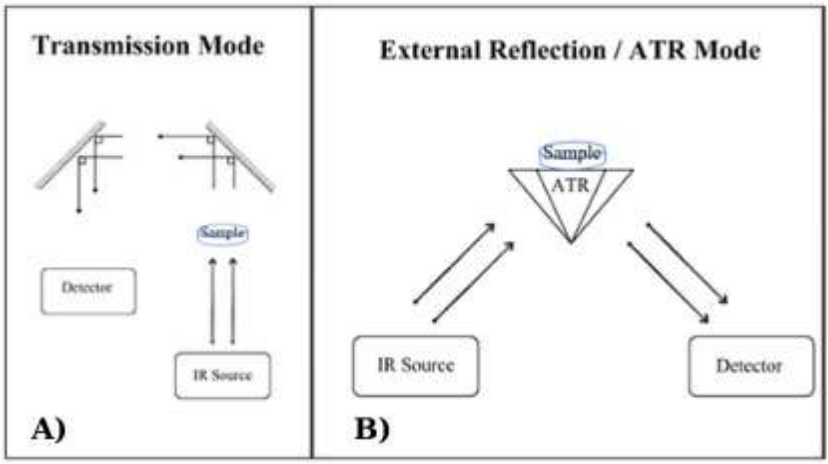
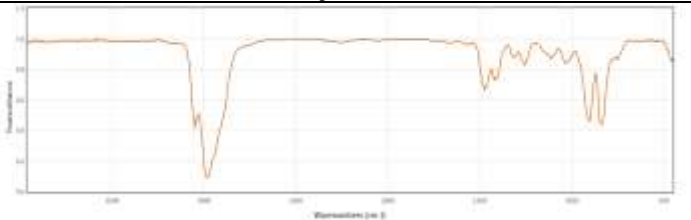
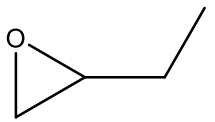
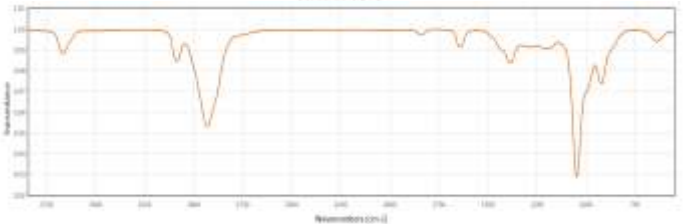
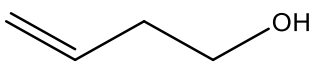
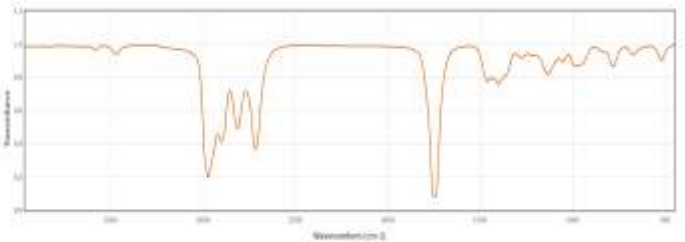
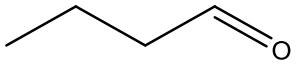


Figure 2: Comparison of FTIR measurement modes: (A) Transmission Mode, where IR radiation passes through the sample and reaches the detector after being absorbed, and (B) External Reflection / ATR Mode, where the IR beam reflects at the interface of an ATR crystal, producing an evanescent wave that interacts with the sample surface.

Even when reference spectra are available, compounds cannot always be definitively identified based solely on their IR spectrum. IR spectroscopy can distinguish certain positional or functional isomers, although some structural isomers may require complementary techniques (Lemmon, et al., 2011). In IR spectroscopy, results are obtained based on a molecule's atomic mass, bond strength, and intramolecular and intermolecular vibrations. Isomeric molecules can also be easily distinguished in IR spectroscopy (Table 4). IR spectra obtained from the NIST Chemistry WebBook were used to understand these differences.

Table 4: Structural isomers of C_4H_8O (1,2-epoxybutane, 3-buten-1-ol, and butanal) along with their IR spectra and molecular representations

Closed formula	IUPAC name	IR spectrum	Molecular formula
C_4H_8O	1,2-Epoxybutane		
C_4H_8O	3-Buten-1-ol		
C_4H_8O	Butanal		

Since factors such as atomic masses, bond strengths, and intramolecular/intermolecular vibrations directly affect a molecule's IR spectrum, each isomer exhibits a unique vibrational fingerprint, allowing them to be distinguished from one another (Silverstein and& Bassler, 1962). IR spectroscopy can reveal the functional groups within a molecule, but it cannot independently determine the bonding order or the full structure (Colthup, 2012). Therefore, structural analyses must be supported by techniques such as Nuclear Magnetic Resonance (NMR) or Mass Spectrometry (MS), UV-Vis Spectroscopy, and Raman Spectroscopy. Furthermore, since symmetric molecules such as N_2 or O_2 are not IR-active, the spectra of these compounds appear as a flat line without any absorption bands.

Among the most important advantages of IR spectroscopy are that the sample can be examined in most physical forms (solid, liquid, gas), it is non-destructive, it does not require detailed sample preparation, it is fast and inexpensive, and it allows analysis with very small amounts of sample. The advantages and disadvantages of IR spectroscopy are listed below (Gullifa et al., 2023:1214825; Huck, 2015:477; Johnson et al., 2023:3215; Türker-Kaya and Huck, 2017:168).

Advantages of IR spectroscopy

- **High Sensitivity and Low Concentration Measurement:** It has the ability to measure very low concentrations (such as 0.005 mg/ml) (Mallah et al., 2015).
- **Fast Analysis:** Data acquisition time can be significantly shorter than one minute and provides rapid results.
- **Simple and Convenient:** As a spectroscopic method, it is generally simpler and less expensive than chromatographic methods.
- **Non-Destructive Analysis:** Does not damage the sample.
- **Simultaneous Multi-Component Analysis:** Allows simultaneous determination of different components (such as active pharmaceutical ingredient (API) and excipient) in the same sample with a single measurement.
- **Monitoring Chemical Interactions:** Can monitor chemical changes (such as polymorphism, hydration, degradation products) by directly measuring molecular bond vibrations.
- **Minimal Preparation:** Solid and liquid samples can be measured without complex preparations, especially with ATR (Attenuated Total Reflection) mode. Minimal pretreatment is sufficient for solid samples.
- **Multifaceted Detection:** Enables monitoring of various physicochemical changes through rich spectral information

- **Enhanced Capabilities with Data Analysis:** Provides reliable degradation indicators when combined with multivariate data analysis (such as PCA/HCA).

Disadvantages of IR spectroscopy

- **Limited Chemical Information:** It cannot provide all information about the chemical composition of the sample and cannot always show characteristic chemical markers.
- **Band Overlap:** The overlap of bands in the IR spectrum can sometimes make it difficult to detect small changes.
- **Structural Identification Limitations:** It cannot determine the exact structure of degradation products or new components; it mainly shows component changes. Therefore, additional methods such as purification and mass spectrometry may be required.
- **Reference Requirement:** Reference templates/standards are required for the identification of newly formed components.
- **Chemical Sensitivity:** Chemical sensitivity may be lower compared to some chromatographic methods.
- **ATR-Specific Disadvantages:** In the ATR sampling method, the dependence of spectrum intensities on wavelength and the occurrence of shifts may affect the interpretation of spectra.

FTIR was initially a spectroscopic technique used to identify the functional groups of chemical compounds, but when combined with computer technology based on advanced software, it has a wide range of applications (Custers et al., 2016:145; Gullifa et al., 2023:1214825). When examining the uses of FTIR in the field of pharmacy,

- Identification of characteristic bands specific to the functional groups of pharmaceutical active ingredients in tablet, capsule, or powder form (Carruthers et al., 2022:145; Handzo et al., 2022)
- Detecting possible interactions between APIs and excipients (Canbay et al., 2019:102; Lupu et al., 2018)
- Distinguishing between different crystal forms (Blanco and Villar, 2000:2311; Roy et al., 2013:976)
- Quality control analyses in preparations containing multiple APIs (Hisada et al., 2020:155; Tao et al., 2024:38)
- Demonstrating API distribution in carrier systems (Carruthers et al., 2022:1862)
- Detection of counterfeit or substandard pharmaceutical products.

2. Counterfeit Drugs

Counterfeit drugs are typically products that contain no active ingredient, contain active ingredients in incorrect doses, contain contaminated components, or are formulated with completely different

chemical contents. As such, they reduce treatment effectiveness and can lead to the development of drug resistance, toxicity, and even fatal outcomes (Blackstone et al., 2014:216). The terms “fake” and “counterfeit” drugs actually encompass substandard, fake, mislabeled, counterfeit, and imitation medical products. These so-called fake drugs also include those that infringe on patents or other intellectual property rights. The World Health Organization estimated in 2017 that one in every ten drugs in low- and middle-income countries failed quality control tests, indicating that the product was substandard or counterfeit, and noted that this rate was even higher for antimalarial drugs, antibiotics, and products used to treat chronic diseases (World Healt Organization, 2024). Both substandard and counterfeit medical products put patients' health at risk. These products accelerate the spread of drug-resistant infections, turning treatable diseases into fatal ones, while also posing a serious economic and health threat that depletes the resources of both families and health systems, undermines trust, and leaves communities vulnerable. Counterfeit drug manufacturers exploit weaknesses in the legal supply chain to reach large populations through online pharmacies, unregulated sales platforms, and illegal distribution networks. Technological advances have made counterfeiting methods more sophisticated; advanced printing techniques, hologram imitations, and packaging similarities have led to the emergence of counterfeit drugs that are difficult to distinguish from the original products (Dégardin et al., 2014:167; World Health Organization, 2017). Therefore, the role of analytical techniques in detecting counterfeiting is extremely important. Instrumental approaches such as FTIR and Raman spectroscopy, LC-MS/MS, GC-MS, and NMR enable the verification of authenticity by extracting the chemical fingerprints of drugs and are widely used by regulatory agencies. Considering all these factors together, it is clear that drug counterfeiting is a complex problem with technical, social, economic, and ethical dimensions, requiring a sustainable public health approach.

3. Analysis of Previous Research on Counterfeit Drug Detection Using IR Techniques

In recent years, numerous studies have been conducted on the detection of counterfeit and substandard drugs using infrared spectroscopy and chemometric models. Within the scope of these studies, sildenafil, tadalafil, acetaminophen, amoxicillin, paracetamol, amlodipine, lisinopril, mRNA COVID-19 vaccines, artemether-lumefantrine, modafinil, and various antibiotics were examined in different formulations such as tablets, capsules, polypills (3D-printed tablets), injection vials, and vaccine vials. Various chemometric analyses (PCA, PLS, PLS-DA, SIMCA, DD-SIMCA, k-NN, SVM, LDA, Gradient Boosting, and Convolutional Neural Network) have been used in combination with infrared spectroscopy. The results obtained in the studies vary depending on the device and algorithm used;

some studies report 100% accuracy and specificity, while others show limited performance, especially in the detection of low doses or substandard products. Overall, these studies demonstrate that portable spectroscopy and chemometric modeling serve as a fast, non-destructive, and effective preliminary screening tool for field applications; however, the reliable detection of substandard products still faces technical limitations.

Maffoli and Anyakora (2025) investigated the performance of a portable, AI-powered handheld NIR spectrometer in detecting counterfeit drugs compared to HPLC. For this study, they analyzed 246 drugs, including analgesics, antimalarials, antibiotics, and antihypertensives, using artificial intelligence. The study defined counterfeit drugs as having active ingredient content outside the 90-110% range. After analyzing the entire API content in the tablets, they compared the actual drug spectrum in the device's library and classified them as match/non-match. These APIs were then measured in the laboratory using HPLC. The study found that the rate of counterfeit or inadequate drugs was 25% in HPLC analyses, while this rate was 22% in classifications performed using a portable NIR spectrophotometer and artificial intelligence. In their study, they reported sensitivity of 37% and specificity of 47% for analgesics. The spectrometer failed to identify any falsified samples (0% sensitivity) but correctly recognized all genuine samples (100% specificity). The method, developed with an NIR spectrophotometer and artificial intelligence, reported a positive predictive value of 13% and a negative predictive value of 71%. The study concluded that the portable NIR spectrometer in its current form is not sufficiently reliable for "real-world applications, including retail pharmacy supply chains and field quality control." The researchers noted that their study was conducted with a limited sample size and encountered limitations related to the hardware and software of the machine learning algorithm used. They recommended that further research be conducted to examine the accuracy of such studies (Maffioli & Anyakora, 2025).

In 2025, Rais et al. used Near-Infrared (NIR) spectroscopy to measure the sildenafil content in counterfeit tablets. In their study, they analyzed 30 original sildenafil tablets and 122 counterfeit sildenafil tablets from 3 different batches. They compared machine learning models developed to measure the sildenafil content in counterfeit tablets with chromatographic methods. They were able to successfully distinguish between counterfeit and genuine tablets with 99% accuracy and determine the amount of sildenafil in the tablets with over 98% accuracy. They reported that Principal Component Analysis (PCA) and Euclidean distance methods achieved 100% accuracy. The total study time being less than 30 seconds makes this method stand out for field applications. The method successfully identified tablets containing incorrect active ingredients, low API levels, or no active ingredient at all. They reported that the developed method is non-destructive and can be performed in the field, with

measurement possible even when the tablets were analyzed directly through the blister packaging. They also report that counterfeit testing can be performed in the field using the blister packs of the products (Rais et al., 2025:116940)

A study was conducted by Kakumba et al. in 2024 to identify counterfeiting and quality control in tadalafil-containing products sold in the Congolese market. They aimed to develop and validate a low-cost, rapid, solvent-free, and complex preparation-free method for counterfeiting and quality control of these products using portable NIR spectroscopy. They preprocessed the collected NIR spectra to reduce variability and improve chemical spectral properties. They used the PCA chemometric method for grouping in spectral analysis. They also used the Partial Least Squares Regression (PLS) method to quantify the amount of active ingredient in tablets. They used the Data-Driven Soft Independent Modeling of Class Analogy (DD-SIMCA) model to classify and validate products by brand. In the study, placebo samples were analyzed with 100% accuracy. The developed method reportedly identified tablets from a specific brand with high accuracy and could distinguish counterfeit/blank samples containing placebo and excipients. They have shown that portable NIR spectroscopy can be an effective, rapid and convenient tool for the detection of counterfeit and inferior drugs in the field, compared to traditional laboratory methods. (Mankulu Kakumba et al., 2024:105)

In a 2022 study by Awotunde et al., researchers developed a model that combined near-infrared (NIR) spectroscopy and machine learning to detect counterfeit or substandard formulations in acetaminophen-containing products. The model was trained using NIR spectra obtained from binary mixtures prepared using only acetaminophen and two different diluents (lactose and ascorbic acid). It was then tested on much more complex ternary mixtures, genuine prescription acetaminophen tablets, and adulterant-containing counterfeit formulations. After preprocessing the acquired spectra, they developed six different models, including PCA, Support-Vector Machine (SVM), and Convolutional Neural Network (CNN), and their combination. They reported that these models performed well on samples similar to the training set but were less reliable when applied to more complex formulations. However, they reported that, by leveraging the strengths of the six models, they predicted correct classification in 93% of cases using a simple voting algorithm. Researchers suggest that different data analysis models or different weighting of individual models in the algorithm could further improve overall performance (Awotunde et al., 2022:12586).

In 2022, Hattori et al. conducted a study to detect counterfeit and substandard amoxicillin capsules. They examined the applicability of portable spectroscopy in counterfeiting and quality control studies by taking spectra with different NIR devices. For this study, 450 FT NIR spectra were

used for the training dataset, while 225 WD NIR measurements were taken for the test dataset. They reported that 8 of a total of 13 different algorithms were selected and tested. In the study, after spectral collection for verification, amoxicillin capsules were removed from their blister packs and the contents were analyzed using an HPLC system. They applied grid search and cross-validation optimization methods to the developed models, and reported that the best results were obtained from k-nearest neighbors (k-NN), extra trees, and gradient boosting classifier models, which were the most suitable for substandard and counterfeit amoxicillin capsules. In this study, they reported that the models provided reliable classification even when measurements were made using hetero-device spectrophotometry. As a result of the study, they reported that combining portable NIR spectroscopy with chemometric models was successful in the rapid and non-destructive detection of counterfeit and low-quality amoxicillin capsules (Hattori et al., 2022:1261).

Assi et al. developed a portable, non-destructive spectroscopic method using NIR (Near-Infrared) spectroscopy to detect counterfeit mRNA vaccines, which were widely used during the COVID-19 pandemic in 2022. In the study, they examined 405 mRNA-based COVID-19 vaccine vials, and the vaccines were measured without any processing after being removed from the glass vials. Multiplicative scatter correction followed by first derivative (MSC-D1) preprocessing was applied to the raw spectral data. They then performed multivariate analysis on the spectral data using PCA to make distinctions. They reported that mRNA-specific bands were dominant in the NIR spectra, while bands belonging to excipient or protein components were not very dominant. Study results reported that portable NIR + chemometric PCA analysis is applicable for rapid, nondestructive, and practical investigation of mRNA-based COVID-19 vaccines (Assiet al., 2022).

Hattori et al. conducted a 2021 study to demonstrate the potential of near-infrared spectroscopy for widespread field detection of counterfeit drugs. For this study, they developed device-independent results using spectra from NIR devices purchased from different manufacturers. They reported that the model they developed demonstrated high accuracy without requiring retraining when a new device was added. After collecting and preprocessing the spectra, they reported using the SVM chemometric algorithm. They reported that the data obtained from measurements in the Wavelength-Dispersive NIR region (780–2500 nm) failed to accurately distinguish substandard amoxicillin capsules due to its low quantitative performance, while the Principal Component (PC) score-based Support Vector Machine model completely identified counterfeit products without any false positives. They also tested this Principal Component (PC) score-based Support Vector Machine model with both a high-performance FT-NIR

and a low-cost WD-NIR device, reporting success in both (Hattori et al., 2021:1251).

In their 2021 study, Caillet et al. examined six portable screening devices (two NIR, two Raman, one MIR, and an analytical paper test device (PAD)) used to detect counterfeit or substandard drugs. Although the primary purpose of this study was to examine the accuracy and expiration dates of the devices used, drugs were included in this review because they were examined for counterfeit or genuine. After preprocessing the measurements, PCA, SVM, and Random Forest chemometric models were created, identifying the results as pass/fail. However, they reported that some devices, by creating an "uncertain" state, did not identify drugs. They reported that the PAD method yielded a high number of erroneous results, while the other five devices were successful in providing "pass/fail" results (Caillet et al., 2021).

In a 2021 study conducted by Lemos and colleagues, they investigated anabolic steroids. They used the attenuated total reflection Fourier-transform infrared microspectroscopy (μATR-FTIR) mapping method to allow measurements directly from the tablet surface without pulverizing it. The study reported that a total of 30 tablet samples seized for counterfeiting or similar reasons were examined. After IR spectra were obtained for analysis, the characteristic absorption bands of each active ingredient were selected and the distribution map method was used. The presence of active ingredients was determined by integrating the identified characteristic bands to produce chemical distribution maps. This allowed information to be collected about the presence and distribution of active ingredients within the tablet. The study reported that 26 of the 30 tablets used in the study contained active ingredients, but the method used for this verification was not disclosed. Five of these 30 samples were classified as counterfeit solely by μATR-FTIR analysis, meaning that μATR-FTIR alone was effective in detecting some counterfeit products compared to other routine tests. The μATR-FTIR mapping method is effective for preliminary discrimination of counterfeit tablets and additionally reveals both the presence and spatial distribution of APIs (Lemos et al., 2021:837).

In a study conducted by Mittal and colleagues (2021), they examined whether ATR-based MIR measurements could easily detect counterfeit antibiotic products using chemometric modeling. The study examined the infrared fingerprint bands obtained from ATR-MIR measurements of powdered products to determine product differentiation. In this study, 57 pharmaceutical products belonging to 27 different antibiotic active ingredients were examined. PCA and Partial Least Squares Discriminant Analysis (PLS-DA) models were applied to classify the spectral data obtained from the IR spectra collected in the study, which examined a total of 481 samples. The results of the study indicated that because each antibiotic brand has a unique spectral fingerprint profile, it can identify drugs

without having to individually identify the underlying chemical components. The overall classification accuracy of the developed PLS-DA model was reported to be 87.33%, and the predictive power of this model for a specific antibiotic was reported to exceed 90%. Furthermore, when the model was applied to counterfeit drugs, these products were correctly identified as "unclassified," meaning they did not belong to any class. In this context, it has been reported that the developed method can be applied as a cost-effective, fast and reliable approach to antibiotic classification and detection of counterfeiting, requiring minimum sample preparation (Mittal et al., 2021:119710).

In a 2021 study conducted in the United States, Zambrzyck et al. investigated the effectiveness of 12 different portable devices (three near-infrared (NIR) spectrometers, two Raman spectrometers, one mid-IR spectrometer, one portable liquid chromatograph (LC), one microfluidic system, one mass spectrometer, one thin-layer chromatography (TLC) kit, a disposable colorimetric assay, and a disposable rapid diagnostic test (RDT)) in detecting counterfeit or substandard drugs. In this study, which used tablets containing varying amounts of API obtained from the market and those used in the laboratory, the devices were evaluated both qualitatively and quantitatively. They identified a range of 90–110% as correct for drug classification. All devices examined reported a high accuracy rate of classification of both false API and non-API tablets, greater than 90%. They reported that the difference between the devices was most pronounced in tablets with API content between 50–80%. In this range, the success rate has been reported to be 0% for some devices, while it has been reported to be 100% for others. They reported that NIR and Raman spectrometers exhibited low sensitivity (5-50%) for substandard samples containing 80% or 50% API. The authors concluded that portable devices are promising in detecting counterfeit drugs, but emphasized that reliably detecting substandard products remains challenging (Zambrzycki et al., 2021).

Yabré et al.published a paper in 2020 in which they developed an analytical method for quality control of Artemether and lumefantrinetablets, an antimalarial drug. They developed a portable near-infrared spectrometry methodto identify trends, similarities, and differences among pharmaceutical samples.In the method they developed, 270 spectra were obtained, and these spectra were subjected to PCA and DD-SIMCA modeling. This modeling aimed to distinguish counterfeit or suspicioustablets. The researchers reported that despite the limited spectral range (approximately 900–1700 nm) and low resolution of the portable NIR device used, the developed PCA + DD-SIMCA model successfully distinguished counterfeit tablets that did not contain the API. Furthermore, the method ensured the correct classification of specific brand tablets by identifying the characteristic spectral signatures of the commercial brands used as references.The method also identified the characteristic spectral signatures of the commercial brands

used as references, ensuring the correct classification of specific brand tablets. Furthermore, the method ensured the accurate classification of specific brand tablets by identifying the characteristic spectral signatures of the referenced commercial brands. According to the modeling results, the combination of PCA and DD-SIMCAachieved 100% sensitivity and 100% specificityfor the samples examined in the study, demonstrating that portable NIR spectroscopycan be a powerful screening tool for detecting pharmaceutical counterfeiting. In the second phase of the study, samples suspected of being counterfeit were quantitatively verified using the developed green RP-HPLC method, confirming that they did not contain API (Yabré et al., 2020: 3397).

In their 2020 study, Assi et al. investigated the possibility of detecting counterfeit drugs in antibiotic tablets by combining portable NIR spectroscopy and chemometric methods. The study examined 23 different antibiotic products, including six different active ingredients (amoxicillin + clavulanic acid, azithromycin, ciprofloxacin, doxycycline, and ofloxacin) in combination. Direct spectra were taken from these tablets "non-destructively" without any pretreatment such as washing, melting, or crushing. After the acquired spectra were preprocessed, they applied PCA and Soft Independent Modeling of Class Analogy (SIMCA) chemometric models. The PCA model distinguished the NIR spectra of different antibiotics, while SIMCA provided more accurate classification except for ciprofloxacin. They reported that SIMCA provided more accurate classification than PCA for all antibiotics except ciprofloxacin, which shares many overlapping excipients. The combination of NIRS with PCA and SIMCA has proven effective in distinguishing branded drugs from generics and tracing the manufacturing sources of drugs (Assi et al., 2021:434).

In a 2020 study, Sroka et al. used a portable consumer-grade Near-Infrared Spectroscopy (NIR) device to determine whether sildenafil tablets were counterfeit or imitation. They reported that while capturing the spectra of the samples, measurements were taken on both sides of the tablets without damaging them, and a total of 78 IR spectral data were collected. The collected spectral data was classified using k-NN, a machine learning algorithm, after pre-processing (smoothing and normalization). The measurements revealed spectral differences between the original and counterfeit products, possibly due to differences in excipients used or different manufacturing steps. Using the K-NN method combined with IR spectroscopy, they classified the tablets as genuine and counterfeit. They reported that the model they developed correctly classified all tablets, meaning they were able to detect counterfeit sildenafil. As a result, they reported that the portable, user-friendly, low-cost and practical miniaturized NIR device can be used as a verification tool in non-laboratory environments (customs, distribution channels, supply chain, counterfeiting inspection) (Sroka et al., 2020:1).

Assi et al. (2020) examined products containing the active ingredient modafinil in their study. Eight products obtained online were analyzed in the study. These products were classified into groups: the group where the active ingredient was diluted with lactose, Samples included diluted modafinil–lactose mixtures, modafinil formulated with various excipients, and tablets adulterated with additional excipients. The researchers developed analytical models using Fourier transform infrared (FTIR), near-infrared (NIR), and Raman spectroscopy, along with partial least squares regression (PLSR), to determine the amount of modafinil in generic drugs. The developed models reported correlation coefficient (R^2) values above 0.94. Furthermore, compared to NIR, tablet-based models obtained using FTIR and Raman spectroscopy were reported to provide higher accuracy than powder mixture-based models (Assi et al., 2020:35).

In a 2020 study, Eady et al. investigated whether counterfeiting could be detected using infrared spectroscopy (NIR) in the formulation of medroxyprogesterone acetate injection, an injectable suspension. Products from three different suppliers were examined, totaling 227 ampoule formulations. Two types of spectroscopy devices were used in the study: a hand-held NIR spectrometer and a laboratory UV–VIS–NIR spectrometer. The developed model was validated with additional positive and negative controls to verify the results. After applying a PCA model to the obtained data, they performed a classification based on Mahalanobis distance (M-dist.). They examined the practicality of the developed model in the simple, rapid, and field-compatible pass/fail detection of counterfeit or adulterated drugs. Paired with open-source software, the handheld device reported 100% product discrimination accuracy. They reported that three brands of injectable DMPA could be distinguished from each other with a handheld spectrometer and that this method could be developed and recommended as a practical pre-screening/quality surveillance tool, especially in countries with limited resources, poor infrastructure, and difficult field conditions (Eady et al., 2021:119917).

In their 2020 study, Trenfield and colleagues developed a method for determining the amount of amlodipine and lisinopril active ingredients in polyprintlets, which are 3D-printed drug forms. The study used laboratory-grade NIR and Raman spectroscopy, processing spectral data using PCA and PLS chemometric models, and measuring the amount of active ingredient. They reported that both devices successfully detected and estimated the amount of active ingredient with the developed method. While no counterfeit or similar drugs were found in this study, it was included because it was believed that the developed model would be able to identify potentially counterfeit or similar drugs (Trenfield et al., 2020:119066).

In a study conducted in 2019, Ciza et al. examined counterfeit and adulterated pharmaceutical products using both NIR and Raman spectroscopy with portable/handheld vibrational spectroscopy devices. In the

study, one of the NIR devices used was a low-cost dispersive handheld NIR, while the other was a laboratory-type FT NIR benchtop device. They used three different chemometric approaches in their study, which involved a total of 58 samples. These methods were the Hierarchical Clustering Algorithm (HCA), DD-SIMCA, and Hit Quality Index (HQI). The drug samples they obtained contained a combination of Artemether-Lumefantrine, paracetamol, and ibuprofen. As a result of their work, they reported that NIR spectroscopy showed better performance in distinguishing counterfeit tablets compared to the Raman system. This study reported that portable NIR devices are quite powerful in counterfeit drug screening. Among the developed chemometric models, they reported that DD-SIMCA showed 100% sensitivity and 100% specificity (Ciza et al., 2019:469).

Wang et al. (2019) investigated the feasibility of low-cost portable optical spectrophotometers for tablets containing dual active ingredients in antimalarial, antiretroviral, and antibiotic drugs. They used four inexpensive NIR spectrophotometers, one MIR spectrophotometer, and a Raman spectrophotometer. Unlike other studies, this study used simulated tablets containing the active ingredient and three commonly used excipients (microcrystalline cellulose, starch, and anhydrous lactose) instead of the actual counterfeit drug. They used different chemometric approaches in the study. They estimated the amount of active ingredient using a Univariate Regression model, followed by PLS regression and PCR (Principal Component Regression). They reported that the best method for estimating the active ingredient was a DLP-NIR device. They also reported that the developed methods predicted the true value with error rates below 6%. They reported that the content of the active ingredients efavirenz and isoniazid was quantitatively estimated with an error of approximately 10%. The study concluded that very low-cost devices were insufficient, but that spectroscopic methods, particularly NIR spectroscopy, could be used as a useful and low-cost screening tool not only for counterfeit but also for screening substandard drugs with sufficient quantitative or semi-quantitative accuracy (Wang et al., 2020:323).

Nurul Syameem Binti Zainal (2019) examined counterfeit paracetamol products in her study. The study first evaluated the product packaging, hologram, and blister analysis; then, the chemical profiles of the products were determined using the ATR-FTIR method, and quantitative analyses were performed using HPLC-DAD. According to the analysis results, although counterfeiting was detected in the packaging of one product, no problems were found in the active ingredient inside. In addition, it was reported that the API content in all products was in the range of 95–105% (Zainal, 2019).

Attori and colleagues (2018) conducted a study using NIR spectroscopy to rapidly detect whether oral solid dosage form pharmaceutical products are counterfeit. In the study, NIR spectra were

obtained from tablets belonging to different commercial brands, and the data obtained were analyzed using chemometric classification methods. This approach enabled a rapid, non-destructive, and reliable distinction between counterfeit and original tablets. The researchers emphasized that NIR spectroscopy demonstrated high accuracy and sensitivity in counterfeit detection and that it is particularly applicable in field or non-laboratory conditions. The study shows that portable NIR devices offer a practical method for rapid identification and separation of counterfeit drugs in pharmaceutical quality control (Hattori et al., 2018:1).

Kahmann et al. (2018) developed an ATR-FTIR-based spectral method to quickly and reliably classify erectile dysfunction drugs as counterfeit or genuine. The study analyzed 300 tadalafil and 177 sildenafil samples, subjected the spectra to preprocessing, and applied chemometric analyses. The developed model achieved over 99% classification accuracy when used on both training and test sets. The study was found to be robust due to its high accuracy rate and non-destructive analysis feature, enabling rapid measurements while preserving the integrity of the samples. However, it was determined that the model's performance depends on the reference data set and device calibration, and that accuracy may vary across different devices or batches. The authors suggested that the method could be particularly useful in field screening and preliminary testing (Kahmann et al., 2018:494).

Ahmad Siti Noratika Binti (2018) developed a method to distinguish commonly used paracetamol products from counterfeit drugs. In the study, PCA and Linear Discriminant Analysis (LDA) were used to determine the differences between samples, achieving over 90% accuracy in detecting counterfeit products. The study enabled the rapid classification of pharmaceutical products by considering both quantitative and qualitative differences and presented a method that can be used outside the laboratory. It was determined that the model is dependent on the reference data set and sample diversity and needs to be recalibrated when different manufacturers and formulations are added. The authors suggest that the method could be used for preliminary screening, particularly in low-resource field conditions (Ahmad, 2018).

Lawson et al. (2018) examined counterfeit or low-quality paracetamol tablets using the ATR-FTIR method and developed a multivariate PLS calibration model to reduce data processing time. The model's R^2 value was determined to be 0.98, and approximately 12% of substandard tablets were detected. The study was found to be robust due to its ability to significantly reduce data processing time and obtain rapid results; samples were preserved thanks to non-destructive analysis. Among the model's limitations were performance variations with different fillers or manufacturer variations and the need for device calibration in field

applications. The authors suggested that the method could be used both in quality control laboratories and in field screening (Lawson et al., 2018).

Guillemain et al. evaluated two different handheld NIR devices in their 2017 study, using a conventional NIR range (cNIR) device and a low-cost short-wavelength NIR range (swNIR) device. They used two portable NIR spectrometers to detect counterfeit pharmaceutical tablets. After measuring 48 different counterfeit and generic samples with both devices, the spectral information of the real drugs was collected. Subsequently, supervised classification models were applied to this data. SVM analysis was performed for swNIR, and LDA for cNIR. All developed models showed over 90% success, while the swNIR + SVM model reportedly achieved 100% accuracy for calibration (learning). They reported that both devices can be used for tablet identification and counterfeit product detection (Guillemain et al., 2017:632).

Neves et al. (2017) developed a method to detect whether anabolic steroids are counterfeit in their study. The study examined 49 original and 47 counterfeit drugs, and the analyses were performed using gas chromatography-mass spectrometry (GC-MS) and FTIR. PLS and PLS-DA methods were applied to the obtained data to investigate the separation of the groups. The developed PLS-DA method successfully classified all samples in the test subset with 100% accuracy (Neves et al., 2017:1288).

Neto and Lisboa (2017) investigated the counterfeit nature of tablets containing Sildenafil and Tadalafil using the FTIR method. The study analyzed genuine sildenafil, genuine tadalafil, their counterfeit versions, and tablets with unknown contents. During the analysis, multi-component spectral matching (deconvolution), objective visual comparison, and correlation tests were used. The results obtained showed that the contribution of excipients and adjuvants to the spectral profile of each sample was effective not only in distinguishing between original and counterfeit products but also in distinguishing between original tablets from different manufacturers. This method enables the simple and rapid identification of counterfeit products and can distinguish between the strong spectral similarities of generic and branded tablets using the same active ingredient; it has also been reported to be able to distinguish between samples produced by the same manufacturer, whether generic or not (Neto and Lisboa, 2017:283).

Dégardin and colleagues (2016) conducted a study on detecting counterfeiting in pharmaceutical tablets using near-infrared (NIR) spectroscopy. In the study, a large tablet database covering tablets from different manufacturers and brands was created, and the NIR spectrum of each tablet was recorded. The spectral data obtained were analyzed using chemometric classification methods to distinguish between counterfeit and original tablets. After first examining the distribution of the data using PCA, the researchers applied k-NN, SVM, and Discriminant Analysis (DA)

classification tools. The study reported 100% accurate classification. The research results demonstrated that NIR spectroscopy can be used as a fast, non-destructive, and reliable screening tool. The use of a large database increased the model's generalizability and ensured high accuracy in detecting counterfeits for different tablet brands and variants, demonstrating the applicability of NIR spectroscopy as a scalable and practical method in pharmaceutical quality control and counterfeit drug detection (Dégardin et al., 2016:89).

Custers et al. (2016) developed a simple and rapid method to distinguish between genuine and counterfeit samples of Tadalafil and Sildenafil in their study. The study used 4 genuine and 32 counterfeit Sildenafil samples, and 5 genuine and 19 counterfeit Tadalafil samples. Using NIR (Near Infrared) and mid-IR spectroscopy results, SIMCA and PLS-DA models were developed. The results obtained showed that the discrimination power of the models was above 99%. Furthermore, it was reported that correct classification between original and counterfeit drugs was possible even when the tablets were in blister form, i.e., without crushing or opening the blister (Custers et al., 2016:378).

Custers et al. (2015) conducted a study on the detection of counterfeit drugs containing Sildenafil and Tadalafil. A total of 209 samples were analyzed in the study, and 68 of them were identified as counterfeit. The analyses were performed using the ATR-FTIR method, and the obtained spectra were examined using PCA, k-NN, Classification and Regression Trees (CART), and SIMCA. According to the results obtained, the SIMCA method showed the best performance. While the k-NN model could not achieve the desired accuracy in distinguishing between genuine and counterfeit samples, the SIMCA method was able to classify them with 100% accuracy. The researchers emphasized that chemometric analysis of ATR-FTIR fingerprints is a valuable tool for distinguishing genuine samples from counterfeit products and classifying counterfeit drugs (Custers et al., 2015:181).

Liu et al. (2015) conducted a study on the quality control of Limonitum, a Chinese medicinal herb. The study examined the raw form, processed form, and counterfeit samples of the herb. Analyses were performed on 18 batches of Limonitum obtained from different regions. Using FTIR fingerprint analysis, the compositional characteristics of Limonitum were evaluated, and it was reported that raw, processed, and counterfeit samples could be distinguished simply and quickly (Liu et al., 2015:909).

DISCUSSION

Literature reviews conducted over the past decade (2015-2025) have demonstrated that various forms of IR spectroscopy are technically feasible for use in first-line screening and field practical investigations against

counterfeit and substandard drugs. This discussion synthesizes the key evidence, addresses the critical challenges, and outlines a pathway for future advancements in the field.

Synthesis of Key Findings and Established Strengths

The collective evidence underscores several irrefutable strengths that make IR spectroscopy a preferred screening tool:

IR spectroscopy captures chemical fingerprints that not only detect the presence of AIPs in counterfeit or substandard products, but also provide information about the chemical composition of the entire dosage form. As evidenced by numerous studies on antibiotics, erectile dysfunction drugs, and painkillers, these studies are sensitive not only to the presence of APIs but also to specific excipients and the manufacturing process. Studies (e.g., Neto and Lisboa, 2017; Mittal et al., 2021) show that even if counterfeit drugs contain the correct dose of API, differences in formulations can be detected to distinguish between original and counterfeit products.

Recent studies using advanced chemometric methods have demonstrated that portable NIR and handheld ATR-FTIR devices have been successfully validated for the detection of counterfeiting. Studies by Ciza et al. (2019), Assi et al. (2020, 2021), and Hattori et al. (2021, 2022) show that when combined with robust chemometric models, these devices can achieve discrimination accuracies exceeding 90-100% under field-like conditions. The studies show that samples can be analyzed without pretreatment such as sample preparation or solvent use. In fact, Assi et al. (2025) reported that samples can even be analyzed without opening the blister packs. Thanks to these advantages, the developed methods offer significant benefits for use at various supply chain control points.

Spectral data obtained from devices are complex due to the large amount of information they contain. Our study confirms that the true power of IR spectroscopy can be enhanced through chemometric and machine learning algorithms. Although Principal Component Analysis (PCA) is invaluable for exploratory data analysis and visualization, relatively more advanced chemometric models and their combinations, such as SIMCA (and its powerful variant DD-SIMCA), PLS-DA, and Support Vector Machines (SVM) are useful for creating predictive, automatic “pass/fail” models.

The reviewed literature confirms the versatility of IR techniques, having been successfully applied to a wide array of products: from solid oral dosage forms (tablets, capsules) to injectable suspensions (Eady et al., 2020) and even complex biological products like mRNA vaccines (Assi et al., 2022). This broad applicability highlights the technique's utility for national regulatory authorities dealing with diverse drug portfolios. With these broad applicability advantages, spectroscopic techniques are useful in various pharmaceutical industries.

Confronting Limitations and Resolving Contradictions

Despite its strengths, the field must honestly address persistent limitations to ensure reliable implementation:

As demonstrated in the studies by Zambrzycki et al. (2021) and Maffioli & Anyakora (2025), IR spectroscopy shows superior performance in detecting counterfeit drugs (those containing incorrect or no API), while its performance in measuring API content to identify substandard drugs (those with insufficient API, e.g., 50-80% of the claimed amount) in terms of the reliability of its performance in measuring API content. Portable devices, in particular, often struggle to achieve the sensitivity and accuracy required for the detection of counterfeit or substandard drugs, leading to false negative results. As noted by Wang et al. (2019), low-cost devices may be inadequate for precise quantitative studies.

Although it is reported that the developed models show high success in classification, the power of the developed models depends on the data set used. The accuracy of any chemometric model is directly linked to the quality and scope of the reference spectral library used to train it. Models trained on a manufacturer's limited number of original products may fail to correctly classify legitimate generic drugs from another source due to excipient variations (Guillemain et al., 2017). Therefore, it is necessary to keep the databases used constantly up to date and expand them when required.

Although the developed methods are fast enough to produce results in a few seconds, the robustness and validation of the developed chemometric model require expertise in data science and spectroscopy. Furthermore, as noted by Caillet et al. (2021), variability between devices, even within the same technology class, can affect spectral reproducibility and may require device-independent modeling approaches or regular recalibration.

Future Perspectives and Recommendations

To transition from a powerful screening tool to a definitive regulatory technology, the field should focus on the following future directions:

To make the developed models more robust, the development of Universal, Shared Spectral Databases is necessary. A global initiative to create carefully selected, open-access spectral libraries for essential drugs would be beneficial. These databases should include the spectra of multiple original manufacturers (taking into account legitimate generic variations), common excipients, and known counterfeit profiles. The first step, using portable devices and simple models (e.g., HQI, k-NN), will perform an ultra-fast IR scan to flag suspicious samples. These will then be subjected to more advanced, cloud-based models or Hybrid and Hierarchical Analysis Models for definitive quantification and structural explanation, thereby increasing the accuracy of the studies. Moving beyond traditional chemometrics, deep

learning algorithms like Convolutional Neural Networks (CNNs) can automatically extract subtle, discriminative features from raw spectra, potentially improving accuracy on complex mixtures (Awotunde et al., 2022). Furthermore, data fusion strategies that combine NIR, MIR, and Raman spectra from the same sample could provide a more comprehensive and resilient chemical profile, making counterfeiting even more difficult.

CONCLUCION

The body of research examined from 2015 to 2025 substantiates infrared (IR) spectroscopy as an indispensable and rapidly evolving tool in the against pharmaceutical falsification. Its principal advantage lies in its capacity to provide a rapid, non-destructive molecular fingerprint that is sensitive to both the active pharmaceutical ingredient and the complex matrix of excipients, enabling the detection of sophisticated counterfeits that may pass visual inspection. The maturation of portable NIR and ATR-FTIR technologies, in particular, represents a paradigm shift, facilitating deployment at critical nodes within the supply chain for real-time screening.

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Abuse and Neglect in Hearing-Impaired Children and the Responsibilities of Nurses

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ABSTRACT

Among children with disabilities, hearing-impaired children are a vulnerable group at high risk of child abuse and neglect and, therefore, deserve special attention to reduce this risk. Identifying suspected abuse and neglect is essential not only to treat the current situation but also to protect the child from further, perhaps more serious, injury. Developing a multidisciplinary approach in collaboration with the caregiver or parents is crucial. Caregivers rarely intend to harm the child. Nurses and other healthcare professionals recognize that hearing-impaired children are at significantly higher risk and play an active role in preventing, identifying, and assessing potential neglect and abuse for these children.

Keywords – Abuse; neglect; hearing-impaired children; nurses

Introductıon

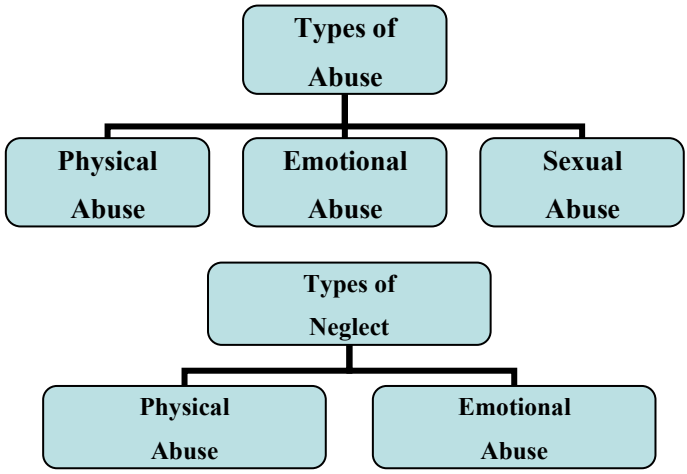
Children with disabilities include all children and adolescents with significant impairments in any area of motor, sensory, communication, social, cognitive, or emotional functioning. Children with disabilities are at higher risk for neglect, physical/emotional abuse, and sexual abuse than children without disabilities (McDonnell et al., 2019). There are concerns that cases of child abuse and neglect are underreported, particularly because many hearing-impaired children have communication difficulties and are unable to report problems directly (Hibbard and Desch, 2007). A UNICEF study on child abuse and domestic violence in Turkey found that emotional abuse, physical abuse, and sexual abuse were reported at rates of 51%, 43%, and 3% among children aged 7-18 (UNICEF, 2010). Furthermore, UNICEF reported in 2021 that approximately 240 million children with disabilities (one in 10) worldwide are deprived of their fundamental rights (UNICEF, 2021). Neglect and abuse of children with hearing impairments, including children with disabilities, is a critical public health problem. However, reliable, easily accessible, and internationally standardized data on the prevalence of neglect and abuse is lacking.

What is Abuse and Neglect?

Abuse and neglect are seen in all societies and are even among the causes of death. Child abuse is any action that physically, emotionally, mentally, sexually, or socially impedes a child's development. Child neglect, on the other hand, is the failure to meet a child's developmental, physical, and emotional needs and to implement and fulfill them. The most important factor distinguishing neglect from abuse is that abuse is an active phenomenon, while neglect is passive (Gönener, 2010; Kavaklı et al., 1998). Child abuse remains a significant social problem in all societies and at every socio-economic level (Gönener, 2010; Çatık and Çam, 2006).

Types of Abuse and Neglect

Types of abuse and neglect are shown in Figure 1 and Figure 2 (Gönener, 2010; Kavaklı et al., 1998).



What is a Hearing-Impaired Child?

Hearing impairment is defined as a loss of hearing in both ears that prevents the understanding of speech (Kırman and Sarı, 2011).

According to the World Health Organization (WHO), individuals with hearing loss of 41 dB or more in the better-hearing ear in adults, or 31 dB or more in children up to 15 years of age, are defined as hearing impaired (the smallest sound intensity heard by the human ear is 20 dB) (Kırman and Sarı, 2011; Primary ear and hearing care training resource, World Health Organization, 2006; Çakır, 1999).

According to the criteria of the American Speech-Language-Hearing Association, the degrees of hearing loss are as follows (Kırman and Sarı, 2011; Özsoy, 2007):

0-15 dB HL; Normal hearing
16-40 dB HL; Very mild hearing loss
41-55 dB HL; Mild hearing loss
56-70 dB HL; Moderate hearing loss
71-90 dB HL; Severe hearing loss
91 dB HL and above; Profound hearing loss

The Risk Factors of Abuse and Neglect on Children with Hearing Impairments

Risk factors include: the child's challenging behaviors (e.g., aggression, disobedience), the need for specialized care and the requirement for constant supervision, the need for specialized education and care services, limited opportunities for recreational activities for the child and family, increased child care costs and financial difficulties arising from reduced career advancement opportunities for parents, limited time for parents or caregivers to rest, and, especially for single parents Şen and Çarman, 2022; Legano et al., 2021).

Due to their vulnerability, there are also different risk factors; children with disabilities who have limited communication skills may be perceived as “easy targets” because of their limited ability to disclose abuse. In addition, children with limited communication skills may not respond to behaviors such as praise or verbal reprimands.
In this case, parents or other caregivers may interpret this lack of response as deliberate non-response and resort to physical discipline methods (Şen and Çarman, 2022).

Being exposed to multiple caregivers in multiple settings increases the likelihood of abuse, including sexual abuse, and reduces a child's ability to develop a trusting relationship with an adult to whom they can disclose mistreatment. Limited capacity for legitimate self-defense and inability to access personal safety information are risk factors for children with disabilities. It may hinder the ability to understand personal safety information; it may prevent the use of self-defense techniques or the ability to distance oneself from angry perpetrators. The sociocultural attitudes of peers may also increase the risk of bullying, teasing, or violent acts against children with disabilities (Şen and Çarman, 2022; Legano et al., 2021).

In addition, children in foster care may be at high risk of abuse or neglect if their foster parents do not have sufficient knowledge about the child's medical or emotional issues and are not prepared to deal with certain problems (Şen and Çarman, 2022).

Management of Abuse and Neglect in Children with Hearing Impairments

Child abuse or neglect is not only a medical diagnosis but also a criminal offense. Healthcare facilities that care for children must be professional in identifying and protecting children who may be victims of abuse and neglect and often have a legal obligation to do so. Identifying suspected neglect and abuse is urgently necessary not only to treat the current situation but also to protect the disabled child from subsequent, perhaps more serious injury (Keeshin and Dubowitz, 2013).

Detecting, preventing, and addressing manipulation, abuse, and/or neglect of children requires careful observation, sensitivity, and a high level of suspicion. Healthcare professionals, especially when abuse is involved, should always keep in mind their duty to report suspected maltreatment to child protective services after ensuring the child's safety, particularly when there is a risk of other types of abuse (Keeshin and Dubowitz, 2013). When reporting suspected child neglect or abuse, photographic documentation should be recorded for physical injuries (e.g., bruises, scrapes, patterned injuries, burns), the child's appearance and hygiene, nutritional status, and skin condition (including wounds, infections, and ulcers). The medical history of the hearing-impaired child should also be added to the records (Şen and Çarman, 2022).

Preventing Abuse and Neglect of Children with Hearing Impairments

A three-stage public health framework (primary, secondary, and tertiary) is used to prevent child neglect and abuse (Ashraf, et.al., 2020). The primary stage is achieved through the establishment of the concept of human rights, child rights-based approaches, educational programs, and legal reforms (Şen and Çarman, 2022). The secondary stage aims to identify high-risk groups, such as children with disabilities, and ensure their access to and effective use of existing services (Dinleyici, 2022). The tertiary stage involves preventing adverse outcomes that may arise from neglect and abuse, identifying and eliminating risk factors, and providing complete and appropriate treatment and rehabilitation services (Dinleyici, 2022).

Abuse and Neglect in Hearing-Impaired Children and the Responsibilities of Nurses

In order to solve the problems of families in cases of abuse and neglect, short-term and long-term goals are created together with the family, and nursing interventions are planned and implemented in accordance with the goals and the results are evaluated (Birol, 2002).

Nurses fulfill their responsibilities in healthcare by exercising their caring role. While fulfilling their caring role, they utilize their relevant knowledge and skills, communication and leadership skills, and professional qualities such as autonomous behavior, self-confidence, critical thinking, and

decision-making. Furthermore, it is crucial for nurses to enjoy working with children with intellectual disabilities to provide quality care (Gönener et al., 2010).

Child sexual abuse is a very serious issue for both families and society. Before a diagnosis of sexual abuse is made, all data must be adequately collected, a very careful approach must be taken, and children and parents must not be placed in a difficult situation during the data collection, diagnosis, and treatment phases (Ceylan et al., 2009).

The nurse should respect the child's privacy during the physical assessment and be mindful that the child may be distressed by their current situation. Because the abuse itself can be difficult to explain, the nurse conducting the interview should write down the child's statements verbatim. It should be noted that this report will serve as a record and evidence for future legal proceedings. Explaining and assessing such a sensitive and negative situation solely based on information obtained from the child may not be sufficient. Therefore, testimony from the child's teachers, parents, close relatives, long-term friends, and members of their immediate circle may also be necessary (Carpenito-Moyet, 2005).

During communication, the child's trust should be gained calmly and all questions answered. A judgmental attitude should be avoided in communication with the child, and a supportive and protective approach should be adopted. The nurse's supportive approach is crucial in identifying the child's low self-esteem, feelings of inadequacy, and fears (McKinney et.al., 2000).

To ensure the highest quality of nursing services, nurses must utilize all methods used in professional nursing care. The care plan process must be developed and evaluated using a holistic approach, taking the child's history (Gönener ve ark., 2010).

The child may not want to talk about what they are experiencing or feeling. A psychiatric nurse can create a therapeutic environment for the child, allowing them to express their experiences and feelings through drawings, games, and stories (Demir et.al., 2024; Viedebeck, 2020; Oflaz, 2015; Yılmaz, 2013).

Unauthorized touching can frighten and cause anxiety in abused children. Nurses should be aware of this sensitivity and obtain permission from children before touching them (Demir et.al., 2024; Özbaş, 2017; Oflaz, 2015). In this context, it may be beneficial for the nurse providing care to have the same gender as the child (Demir et.al., 2024; Özbaş, 2017).

Considering the effects of child abuse on child and adult mental health, it is important for psychiatric nurses, as well as pediatric nurses, to understand the types of child abuse, its risk factors, symptoms, effects on mental health, and the nursing approach to abuse (Demir et.al., 2024; Özbaş, 2017). Psychiatric nurses should be aware that all forms of abuse pose a threat to a child's mental health and that abuse is a traumatic experience for

the child. Therefore, psychiatric nurses should first develop a trusting relationship with the child in cases of abuse. Developing a trusting relationship between the nurse and the child will help the child cope with the trauma they have experienced, regain a sense of trust, and express their feelings (Demir et.al., 2024; Viedebeck, 2020; Erkut and Gözen, 2019;).

Nurses should collaborate with professionals such as teachers, social workers, and psychologists regarding family relationships, social environment, peer relationships, and school-related matters for treatment and rehabilitation. This nursing approach will ensure the child continues his/her education, integrates into society, and develops expectations for the future (Demir et.al., 2024; Özbaş, 2017; Yılmaz, 2013).

Conclusion

The abuse and neglect of hearing-impaired children is a problem that concerns the entire society. Nurses play a crucial role in the care of these children. In fulfilling this role, nurses should consider the child as a whole, including their family and environment, and should understand the growth and developmental processes of children and the effects of neglect and abuse on children and their families. They should utilize all methods used in professional nursing services, including data collection, analysis, history-taking, observation, communication, problem identification, short- and long-term goal setting, and planning, implementation, and evaluation of interventions, all of which are scientifically oriented toward problem-solving, and should record all procedures performed.

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Effects of Luteolin in Diabetes Mellitus: A Review of the Current Literature

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ABSTRACT

In this section, the potential therapeutic effects of luteolin on diabetes mellitus are examined in detail in light of molecular mechanisms, experimental findings, and current clinical data.
Diabetes mellitus is a metabolic disease characterized by chronic hyperglycemia resulting from impairments in insulin secretion, insulin action, or both. Oxidative stress, chronic inflammation, and cellular dysfunction resulting from hyperglycemia play a significant role in the progression of the disease and the development of its complications.
Luteolin is a flavonoid noted for its antioxidant, anti-inflammatory, hypoglycemic, and metabolic regulatory properties. Luteolin (3′,4′,5,7-tetrahydroxyflavanone) is a natural flavonoid found in many fruits and vegetables. In recent years, it has been extensively researched for its antioxidant, anti-inflammatory, lipid/glucose metabolism-regulating properties in diabetes and diabetic complications. This review discusses the effects of luteolin on diabetes, including its possible molecular mechanisms, experimental and clinical evidence, limitations, and therapeutic potential.
More than 20 scientific studies published after 2018 were evaluated.
The obtained data suggest that luteolin may have protective effects, particularly on insulin resistance, pancreatic β-cell function, inflammatory cytokines, AGE accumulation, oxidative stress, and the development of complications.

Keywords: Diabetes Mellitus, Luteolin, Flavonoid, Oxidative Stress, İnflammation, İnsulin Resistance.

INTRODUCTION

1. General Mechanısm Of Dıabetes Mellıtus

Diabetes mellitus, also known as diabetes mellitus, is a chronic disease that causes high blood sugar levels to accumulate due to the pancreas' inability to produce sufficient insulin or the body's inability to use insulin properly (Lisco et al.,2025:154). This affects how the body converts food into energy, resulting in elevated blood sugar levels. The energy the body needs is provided by the essential nutrients protein, fat, and carbohydrates(Hantzidiamantis et al.,2024). The most important of these nutrients is glucose, or simple sugar. The blood transports glucose to all cells in the body for use as energy. The importance of glucose stems from its role

as the body's primary energy source, especially in the brain. Cells use the glucose they need with the help of the hormone insulin, secreted by the pancreas. If the body cannot produce insulin, glucose cannot be used as energy, and blood sugar levels will rise(Gillespie et al., 2023:889).

Long-term hyperglycemia leads to microvascular and macrovascular complications such as cardiovascular diseases, neuropathy, nephropathy, and retinopathy(Zhao et al.,2023). The prevalence of diabetes mellitus is increasing both in Turkey and globally(Zakir et al., 2023). The inability to maintain adequate glycemic control despite modern treatments has increased interest in phytochemical compounds. In recent years, the role of natural compounds in diabetes management has become increasingly important (Onukwuli et al., 2024).

2. What Is Luteolin?

Luteolin is a flavonoid with a 3',4',5,7-tetrahydroxyflavone structure that occurs naturally in parsley, celery, sage, peppers, onions, and medicinal plants(Saleem et al., 2021 :509). Recent studies have shown that luteolin has the potential to improve glycemic control, reduce insulin resistance, protect pancreatic β-cells, and alleviate diabetic complications(Papuc et al., 2021:325). It particularly exerts multifaceted effects on fundamental mechanisms of diabetes pathophysiology, such as oxidative stress, inflammatory response, and glucose and lipid metabolism (Hou et al.,2025).

Luteolin exhibits antidiabetic activity through pleiotropic mechanisms, including regulation of intestinal microenvironmental homeostasis, lipogenesis and catabolism, and carbohydrate absorption(Wang et al.,2021:1441). The compound regulates key pathological processes (inflammation, oxidative stress, apoptosis, and autophagy) that contribute to both the pathogenesis and complications of diabetes(Chen et al.,2025:15). Evidence suggests therapeutic potential for diabetic cardiomyopathy, nephropathy, and osteopathy through these cellular mechanisms ((Pradhan et al., 2025).

It has regulatory effects on many critical pathways in diabetes, such as NF-κB, STAT3, IRS-1/PI3K/Akt, and AMPK It has become popular in diabetes research due to its anti-inflammatory and antioxidant properties.
The antidiabetic effect of luteolin is associated with increased PPARγ and GLUT expression(Shehnaz et al.,2023:9129).

Various in vitro and in vivo studies have been conducted to investigate the effects of luteolin on diabetic complications, and it has also been shown to have a significant impact in their management(Kahksha et al., 2023 : 126).

Luteolin is a flavone compound with potent antioxidant activity found in a wide range of plants. It is absorbed in the intestine, and its metabolism proceeds via glucuronidation and sulfation pathways(Tian et al., 2021 : 257–264)

Luteolin is a polyphenolic flavonoid with a high free radical scavenging capacity. Numerous studies have shown that luteolin regulates signaling pathways such as NF-κB, MAPK, and Nrf2 ((Mahdiani et al.,2022:744–762).

These properties directly impact oxidative stress and inflammation, which play critical roles in the pathophysiology of diabetes. Luteolin also has regulatory effects on lipid metabolism, glucose homeostasis, and cellular energy production (Wang et al.,2021:1441)

Luteolin administration reduced albuminuria, alleviated glomerular sclerosis, and significantly reduced tubulointerstitial fibrosis in STZ and db/db mouse models. (Zhang et al.,2021:729).

In diabetic neuropathy models, luteolin increased nerve conduction velocity and reduced oxidative damage in peripheral nerves by activating the Nrf2 pathway. In diabetic retinopathy experiments, it suppressed VEGF expression, limited angiogenesis, and reduced retinal oxidative stress (Saikia et al.,2024:2351).

Luteolin activated the AMPK/PGC-1α pathway in high-fat diet-induced hepatic steatosis, increasing mitochondrial biogenesis and reducing steatosis and inflammation. Luteolin-loaded nanoparticles have also been reported to be effective against NAFLD and insulin resistance (Sharma et al., 2025:295).

2.1. Antioxidant effects of luteolin

Increased free radicals in diabetes accelerate cell damage and the development of complications. Luteolin increases the activity of antioxidant enzymes such as SOD, CAT, and GPx, while reducing lipid peroxidation (Sun et al., 2021:1309).

Several studies published after 2018 have shown that luteolin reduces oxidative stress by activating the Nrf2/HO-1 pathway (Yu et al.,2024: 8053).

2.2. Anti-inflammatory effects of luteolin

In Diabetes, inflammatory cytokines (IL-6, TNF-α, IL-1β) both increase insulin resistance and accelerate the development of complications. Luteolin contributes to the reduction of the proinflammatory response by inhibiting the NF-κB pathway (Chen et al.,2023). Experimental studies have shown that luteolin administration improves glucose metabolism and reduces systemic inflammation. (Daily et al.,2021:218). Luteolin reduced the levels

of MDA and 8-OHdG, markers of oxidative stress, in diabetic tissues and increased antioxidant enzymes such as SOD, CAT, and GSH-Px. It was also found to be effective in suppressing proinflammatory cytokines such as IL-1β, TNF-α, and IL-6 (Alsawaf et al., 2022 :1717).

2.3. Effects on insulin resistance

Insulin resistance is the fundamental disorder at the heart of type 2 diabetes. Luteolin has been reported to increase GLUT4 translocation, activate the AMPK pathway, and improve insulin sensitivity by reducing hepatic gluconeogenesis(Miao et al.,2023 :1991)
.
In high-fat diet-induced type 2 diabetes models, luteolin: It reduced hepatic steatosis, suppressed the expression of SREBP-1, a key regulator of lipogenesis, and improved insulin sensitivity. It also lowered HOMA-IR values, reduced visceral fat, and increased adiponectin levels (Shehnaz et al., 2023:9129).

In streptozotocin (STZ)-induced diabetes rats, luteolin reduced fasting glucose and HbA1c levels, increased serum insulin levels, and preserved pancreatic islet structure. In in vitro models, it also significantly reduced palmitate- or high-glucose-induced β-cell apoptosis
β-cell dysfunction plays a critical role in the progression of diabetes.
Luteolin reduces β-cell apoptosis due to oxidative stress and promotes insulin secretion(Al-Keridis et al.,2023 :126).

2.5. Safety and toxicity

Luteolin is generally considered safe. Animal studies have not shown any serious toxicity, even at high doses. However, long-term controlled studies in humans are limited, and drug interactions with high doses should be investigated(Taheri et al., 2021).

6. Conclusion

Current findings demonstrate that luteolin has positive effects on many stages of diabetes pathophysiology.
Studies conducted after 2015 demonstrate that luteolin is a potent natural agent with multiple effects on many stages of diabetes pathophysiology. Luteolin:
• Reduces insulin resistance,
• Protects β-cells,

• Suppresses oxidative stress and inflammation,
• Alleviates complications such as diabetic nephropathy, neuropathy, and retinopathy,
• Reduces hepatic steatosis and cardiovascular damage (26).

Its antioxidant, anti-inflammatory, insulin-sensitizing, and β-cell-protective effects make luteolin a potential complementary therapeutic agent. Luteolin exhibits antidiabetic activity through pleiotropic mechanisms, including regulation of intestinal microenvironmental homeostasis, lipogenesis and catabolism, and carbohydrate absorption. The compound regulates fundamental pathological processes (inflammation, oxidative stress, apoptosis, and autophagy) that contribute to both the pathogenesis and complications of diabetes. Evidence suggests therapeutic potential for diabetic cardiomyopathy, nephropathy, and osteopathy through these cellular mechanisms. However, current evidence does not conclusively establish the clinical efficacy of luteolin in human diabetes. Current data are primarily derived from preclinical studies, and clinical validation is not yet available. Critical gaps remain regarding bioavailability, optimal formulations, safety profiles, synergistic effects, and appropriate dosage regimens. While preclinical evidence supports the antidiabetic properties of luteolin and its effects on complications, the significant translational gap between experimental findings and clinical practice prevents definitive statements about therapeutic benefit in patients with diabetes. Clinical studies are necessary to confirm the efficacy and safety suggested by preclinical studies. However, further research is needed regarding the clinical dose, bioavailability, and safety of luteolin. Future randomized controlled human studies will clarify luteolin's role in diabetes treatment, and more clinical studies are needed.

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Migraine as a Brain Clearance Disorder: The Emerging Role of the Glymphatic System

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ABSTRACT

Migraine is increasingly understood not only as a pain disorder but as a brain condition related to impaired physiological homeostasis and insufficient clearance of inflammatory and metabolic byproducts. Recent insights emphasize that the brain possesses a unique fluid transport system that becomes highly active during deep sleep, allowing the removal of potentially harmful substances that accumulate during wakefulness. In individuals with migraine, this natural clearance mechanism appears to become progressively overloaded or dysregulated, especially as attacks become more frequent or sleep quality deteriorates.

In the early stages of migraine, the brain is often still able to compensate by enhancing its internal cleansing mechanisms. However, when this compensatory capacity begins to fail, migraine no longer remains a purely episodic phenomenon. Instead, the brain remains in a persistent hypersensitive state, even between attacks, which may contribute to cognitive fog, sensory overload, or constant head pressure. This shift marks the transition toward a chronic phase, during which responsiveness to conventional pain-suppressing treatments frequently declines.

For this reason, contemporary therapeutic perspectives emphasize that effective migraine management should aim not only to suppress pain but also to support the brain's intrinsic ability to recover. Protecting deep sleep quality, regulating autonomic balance, and enhancing fluid drainage within the brain are increasingly considered essential strategies to prevent disease progression and maintain long-term neurological resilience.

Keywords – Migraine; glymphatic system; neuroinflammation; brain clearance; neuroimmune modulation

INTRODUCTION

Migraine is currently recognized not merely as a recurrent headache disorder but as a complex brain network disease involving sensory processing, pain modulation, autonomic regulation, and neuroimmune interaction (Ashina, 2020:1–3; Goadsby et al., 2017:553–560). Affecting approximately 15% of the global population, it is one of the leading causes of neurological disability and is increasingly described as a systemic neurophysiological dysregulation rather than a localized pain syndrome (GBD 2016 Collaborators, 2019:121–125). Functional neuroimaging studies have shown that migraine attacks are initiated by early activation in regions such as the hypothalamus and insula even before pain begins indicating a centrally driven premonitory phase (Karsan & Goadsby, 2018:55–60). The trigeminovascular system subsequently acts through meningeal sensory afferents and the trigeminocervical complex to trigger neuropeptide release

and sterile neuroinflammation (Messina et al., 2022:40–46). In aura-positive migraine, this process may be preceded by cortical spreading depression (CSD) a propagating wave of transient neuronal and glial depolarization (Vinogradova, 2015:15–19). In chronic stages, migraine evolves from an episodic event to a persistent "brain state," characterized by maladaptive neuroplastic reorganization within the brainstem, thalamus, cortical sensory networks, and affective–cognitive circuits (Ashina, 2020:1–3; Katsarava et al., 2018:45–49).

The Clinical Nature of Migraine and Contemporary Neurobiological Perspectives

Migraine is now widely recognized not as a mere episodic headache disorder but as a complex, multisystem neurobiological condition affecting over one billion individuals worldwide (Ashina, 2020:1–3). With an estimated global prevalence of 15%, it represents the leading cause of years lived with disability among neurological diseases (GBD 2016 Collaborators, 2019:121–125). Contemporary neuroscience conceptualizes migraine as a dysfunction of interconnected brain networks governing sensory processing, pain modulation, autonomic regulation, and neuroimmune signaling, rather than as an isolated cranial pain phenomenon (Goadsby et al., 2017:553–560).

Pathophysiologically, migraine is a "network disease." In many patients, attacks begin with a prodromal phase, during which functional neuroimaging has demonstrated early activation of the hypothalamus and insula, independent of pain (Karsan & Goadsby, 2018:55–60). This is followed by trigeminovascular activation: trigeminal afferents projecting to the meninges and intracranial vessels transmit nociceptive signals to the trigeminocervical complex (TCC) within the brainstem (Messina et al., 2022:40–46). In patients with aura, this process is preceded by CSD a transient wave of neuronal and glial depolarization that advances across the cortex (Vinogradova, 2015:15–19). Beyond the acute phase, migraine chronification is associated with long-term neuroplastic reorganization involving the brainstem, thalamus, sensory cortices, and affective–cognitive networks (Ashina, 2020:1–3; Katsarava et al., 2018:45–49).

Clinically, migraine is characterized by recurrent attacks lasting 4–72 hours, typically unilateral, pulsating, and aggravated by physical activity, and accompanied by photophobia, phonophobia, and nausea (Headache Classification Committee of IHS, 2018:20–28). Aura fully reversible visual or sensory disturbances occurs in roughly one third of cases (Russell & Olesen, 1996:355–361). Chronic migraine, defined as ≥15 headache days per month, is frequently linked to maladaptive overuse of acute medications (Natoli et al., 2010:271–279). Diagnostic confirmation relies primarily on structured clinical history; validated tools such as ID-Migraine may complement structured evaluation (Lipton et al., 2003:375–382; Hagen et al.,

2008:603–608). Neuroimaging is reserved strictly for red-flag features suggestive of secondary headache (Rasmussen & Olesen, 1992:438–440).

Treatment is divided into acute and preventive strategies. In acute management, NSAIDs are first-line agents; early administration of triptans is recommended when response is insufficient (Diener et al., 2019:101–110). Ergot derivatives and opioids are discouraged due to dependency and medication-overuse headache risks (Olesen et al., 2018:1–5). Preventive therapy is indicated when migraine causes substantial disability despite optimized acute treatment. β-blockers, topiramate, and candesartan remain established first-line preventives (Tfelt-Hansen et al., 2012:91–94). A major therapeutic advance is the advent of calcitonin gene-related peptide (CGRP)-targeted monoclonal antibodies (erenumab, fremanezumab, galcanezumab, eptinezumab), the first migraine-specific mechanism-based biologics, achieving >50% reduction in attack frequency in many patients (Ashina et al., 2021:980–986; Reuter et al., 2018:109–119). Recently, ditans (5-HT1F agonists) and gepants (CGRP receptor antagonists) have emerged as safer alternatives for patients with cardiovascular risk (Puledda et al., 2023:112–119). Moreover, non-invasive neuromodulation techniques (e.g., vagus nerve stimulation, REN, tVNS, tDCS) are gaining importance in refractory or drug-intolerant cases (Yarnitsky et al., 2019:5–10; Renner et al., 2019:40–43).

Long-term success depends heavily on therapeutic education the patient must understand that migraine is not a disease to be "cured," but a chronic neurological condition to be optimally managed (Steiner et al., 2021:20–24). Emerging precision medicine emphasizes individualized trigger profiling (e.g., sleep irregularities, stress, hormonal oscillations), which can reduce attack burden even before pharmacological intervention (Puledda et al., 2023:112–119).

In conclusion, migraine has been redefined from a pain-centric disorder to a dynamic multisystem brain network dysfunction involving electrical, vascular, inflammatory, and neuroimmune mechanisms. This reconceptualization is driving a major therapeutic transition from symptomatic suppression toward early-phase interception and circuit-level neuromodulation, paving the way for future genotype- and endophenotype-matched personalized treatment strategies.

The Brain's Glymphatic Pathway: Structure, Function, and Regulatory Dynamics

Although the central nervous system (CNS) was long believed to lack a conventional lymphatic network, findings from the past decade have radically overturned this view and revealed the existence of a fluid-based waste clearance system unique to the brain the glymphatic system (Iliff et al., 2012). This system, unlike the peripheral lymphatic structures, operates through the direct movement of cerebrospinal fluid (CSF) from arterial

perivascular spaces into the interstitial compartment. It relies heavily on aquaporin-4 (AQP4) water channels densely localized on astrocytic end-feet surrounding cerebral vasculature, which facilitate this directional fluid exchange (Jessen et al., 2015). Through this dynamic transport, the system enables the removal of neurotoxic proteins such as β-amyloid, tau, and α-synuclein, thereby functioning as the brain's intrinsic metabolic detoxification network (Plog & Nedergaard, 2018:379–394).

One of the most striking features of the glymphatic system is its intimate coupling with the sleep–wake cycle. The landmark study by Xie et al. (2013:373–377) demonstrated that glymphatic activity increases up to tenfold during slow-wave non-REM sleep. At this stage, neuronal activity declines, the interstitial space expands, and metabolic waste clearance accelerates. Sleep is therefore no longer viewed as a passive state of rest but rather as an active "detoxification period" essential for maintaining cerebral homeostasis (Xie et al., 2013:373–377). Correspondingly, the heightened risk of Alzheimer's disease observed in patients with chronic sleep disruption is now considered not merely correlative, but mechanistically rooted in glymphatic dysfunction (Rasmussen et al., 2018:1016–1024).

The efficiency of the glymphatic system is governed by several physiological determinants. While arterial pulsatility drives CSF influx, the polarized perivascular distribution of AQP4 channels ensures biomechanically optimized fluid movement (Jessen et al., 2015:2583–2599). However, aging has been shown to disrupt AQP4 polarization, weaken meningeal lymphatic structures, and profoundly impair glymphatic clearance capacity (Kress et al., 2014:845–861). Importantly, this decline is not exclusive to normal aging it is significantly exacerbated by obesity, hypertension, chronic inflammation, traumatic brain injury, and sleep fragmentation (Plog & Nedergaard, 2018:379–394).

Consequently, glymphatic failure is now recognized not as a benign age-related phenomenon but as a shared pathological substrate across neurodegenerative diseases. In Alzheimer's disease, β-amyloid accumulation is known to occur early in the pathogenic cascade; critically, glymphatic clearance rates decline years before overt amyloid deposition becomes clinically detectable, suggesting its role as a pre-symptomatic biomarker (Rasmussen et al., 2018:1016–1024). Similar mechanistic implications have emerged in Parkinson's disease (α-synuclein accumulation), multiple sclerosis (inflammatory edema), and migraine all of which are now being reinterpreted through a glymphatic lens (Iliff et al., 2012; Plog & Nedergaard, 2018:379–394).

More importantly, the glymphatic system is not merely a passive waste disposal mechanism; it also functions as an active neuroimmune interface, mediating molecular communication between the brain and peripheral immune pathways (Louveau et al., 2017:2271–2281). Antigenic molecules carried through perivascular pathways into meningeal lymphatics

are subsequently delivered to cervical lymph nodes, initiating immune signaling. Thus, the glymphatic network represents a central modulator not only of neurodegeneration but also of neuroinflammatory processes (Vittorini et al., 2024).

In conclusion, the discovery of the glymphatic system represents a paradigm shift in our understanding of CNS physiology and pathophysiology. Therapeutic strategies targeting this system including optimization of sleep architecture, preservation of AQP4 channel polarization, enhancement of vascular pulsatility, and application of non-invasive neuromodulation hold great promise as disease-modifying interventions across a broad spectrum of neurological disorders, ranging from Alzheimer's disease to migraine. The glymphatic system is increasingly regarded not merely as a clearance mechanism but as a critical defensive infrastructure safeguarding the brain against aging, inflammation, and neurodegeneration.

The Role of the Glymphatic System in Migraine Pathophysiology

Migraine is increasingly being reframed not merely as a neurovascular pain disorder but as a progressive failure of the brain's intrinsic clearance systems particularly the glymphatic and meningeal lymphatic networks. Rather than originating solely from excessive neural excitation or trigeminovascular activation, migraine appears to emerge when the brain fails to efficiently eliminate neuroinflammatory and vasodilatory molecules such as CGRP, glutamate, potassium ions, lactate, and reactive oxygen species. The glymphatic system, which facilitates CSF influx through periarterial channels and directs metabolic waste toward perivenous and meningeal lymphatic efflux pathways, operates as a nocturnal detoxification mechanism essential for resetting cerebral homeostasis between attacks (Iliff et al., 2012:147). When this system becomes overloaded or structurally impaired, inflammatory burden accumulates in perivascular and interstitial compartments, progressively lowering the neural excitability threshold and prolonging trigeminal sensitization. In this context, migraine can be more accurately described not as transient episodes of abnormal neural firing but as a state of insufficient overnight neurochemical recovery a failure of the brain to clear what it produces.

Current clinical and experimental evidence suggests that the glymphatic system operates most efficiently during deep non-rapid eye movement (NREM Stage 3) sleep, when neuronal activity decreases and astrocytic AQP4 water channels become optimally polarized along vascular boundaries (Xie et al., 2013:373–377). In healthy conditions, this allows CSF to enter the brain via periarterial spaces, mix with interstitial fluid (ISF), and transport accumulated toxins toward meningeal lymphatic pathways for immune processing. However, during a migraine attack—especially when triggered by CSD astrocytic swelling mechanically

constricts periarterial inflow routes within minutes, temporarily shutting down glymphatic circulation (Schain et al., 2017:2904–2915). The consequence is not only intensified pain signaling but also impaired clearance of CGRP and other proinflammatory mediators, which remain trapped in perivascular spaces instead of being eliminated. This leads to prolonged inflammatory signaling, delayed pain resolution, and contributes to the transition from episodic to chronic migraine through cumulative toxic retention.

Mounting human imaging data now support a two-phase model: in early episodic migraine, glymphatic activity may actually increase as a compensatory mechanism to counter rising neuroinflammatory load (Cao et al., 2024; Lee et al., 2022:718–725). However, as attacks become more frequent or sleep quality deteriorates, this compensatory state gives way to an exhausted phase characterized by significantly reduced CSF influx and impaired meningeal lymphatic drainage, especially around the superior sagittal sinus (Wu et al., 2024:583–595). This shift aligns with the emergence of cognitive fog, photophobia hypersensitivity, and decreased treatment responsiveness—symptoms poorly explained by classical pain-centric models. For this reason, migraine is increasingly recognized not as a paroxysmal pain phenomenon but as a progressive neurofluid clearance disorder in which the brain becomes unable to fully reset its biochemical environment between episodes.

The role of AQP4 polarization is central in this pathophysiological model. AQP4 is a water channel densely expressed on astrocytic endfeet that line cerebral blood vessels, and its correct perivascular localization acts as a structural gatekeeper for CSF entry into the interstitial space. In healthy brains, AQP4 polarity allows CSF to be driven deep into perivascular corridors, accelerating the clearance of neurotoxic metabolites. However, repeated exposure to csd, oxidative stress, inflammatory cytokines, and mechanical glymphatic overload can progressively disrupt this perivascular AQP4 alignment a phenomenon termed AQP4 depolarization (Huang et al., 2023:64–74). When AQP4 polarity collapses, the physical efficiency of glymphatic inflow declines sharply, resulting in a measurable slowdown in interstitial waste removal. This dynamic partly explains why chronic migraine is more than just “more attacks”; it represents a structural and functional transition into a clearance-impaired neurological state.

Notably, recent imaging evidence demonstrates that this glymphatic deterioration becomes measurable even between attacks, in the so-called interictal period. In chronic migraine patients, diffusion tensor imaging–analysis along perivascular spaces (DTI-ALPS) has revealed significantly lower glymphatic inflow indices compared to both episodic migraine patients and healthy controls (Wu et al., 2024:583–595). Likewise, contrast-enhanced MRI has visualized sluggish meningeal lymphatic outflow, particularly around the superior sagittal sinus, where residual contrast

material persists longer in chronic migraine than in either episodic migraine or non-migraine subjects. These findings are not simply acute-attack phenomena; they indicate a chronically impaired neurofluid clearance state, present even in the absence of pain. This supports the growing understanding that migraine should not be defined purely by attack frequency but by the brain's ability or failure to clear inflammatory load between attacks.

Furthermore, objective correlations have emerged between glymphatic function and sleep quality, assessed through tools such as the Pittsburgh Sleep Quality Index (PSQI). In chronic migraine populations, lower DTI-ALPS scores significantly correlate with poorer PSQI scores, even after statistical correction (Wu et al., 2024:583–595). This aligns with experimental evidence demonstrating that glymphatic flow increases two- to four-fold during slow-wave sleep, particularly during NREM Stage 3, when EEG delta power is highest (Xie et al., 2013:373–377; Hablitz et al., 2019). Accordingly, impaired sleep is not merely a comorbidity in migraine it is a direct mechanistic driver of neuroinflammatory accumulation, contributing to transition into chronic disease. These findings together redefine migraine not only as a neurovascular or neuroinflammatory disease but as a neurophysiological clearance disorder, one in which the brain fails to adequately reset its inflammatory and metabolic microenvironment.

Under these conditions, it becomes evident that migraine is not simply an episodic pain phenomenon, but rather a progressive failure of nightly neurotoxin clearance, in which each insufficiently resolved attack contributes to a compounding biochemical burden. This mechanistic reframing explains several previously paradoxical observations: why some patients experience a persistent "postdromal fatigue" even after their pain subsides, why chronic migraine is associated with cognitive fog and hypersensory states even between attacks, and crucially, why the efficacy of CGRP antagonists and triptans diminishes over time not because the target molecule changes, but because the brain's foundational ability to eliminate the byproducts of the attack has collapsed (Vittorini et al., 2024).

What further reinforces this model is evidence showing that glymphatic function does not deteriorate linearly, but rather transitions through a compensatory hyperactive phase before reaching exhaustion. In episodic migraine, imaging studies using DKI-ALPS have sometimes demonstrated increased glymphatic flow, interpreted as a temporary overcompensatory attempt to accelerate clearance under rising neuroinflammatory load (Cao et al., 2024). During this phase, AQP4 polarity is still largely preserved. The brain is not yet failing, it is fighting. However, if attacks become too frequent, sleep remains fragmented, or pharmacological overuse suppresses pain but not neurotoxin load, the system shifts into a decompensated state. At that point, glymphatic inflow slows (measured as reduced ALPS index), meningeal lymphatic efflux is delayed,

and astrocytic AQP4 becomes mislocalized away from perivascular borders the hallmark of irreversible structural transition (Huang et al., 2023:64–74).

This evolving model allows migraine to be more accurately understood in three fluid-physiological stages, rather than a binary episodic–chronic classification:

Tablo 1: Functional Staging of Glymphatic Activity in Migraine

Glymphatic Phase	Functional Status	AQP4 Polarity	Clinical State
Phase I - Stable	Normal clearance	Preserved	Episodic, reversible
Phase II - Compensatory Hyperactive	Accelerated clearance (overworking)	Stressed but intact	"Migraine building but brain coping"
Phase III - Exhaustional Failure	Reduced inflow + impaired efflux	Depolarized	Chronic, drug-resistant state

Reference: Wu et al., 2024:583–595

This model is clinically far more precise because it explains not only what the patient feels, but what the brain is failing to do namely, reset its biochemical environment between attacks. It also clarifies why early therapeutic strategies must prioritize preservation of glymphatic resilience, rather than focusing exclusively on short-term nociceptive suppression.

Given this framework, it becomes evident that the failure in migraine is not the excessive production of neuroinflammatory molecules alone, but the inadequate elimination of those molecules once they have been produced. In other words, migraine is not only a disorder of "neural activation," but fundamentally a disorder of "neural clearance." This explains why traditional pharmacological approaches triptans, NSAIDs, gepants, even CGRP monoclonal antibodies are often effective in the early compensatory phase, yet gradually lose power as the brain transitions into the exhaustional phase of glymphatic collapse. These drugs silence nociceptive output, but they do not restore the brain's ability to clean itself. Therefore, the symptom appears resolved, while the underlying biochemical burden remains accumulating silently, attack after attack (Vittorini et al., 2024).

One of the strongest arguments supporting this model is the emerging recognition of "interictal migraine impairment" a state in which patients do not necessarily report pain but continue to exhibit brain fog, sensory hypersensitivity, irritability, or fatigue. These symptoms align poorly with the classical pain-based view, yet perfectly with partial glymphatic–lymphatic failure. In fact, Wu et al. (2024) demonstrated that chronic migraineurs showed significantly impaired glymphatic inflow even when scanned in a pain-free state, indicating that the pathophysiology is not

confined to attacks but persists continuously at the clearance level. Furthermore, lower ALPS index values correlated with worse sleep quality, higher disease chronicity, and increased disability scores, providing strong evidence that migraine burden reflects cumulative clearance failure rather than frequency of electrical attack events alone.

This paradigm also reframes sleep not as a lifestyle modifier, but as a neurophysiological determinant of recovery capacity. Deep sleep specifically NREM Stage 3 is the primary time window during which glymphatic clearance rapidly accelerates, increasing CSF–interstitial fluid exchange by up to 4-fold compared to wakefulness (Xie et al., 2013:373–377). Thus, when patients report that poor sleep "automatically triggers a worse migraine day", this is not subjective correlation; it is biologically mechanistic. Impaired sleep does not merely sensitize the trigeminal system it prevents the brain from flushing out the previous day's inflammatory waste. The attack that follows is therefore not simply "triggered," but biochemically enabled by insufficient overnight cleansing (Burgos et al., 2024:517-225).

This understanding fundamentally shifts the clinical objective in migraine management: the goal is no longer just to stop the pain, but to preserve or restore the brain's nightly clearance capacity before it collapses. Pain suppression alone particularly without supporting clearance may paradoxically accelerate progression toward chronic migraine. Wu et al. (2024) demonstrated that patients with medication-overuse headache (MOH), who chronically suppress pain pharmacologically, exhibited the most severely impaired glymphatic and meningeal lymphatic function of all migraine subgroups. This suggests that current "aggressive pain control" strategies, when not coupled with clearance-supportive interventions, may unintentionally silence the alarm while allowing the fire to spread beneath the surface.

In contrast, therapeutic strategies that directly enhance glymphatic and lymphatic function show potential to modify disease trajectory rather than simply mute symptoms. These include sleep-based interventions (optimizing NREM Stage 3 depth), vagus nerve stimulation (which boosts parasympathetic tone and CSF pulsatility), postural and respiratory modulation techniques (enhancing meningeal outflow dynamics), and pharmacological AQP4 polarity stabilizers currently under investigation. Importantly, these are not alternative or holistic therapies, but mechanism-corrective strategies that align directly with the emerging pathophysiology targeting not the output (pain), but the system failure that eventually produces it.

This framework also offers a more accurate sequencing model for clinical progression. Migraine is no longer simply "episodic versus chronic," but rather fluid-pathophysiologically staged:

Stage I: Stable: Glymphatic function intact, AQP4 polarity preserved

Stage II Compensatory Hyperactive: Glymphatic system overworking to keep up with excessive inflammatory load

Stage III Exhaustional Failure: Glymphatic inflow and lymphatic efflux both impaired; AQP4 polarity disrupted; pharmacoresistance begins

This staging system more accurately reflects the true reversibility window of migraine. When intervention occurs before AQP4 polarity is lost most often during the compensatory hyperactive phase complete reversal of disease trajectory may still be possible. Once the system enters the exhaustional phase, however, the damage becomes structurally anchored, and treatment transitions from reversal to containment (Burgos et al., 2024:517-225).

This multi-phase clearance model also clarifies why not all migraine patients respond equally to identical treatments and why some therapies appear to "suddenly stop working" after initially showing strong benefit. In the compensatory hyperactive phase, CGRP antagonists, triptans, and NSAIDs often appear remarkably effective, because the brain's clearance machinery is still intact and only temporarily overburdened. Pain suppression coincides with active glymphatic cooperation, allowing the system to recover after each attack. However, once the brain transitions into the exhaustional failure phase, where AQP4 polarity collapses and perivenous drainage slows, these same medications become pharmacologically muted, not because their molecular targets change but because the brain's self-cleaning system is no longer functioning well enough to complete recovery once pain is suppressed. In essence, pain-blocking treatments become like "turning off the alarm while the building is still filling with smoke."

This explains why chronic migraine is not just more frequent migraine it is biologically different. It represents a shift from neural hyperreactivity to neurofluidic insufficiency. The system that should eliminate CGRP, lactate, potassium ions, inflammatory cytokines, and extracellular glutamate never fully resets between attacks, leaving the brain in a persistently "primed" state of hypersensitivity and energetic inefficiency. This pathophysiological persistence aligns with patient-reported symptoms such as "brain pressure," "feeling swollen inside the head," "head never feels clean," or "the migraine never fully goes away" descriptions that rarely make sense within a purely vasodilatory or neurotransmitter-based framework, yet map perfectly onto impaired glymphatic–lymphatic physiology.

Moreover, this new framework reframes sleep optimization from a lifestyle suggestion into a frontline therapeutic necessity. Deep sleep is not simply restorative it is the primary metabolic wash cycle of the brain. When deep NREM3 sleep is fragmented or suppressed, glymphatic flow decreases immediately and dramatically (Xie et al., 2013:373–377). Likewise, vagus

nerve activity, which increases during parasympathetic dominance, enhances CSF pulsatility and therefore accelerates clearance. Therefore, interventions that improve deep sleep quality, enhance parasympathetic tone, and prevent AQP4 depolarization are not supportive extras they are potential disease-stopping interventions if introduced early enough (Vittorini et al., 2024).

For this reason, the future of migraine therapy is unlikely to be defined by more potent pain-blocking molecules, but rather by treatments that restore the brain's clearance infrastructure. The central therapeutic question is shifting from "How do we stop the migraine attack?" to "How do we prevent the brain from entering a state where attacks become biochemically inevitable?" This is a decisive strategic pivot: from symptomatic interruption to physiological preservation.

Emerging treatment strategies aligned with this paradigm include:

• AQP4 polarity preservation, preventing astrocytic mislocalization before glymphatic inflow collapses (Huang et al., 2023:64–74)

• Non-pharmacological deep-sleep enhancement, structured NREM3 amplification via targeted routines or neuromodulation

• Transcutaneous auricular vagus nerve stimulation (taVNS), parasympathetic activation shown to augment CSF pulsatility

• Postural and respiratory cerebrofluid mechanics, including side-sleeping, diaphragmatic breathing, jugular outflow optimization

• Meningeal lymphatic drainage facilitation, mechanical or positional strategies to reduce efflux resistance (Mestre et al., 2018:4878)

These therapies do not merely reduce nociceptive firing they enable the brain to actually recover. They target the "why it recurs", not just "when it hurts." Their objective is not immediate silence, but long-term remission. In this sense, glymphatic-supportive and lymphatic-optimizing strategies have the potential to move migraine from a managed condition to a preventable neurobiological failure.

A Paradigm Reversal: From Pain-Centric Medicine to Clearance-Centric Medicine

The implications of this reconceptualization are profound. If migraine is truly a disorder of neurofluidic insufficiency, then the primary clinical failure is not pain it is the brain's inability to cleanse itself. Pain is the alarm, not the pathology. The true pathology lies in the failure of the glymphatic and meningeal lymphatic systems to restore biochemical equilibrium after each attack.

This paradigm also explains one of the most clinically frustrating realities: even when migraine pain is successfully suppressed, the brain often does not "feel normal." Patients may report lingering cognitive drag, visual sensitivity, fatigue, irritability, or an indefinable internal "pressure." Classical neurology has struggled to account for this interictal disturbance yet it is entirely consistent with a slowly drowning brain: one attempting to

operate while still partially immersed in its own unresolved neuroinflammatory waste (Huang et al., 2023:64–74).

This is why chronic migraine patients overwhelmingly describe the condition not as "repeated individual attacks," but as a continuous brain-state abnormality with fluctuating flare-ups. The migraine, in their lived experience, is not something that "comes and goes," but something that never fully leaves. This is not a failure of perception it is a failure of clearance. If this is true, then the true point of no return in migraine is not when attacks become more frequent it is when the glymphatic system loses its ability to compensate.

Once AQP4 polarity is significantly disrupted and meningeal efflux slows, migraine becomes a self-perpetuating state and pharmacoresistance becomes predictable rather than unexpected.

At this stage, the clinical objective must shift dramatically not to silence attacks, but to restore the brain's detoxification infrastructure. Failure to do so guarantees symptomatic recurrence, regardless of how effective acute therapies may appear (Cao et al., 2024).

Redefining Clinical Strategy: Migraine as a Preventable Failure of Brain Clearance

If migraine is fundamentally a progressive impairment of neurofluid clearance, then the future of clinical management must shift away from merely reducing pain frequency and instead focus on preserving glymphatic and meningeal lymphatic resilience before collapse occurs. This shift demands that migraine no longer be staged solely by attack count, but rather by clearance integrity a far more biologically relevant and prognostically powerful metric.

Under this upgraded framework, migraine should be clinically categorized into three physiological stages not two:

Tablo 2: Glymphatic Functional Staging and Clinical Intervention Priorities in Migraine

Stage	Glymphatic Status	Clinical Meaning	Intervention Priority
Stage I - Stable	Normal clearance	Episodic, reversible	Early lifestyle & sleep protection
Stage II - Compensatory Hyperactive	Overworking but coping	High attack reactivity but still recoverable	Critical intervention window preservation phase
Stage III - Exhaustional Failure	Reduced inflow & delayed efflux	Chronic, pharmacoresistant state	Focus shifts to damage control & reversal support

Reference: Zhang et al., 2023

This classification is more than theoretical it has direct therapeutic consequences:

• **In Stage I,** the goal is not aggressive medication but protection of sleep quality, autonomic balance, and AQP4 polarity before it is threatened. Preventive care begins before pain escalates.

• **In Stage II,** conventional pharmacotherapy can still work, but must be combined with clearance-preserving interventions sleep-phase stabilization, vagus stimulation, anti-inflammatory neurofluid regulation or collapse into Stage III becomes inevitable.

• **In Stage III,** attempting to "treat harder with the same method" often fails, because the target has moved the pain is no longer the disease, the clearance failure is. At this point, only system-rebuilding strategies have true disease-modifying potential.

In other words, migraine is only fully treatable while the glymphatic system is still able to fight back. The urgency is not determined by pain severity but by whether the brain still clears what it inflames.

Therapeutic Reorientation: Cleaning Before Suppressing

The treatment philosophy for migraine must therefore undergo a fundamental reversal. Instead of trying to suppress what the brain is producing, we must first restore the brain's ability to clear what it has already produced. Pain is not the primary therapeutic target the failure to eliminate pain-driving metabolites is.

This distinction is critical:

• Pain-suppressive strategies (triptans, NSAIDs, CGRP mAbs) silence nociceptive output but leave the toxic biochemical load intact. They are neurological mute buttons not detoxifiers.

• Clearance-restorative strategies (deep sleep enhancement, AQP4 polarity protection, vagus-driven glymphatic stimulation, optimized respiration/posture) support the brain's intrinsic self-cleaning systems, reducing the likelihood that nociceptive signals will need to be generated again.

In this paradigm, the central therapeutic question is no longer:
"How do we stop today's migraine?"
but rather:
"How do we prevent the brain from entering a metabolic state where migraine becomes inevitable tomorrow?"
This aligns with a deeper biological law observed across neurodegenerative research:
A brain that fails to clean itself will eventually fail to regulate itself.

Migraine, like Alzheimer's and Parkinson's, may ultimately belong to a spectrum of neuroclearance failure disorders, differing only in chronicity, molecular burden, and network vulnerability (Cao et al., 2024).

Practical Clinical Implications: How Migraine Treatment Must Be Reconstructed

If we accept that migraine is a failure of brain clearance before it is a failure of pain control, then the therapeutic hierarchy must be radically reorganized not by discarding current medications, but by reordering clinical priorities to align with the true biological failure timeline (Burgos et al., 2024:517-225).

The future migraine treatment algorithm must follow this order:

1. **Preserve and optimize glymphatic function**
    - prioritizing deep NREM3 sleep quality,
    - protecting AQP4 astrocytic polarity,
    - supporting parasympathetic tone (especially via vagus modulation).
2. **Prevent inflammatory accumulation between attacks**
    - enhance meningeal lymphatic efflux via posture and respiratory techniques,
    - reduce neuroimmune burden before it reaches exhaustion threshold,
    - treat the "interictal toxic load" not only the ictal attack.
3. **Only then suppress pain if necessary**
    - triptans, NSAIDs, CGRP inhibitors remain valuable,
    - but now positioned as secondary, not primary,
    - always combined with clearance-preserving strategies.
4. **Do not allow Stage II to silently convert into Stage III**
    - chronic migraine begins before attack frequency increases,
    - its earliest signature is loss of nightly recovery, not increase in pain days.
    - Intervention must occur while the system is still compensating, not after it has collapsed.

Thus, the central clinical command becomes clear:

Clean the brain first suppress the pain second. Because a brain that is not allowed to clean will keep creating pain, no matter how often we suppress it. This single reversal from suppression-first to clearance-first may represent the most critical paradigm shift in migraine medicine in 50 years (Huang et al., 2023:64–74; Cao et al., 2024).

Practical Clinical Implications: How Migraine Treatment Must Be Reconstructed

If migraine is fundamentally a failure of the brain's clearance mechanisms rather than merely a failure of pain control, then treatment priorities must be reorganized not by eliminating existing medications, but by placing them in the correct biological sequence. The first and most important priority is to preserve and optimize glymphatic function. This

requires maintaining deep NREM Stage 3 sleep, protecting astrocytic AQP4 polarity, and supporting parasympathetic tone, particularly through vagus nerve modulation. These measures ensure that the brain is still capable of clearing inflammatory waste during the night (Vittorini et al., 2024).

The second objective is to prevent the accumulation of inflammatory molecules between attacks, rather than waiting for the next episode to occur. This includes enhancing meningeal lymphatic efflux through postural and respiratory optimization, reducing neuroimmune burden before it reaches the exhaustion point, and addressing the interictal inflammatory load, not only the ictal (pain-phase) symptoms (Cao et al., 2024).

Only after these clearance mechanisms are protected should pain suppression be introduced, using agents such as triptans, NSAIDs or CGRP inhibitors. These medications remain clinically valuable but in this new hierarchy, they become secondary rather than primary tools, and must always be combined with clearance-preserving strategies rather than being used in isolation.

A critical clinical warning arises from this model: Stage II (compensatory hyperactive phase) must not be allowed to silently progress into Stage III (exhaustional failure). Chronic migraine begins before attack frequency increases, and its earliest warning sign is the loss of full nightly recovery, not necessarily the rise in pain days. Intervention must therefore occur while the system is still compensating, before the point of irreversible decline (Burgos et al., 2024:517-225).

For this reason, the central clinical command becomes clear: Clean the brain first suppress the pain second. A brain that is not allowed to clean itself will inevitably keep producing pain, no matter how aggressively we suppress its signals. This shift from suppression-first to clearance-first represents perhaps the most critical paradigm change in migraine medicine in the past fifty years (Huang et al., 2023:64–74).

CONCLUSION

The evolving understanding of migraine has made one fact clear: migraine is not primarily a disorder of excessive neural activation, but rather a disorder of insufficient brain clearance. Pain is not the disease itself it is a symptom of the brain's failure to effectively eliminate pro-inflammatory and metabolically toxic substances. As long as the brain retains its ability to clean itself, migraine remains episodic, reversible, and responsive to pharmacological therapies. However, once the glymphatic–meningeal lymphatic system becomes exhausted, migraine transitions into a persistent and treatment-resistant state not because the pain necessarily intensifies, but because the brain can no longer return to its physiological baseline.

This marks a critical turning point in clinical neurology. Migraine must no longer be treated simply as a storm to be silenced, but as a

physiological clearance system to be preserved. The timing of intervention is therefore essential. Treatment should not begin only when pain becomes stronger it should begin at the moment the brain begins to fail in its ability to clear itself, even if the pain is not yet severe. This is not merely a therapeutic suggestion; it is a neurophysiological necessity.

A brain that maintains efficient clearance does not remain in pain. Conversely, a brain that fails to clear its inflammatory burden will eventually lose the capacity to recover, regardless of how aggressively symptoms are suppressed.

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Heat- Induced Acrylamide Formation in Cereal-Based Foods: Analytical Approaches and RP-HPLC Application to Kavurga

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ABSTRACT

Acrylamide is recognized as a chemical compound that emerges during thermal processing of foods, particularly when carbohydrate-rich matrices are exposed to elevated temperatures through processes such as frying, baking, or roasting. Owing to experimental evidence indicating neurotoxic, genotoxic, and carcinogenic potential, acrylamide has been classified by the International Agency for Research on Cancer as a Group 2A substance, indicating probable carcinogenicity to humans. Dietary exposure to acrylamide is largely associated with cereals and cereal-derived products; however, research has predominantly focused on industrially processed foods, while traditional products remain underrepresented in the literature. Kavurga, a traditional Turkish food obtained by dry roasting of wheat grains at high temperatures, constitutes a potentially relevant but insufficiently studied source of dietary acrylamide exposure. This book chapter presents a comprehensive overview of acrylamide formation mechanisms, influencing factors, health effects, and analytical determination methods, with particular emphasis on chromatographic techniques. In addition, a simple, reliable, and cost-effective reversed-phase high-performance liquid chromatography (RP-HPLC) method with UV detection was developed and validated for the determination of acrylamide in roasted wheat (kavurga). Sample preparation involved aqueous extraction followed by matrix clarification using Carrez reagents. Chromatographic separation was achieved on a C18 column using a water–methanol mobile phase, with detection performed at 210 nm. The developed method demonstrated excellent linearity over the studied concentration range ($R^2 = 0.9995$), with limits of detection and quantification of 4 ppm and 5 ppm, respectively. Intra-day and inter-day validation studies showed satisfactory accuracy and precision. Application to real samples revealed no detectable acrylamide in lightly roasted wheat, whereas 5 ppm acrylamide was detected in dark-roasted kavurga, highlighting the strong dependence of acrylamide formation on roasting severity conditions.

Keywords – Acrylamide, RP-HPLC, Kavurga,

INTRODUCTION

Wheat (Triticum aestivum) is an annual herbaceous plant species belonging to the genus *Triticum* and is the only wheat species that has been extensively bred worldwide. Wheat is one of the most widely cultivated cereals globally and plays a fundamental role in human nutrition. Together with maize and rice, it accounts for approximately one-third of the global caloric intake. Due to its ease of cultivation, high adaptability, and ability to grow under a wide range of climatic conditions, wheat has been regarded as a staple food for thousands of years.

Wheat is a biologically and nutritionally rich cereal grain. It provides nearly half of the calories consumed worldwide and is an important source of carbohydrates (60–70%, mainly starch), proteins (10–15%, primarily gluten proteins), minerals (Cu, Mg, Zn, P, and Fe), vitamins (B-complex and E), riboflavin, niacin, thiamine, and dietary fiber (2–3%). Consumption of whole wheat is particularly important for intestinal health, glycemic control, and cardiovascular health (Shewry, 2009; Kumar et al., 2013; USDA, 2022; Halid, Hameed, and Tahir, 2023).

The origin of wheat traces back to present-day Southeastern Anatolia. Archaeological evidence indicates that wild wheat species were domesticated approximately 10,000 years ago around the Karacadağ region, reinforcing the role of Mesopotamia in the agricultural revolution. Anatolia is considered one of the most important centers in the world in terms of both the genetic diversity of wheat and early agricultural activities (Killian et al., 2009; Velimirovic et al., 2009; Aktaş et al., 2018).

Processing and Uses of Wheat

Depending on its intended use, wheat undergoes various processing methods (Khan and Shewry, 2009; Shewry, 2010; Özkaya and Özkaya, 2019):

- **Milling:** Production of flour, bran, and semolina
- **Fermentation:** Bread and bakery products
- **Roasting:** Kavurga and similar roasted cereal products
- **Boiling:** Bulgur and wheat for traditional desserts

In addition, wheat is used in alcohol production (beer, whisky), starch derivatives, and animal feed.

What Is Roasted Wheat (Kavurga)?

Kavurga is a traditional Turkish roasted cereal product obtained by roasting or lightly toasting wheat grains on a metal plate, pan, or a suitable heated surface. It is a type of traditional snack that is characteristic of many regions of Anatolia. Historically, kavurga has been considered an important food source in nomadic cultures due to its long shelf life and high energy content. Today, it continues to be widely consumed as both a reflection of traditional dietary habits and a natural snack option, particularly in Eastern Anatolia, Central Anatolia, and the Black Sea regions.

From a nutritional perspective, kavurga largely preserves the natural constituents of wheat grains. Wheat is a cereal rich in protein, vitamin E, starch, essential amino acids, phenolic compounds, and dietary fiber. For this reason, kavurga has traditionally been preferred in regions with harsh winter conditions, as it provides prolonged satiety and high energy intake (Bayram, 2005; Demir and Kılınç, 2009; Özbek, 2010; Işıklı et al., 2014).

The roasting or toasting process enhances the aromatic properties of the grains, increases crispness, and improves the sensory quality of the product. However, thermal processing may also induce chemical reactions

such as Maillard reactions, caramelization, lipid oxidation, and degradation of certain phenolic compounds. While these reactions contribute positively to flavor and color development, they may also lead to the formation of undesirable compounds, including heat-induced processing contaminants such as acrylamide. Therefore, controlling roasting temperature and time is critical to maintaining product quality and safety (Kayahan, 2003; Bayram, 2005; Shewry, 2010; Işıklı et al., 2014).

During the thermal processing of foods, particularly those containing both amino acids and reducing sugars, heat-induced chemical compounds such as acrylamide and 5-hydroxymethylfurfural (HMF) are formed as a result of the Maillard reaction (Surh et al., 1994; Stadler et al., 2002; Charnock, 2022). The Maillard reaction accelerates at temperatures above 120 °C and becomes especially pronounced in foods subjected to frying or baking at temperatures between 140 and 180 °C, such as potato chips, biscuits, bread, crackers, and breakfast cereals. The formation of these reaction products has raised significant global food safety concerns due to their potential genotoxic and neurotoxic effects (Longhua et al., 2012). Among these compounds, acrylamide and HMF are widely recognized in the literature as major heat-induced food contaminants and are considered critical from both food processing and consumer health perspectives (Capuano and Fogliano, 2011).

Acrylamide: Formation, Mechanism, and Health Effects

Thermal treatments are commonly employed in food processing to improve microbial safety and prolong product stability (Capuano and Fogliano, 2011). Nevertheless, exposure of carbohydrate-rich foods to elevated temperatures may result in the formation of acrylamide, particularly in products such as potatoes, biscuits, and fried or baked snacks. Acrylamide formation is closely associated with temperature-dependent non-enzymatic browning reactions occurring during food processing.

At the molecular level, acrylamide originates mainly from the interaction between free amino acids, especially L-asparagine, and reducing sugars including glucose and fructose. Upon heating, asparagine initially reacts with carbonyl compounds to form unstable intermediates, which subsequently undergo rearrangement and degradation reactions. These thermal degradation pathways promote the generation of acrylamide together with other low-molecular-weight compounds and volatile by-products.

The rate of acrylamide formation increases markedly above 120 °C and becomes most pronounced within the temperature range of 140–180 °C. As a result, dry heat processing techniques such as frying, baking, and roasting are considered the most critical operations contributing to acrylamide occurrence. Foods characterized by high carbohydrate availability, elevated asparagine content, and low moisture conditions are therefore more susceptible to acrylamide formation during thermal

processing (Capuano and Fogliano, 2011; Charnock, 2022; Adimas et al., 2024).

Factors Affecting Acrylamide Formation

The amount of acrylamide formed varies depending on the food composition and processing conditions. The main influencing factors are summarized below (Rydberg et al., 2005; Ciesarova et al., 2006; Adimas et al., 2024):

1. Chemical Composition of the Food

- **Asparagine level:** The main precursor of acrylamide; wheat, barley, and potatoes contain high levels of asparagine.
- **Reducing sugar content:** Increased glucose and fructose levels lead to higher acrylamide formation.
- **Moisture content:** Low moisture accelerates the Maillard reaction.
- **pH:** Acidic conditions reduce acrylamide formation, whereas alkaline conditions enhance it.

2. Thermal Processing Conditions

- **Temperature:** Rapid increase above 120 °C, with maximum formation between 140 and 180 °C.
- **Time:** Prolonged cooking or roasting increases acrylamide levels.
- **Cooking method:**

  Frying > Baking > Dry roasting > Boiling (no acrylamide formation during boiling).
- **Color development:** Darker product coloration is generally associated with higher acrylamide levels.

Health Effects of Acrylamide

Acrylamide (CH_2=CH–$CONH_2$, Figure 1) is a colorless, odorless, and water-soluble crystalline chemical compound, with a solubility of 215.5 g/100 mL at 30 °C and a molecular weight of 71.08 g/mol. It is a widely used industrial chemical worldwide and has recently been identified as a compound that is naturally formed in foods subjected to high-temperature cooking processes.

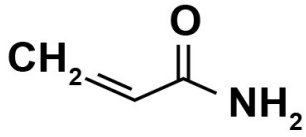

Figure 1. Chemical structure of acrylamide

Experimental studies have demonstrated that acrylamide exhibits neurotoxic, reproductive toxic, and carcinogenic properties in several animal models. In humans, however, neurotoxic manifestations have been reported

mainly under conditions of high-level exposure, particularly in occupational environments. The biological pathways responsible for acrylamide-induced neurotoxicity are thought to play a central role in mediating other adverse effects observed in experimental animals.

At the cellular level, acrylamide interferes with kinesin-related motor proteins involved in intracellular transport within neurons, as well as with proteins that regulate vesicle fusion at nerve terminals. Disruption of these processes can impair neuronal function and ultimately result in neuronal degeneration. Such neurotoxic effects, together with associated behavioral changes, may negatively influence the reproductive capacity of exposed laboratory animals.

Kinesin motor proteins are also essential for normal sperm motility; therefore, their inhibition by acrylamide may lead to altered reproductive outcomes and reduced fertility. Furthermore, the interaction of acrylamide with kinesin-associated proteins provides a plausible explanation for some of its reported genotoxic effects. These proteins contribute to the assembly of the mitotic spindle during cell division, which is critical for accurate chromosome segregation. Disturbance of spindle formation may underlie the clastogenic effects observed in genotoxicity assays and sperm cell damage tests following acrylamide exposure.

Other mechanisms underlying ACR-induced carcinogenesis or neurotoxicity are likely related to its affinity for sulfhydryl groups in proteins. Binding to sulfhydryl groups can inactivate proteins and enzymes involved in DNA repair and other critical cellular functions. Direct interaction with DNA may or may not be a major mechanism of cancer induction in animals. The DNA adducts formed are not specifically associated with tumor sites, and acrylamide generally yields negative results in gene mutation assays, except at high doses that are unlikely to be achieved through dietary exposure. Furthermore, epidemiological studies have not demonstrated an increased cancer risk associated with either high occupational exposure or low-level dietary intake of acrylamide (Exon, 2006; Pedreschi, 2007; Michalak et al., 2013; Pennisi et al., 2013; Erkekoglu and Baydar, 2014; Fan et al., 2023).

Analytical Methods for Acrylamide Determination

Acrylamide, which is classified as a *probably carcinogenic compound to humans* by the International Agency for Research on Cancer (IARC), has been shown in numerous studies to be formed in starch-rich foods subjected to high-temperature cooking, including various baked and fried products. Consequently, extensive research has been conducted using different analytical approaches to determine acrylamide levels in foods exposed to high temperatures.

In early studies, due to the high polarity of acrylamide, gas chromatography–mass spectrometry (GC–MS) methods involving a

derivatization (bromination) step were predominantly developed. In these approaches, acrylamide is converted into more volatile derivatives, such as 2,3-dibromopropionamide, followed by determination using isotope dilution—typically with a ^{13}C-labeled acrylamide internal standard—in selected ion monitoring (SIM) mode (Nemoto et al., 2002; Roach et al., 2003; Castle and Eriksson, 2005). These methods have been successfully applied to various food categories, including potato chips, fried potatoes, cereal-based products, and breakfast cereals, achieving limits of detection and quantification at the μg/kg level. The use of GC–MS/MS has further enhanced selectivity and accuracy, with low background noise and good repeatability particularly reported for potato-based products (Negoita et al., 2020).

A report by Barber et al. (2001) on the determination of underivatized acrylamide and glycidamide in rat plasma using reversed-phase high-performance liquid chromatography provided a foundation for the development of liquid chromatography–mass spectrometry (LC–MS) methods for acrylamide analysis in foods. LC–MS/MS rapidly became the preferred analytical technique. The Swedish National Food Agency developed an LC–MS/MS method for acrylamide determination in potato and cereal products over a concentration range of 30–10,000 ppb. This method monitored five acrylamide product ions along with one labeled acrylamide product ion for identification and quantification in food extracts (Rosen and Hellenas, 2002). The Stockholm University research group further investigated the sources of acrylamide formation during cooking using GC–MS and LC–MS/MS, reporting moderate levels in protein-rich foods and higher levels in carbohydrate-rich foods (Tareke et al., 2002).

Terada and Tamura (2003) developed a simple and reliable column-switching HPLC method with UV detection for the determination of acrylamide in processed foods. Following aqueous ultrasonic extraction and clean-up using Oasis HLB cartridges, acrylamide was separated using a two-column HPLC system. The method demonstrated a low detection limit (10 μg/kg), high recoveries (93.1–101.5%), and good repeatability, and was successfully applied to potato-based products and instant noodles.

Bebius et al. (2024) validated an LC–MS/MS method under AOAC “First Action 2023.01” guidelines for a wide range of matrices, including coffee, cereal products, infant foods, dried fruits, roasted nuts, snacks, spices, and dry pet foods, and performed single-laboratory validation.

Wheat-based products such as bread, biscuits, and breakfast cereals represent major sources of dietary acrylamide exposure; therefore, controlling acrylamide formation in these foods is critical to reducing dietary intake and associated health risks. As a result, numerous studies have focused on acrylamide analysis in such products. In particular, acrylamide has been evaluated both quantitatively and through non-target screening using high-resolution mass spectrometry (HRMS) in biscuits and baked

products (Fernandes et al., 2019). Alpözen et al. (2014) developed an LC–MS/MS method for acrylamide determination in three traditional bread types—white bread, bran bread, and whole wheat bread—analyzing the crust portions of 85 bread samples.

Mesías et al. (2022) presented a comprehensive review summarizing acrylamide levels in cereal products and the associated measurement methodologies, evaluating the effects of different formulations and processing conditions (e.g., whole grain, fiber addition, fortification) on acrylamide content. Ölmez et al. (2008) reported an overview of acrylamide contents in 311 processed and traditional Turkish foods, finding average acrylamide levels of 247, 198, and 152 µg/kg in crackers, biscuits, and infant biscuits, respectively.

Şenyuva and Gökmen (2005) analyzed acrylamide levels in 120 retail food products randomly collected from markets in Türkiye, reporting average acrylamide concentrations across food groups in the following order: crackers > potato chips > biscuits > cakes > infant foods > corn chips > cookies > breakfast cereals > bread > grilled vegetables > wafers > chocolate. While no acrylamide was detected in bread crumb, significant levels were observed in bread crust. Gündüz et al. (2017) investigated acrylamide levels in 90 commercial cracker, biscuit, and infant biscuit samples sold in Türkiye using GC–MS following bromination derivatization, reporting mean acrylamide concentrations of 604, 495, and 153 µg/kg in crackers, biscuits, and infant biscuits, respectively.

Geng et al. (2011) developed a novel derivatization-based analytical method for acrylamide determination in starch-based foods, involving aqueous extraction, defatting with hexane, derivatization with $KBrO_3$/KBr, and liquid–liquid extraction. The resulting 2-bromopropenamide was quantitatively determined by HPLC-DAD and confirmed by GC–MS. Longhu et al. (2012) developed an SPE-based HPLC method using a reversed-phase C18 column for the sensitive determination of acrylamide in food samples, achieving low detection limits, high enrichment factors, and good repeatability, with recoveries of 88.9–89.5% in fortified potato samples and successful application to potato peel and potato chips.

In another study, the applicability of an ion-pair RP-HPLC method coupled with diode-array detection (DAD) at 200 nm was evaluated for the determination of acrylamide concentrations in cereal-based infant foods. The optimal sample preparation procedure included extraction with 80% methanol in water, defatting with hexane, a freezing step, and clean-up using Oasis HLB solid-phase extraction cartridges (Michalak et al., 2013). Atdekani et al. (2015) investigated acrylamide levels in Sangak bread produced in Shiraz, Iran, using an SPE-HPLC method. Acrylamide concentrations ranging from 10 to 12 ppm were determined in 60 bread samples, with no statistically significant correlation observed between

acrylamide formation and flour-related parameters such as moisture, protein, gluten, and ash content.

Bagheri et al. (2019) reported the fabrication of a magnetic molecularly imprinted polymer in which propionamide was employed as a template to form a chitosan-based imprinted layer on an Fe_3O_4@PEG magnetic core under aqueous conditions. The resulting dual-template MIP material was coupled with HPLC and successfully applied for the selective determination of acrylamide in biscuit samples, demonstrating the potential of imprinting-based approaches for complex food matrices.

In a more recent contribution, Wei and Xiong (2024) proposed a reversed-phase HPLC method with UV detection for acrylamide analysis in starch-rich foods. Their procedure involved lipid removal using hexane, followed by ultrasonic extraction with chloroform prior to chromatographic analysis. Separation was performed on a C18 column under isocratic conditions. The method showed strong analytical performance, characterized by excellent linearity ($r = 0.9991$) and acceptable recovery values ranging from 87.6% to 97.8%.

Determination of Acrylamide in Roasted Wheat (Kavurga) by RP-HPLC

This work focuses on evaluating acrylamide occurrence in kavurga, a traditional wheat-based cereal product produced by high-temperature roasting, and on establishing a reversed-phase high-performance liquid chromatography (RP-HPLC) method suitable for its quantitative determination. The proposed analytical approach was systematically developed and validated to ensure reliable, sensitive, and reproducible measurement of acrylamide. Method performance was assessed through standard validation parameters, including accuracy, precision, recovery, and detection capability. Furthermore, the validated method was applied to kavurga samples in order to quantify the acrylamide levels formed under different roasting conditions.

Preparation of Solutions

An acrylamide stock solution with a nominal concentration of 100 mg/mL was freshly prepared using deionized water as the solvent. From this stock, a series of working calibration standards covering the concentration range of 5–300 ppm (5, 15, 40, 60, 100, 200, and 300 ppm) were obtained by stepwise dilution with deionized water. In addition, quality control samples were prepared independently at three concentration levels (20, 60, and 250 ppm) following the same dilution strategy to evaluate method performance.

Carrez I Solution

Fifteen grams of potassium hexacyanoferrate (II) trihydrate [$K_4[Fe(CN)_6]\cdot 3H_2O$] were weighed and transferred into a 100 mL volumetric flask. Eighty milliliters of distilled water were added, and the solution was thoroughly mixed until complete dissolution. The volume was then adjusted

to 100 mL with distilled water. The solution was stored in an amber bottle at refrigerator temperature until use.

Carrez II Solution

Thirty grams of zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) were weighed and transferred into a 100 mL volumetric flask. Eighty milliliters of distilled water were added, and the solution was thoroughly mixed until complete dissolution. The volume was then adjusted to 100 mL with distilled water. The solution was stored in an amber bottle at refrigerator temperature until use.

Extraction Procedure

Roasted wheat grains (kavurga) were first ground to obtain a uniform and fine powder. From the homogenized material, an accurately weighed 5.00 g aliquot was placed into a 50 mL conical centrifuge tube. Deionized water (15 mL) was added, and the suspension was mixed thoroughly using a vortex mixer for 2 min. The mixture was subsequently subjected to ultrasonic extraction in a water bath maintained at 50 °C for 30 min. After the extract was allowed to cool to ambient temperature, clarification was achieved by sequential addition of Carrez reagents: 2.0 mL of Carrez I solution (15% $K_4[Fe(CN)_6]$) followed by vortex mixing for 1 min, and then 2.0 mL of Carrez II solution (30% $ZnSO_4 \cdot 7H_2O$) with an additional 1 min of mixing.

The formed precipitate was removed by centrifugation at 10,000 rpm for 10 min, and the clear supernatant was carefully collected. The resulting extract was concentrated to dryness and subsequently redissolved in deionized water prior to chromatographic determination.

Chromatographic Conditions and Calibration Curve

Chromatographic measurements were carried out on an Agilent Technologies 1200 Series high-performance liquid chromatography system. The separation of acrylamide was accomplished using a reversed-phase C18 column (250 × 4.6 mm, 5 μm). A mixture of water and methanol (90:10, v/v) was employed as the mobile phase and delivered at a constant flow rate of 2.0 mL/min. UV detection was performed at 210 nm, and a sample volume of 15 μL was injected into the system for each analysis.

Chromatographic runs of the prepared working standards were carried out, and the corresponding peak responses were evaluated based on integrated peak areas. Acrylamide was consistently eluted at a retention time of approximately 2.7 min. Quantitative evaluation was performed using an external calibration approach by correlating peak area responses with the respective analyte concentrations, as illustrated in Figure 2.

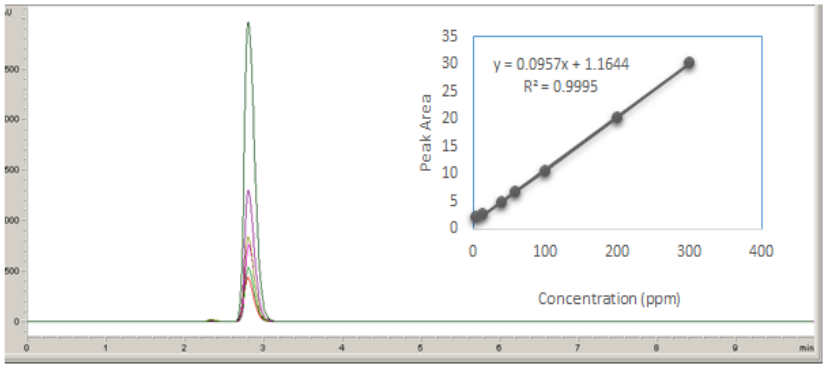

Figure 2. RP-HPLC chromatogram of acrylamide showing the retention time (2.7 min) and the calibration curve constructed from peak area versus concentration under optimized conditions.

Calibration Curve, LOD, and LOQ

The obtained calibration curve showed excellent linearity and was described by the equation $y = 0.09557x + 1.1644$ with a determination coefficient of $R^2 = 0.9995$, where *y* represents the peak area and *x* represents the concentration (ppm).

The limit of detection (LOD), defined as the concentration corresponding to a signal-to-noise (S/N) ratio of 3, was determined to be 4 ppm, while the limit of quantification (LOQ), corresponding to an S/N ratio of 10, was determined to be 5 ppm.

Accuracy and Precision

The accuracy and precision of the method were evaluated in terms of intra-day and inter-day variability. Intra-day and inter-day experiments were performed using acrylamide solutions prepared at three concentration levels (10, 65, and 250 ppm) within the calibration range. Chromatograms were recorded, and six replicate analyses were performed at each concentration level. The mean concentration, standard deviation (SD), relative standard deviation (RSD), and relative error (RE) were calculated from the obtained peak areas.

Method accuracy was evaluated using percentage relative error (RE%), whereas method precision was assessed on the basis of percentage relative standard deviation (RSD%). The calculated RE% and RSD% values obtained from both intra-day and inter-day experiments are presented in Table 1.

Table 1. Acuuracy and Precision of Proposed Method

	Intra-day			Inter-day		
Added (ppm)	Mean±SD (ppm)	Accuracy RE%	Precision RSD%	Mean±SD (ppm)	Accuracy RE%	Precision RSD%
10	9.67±0.04	-3.30	0.41	10.11±0.05	1.10	0.49
65	66.10±0.08	1.69	0.12	63.66±0.09	2.06	0.14
250	248.88±1.01	-0.45	0.41	251.45±1.22	0.58	0.49

SD: Standard Deviation, RSD: Relative Standart Deviation, RE:Relative Error

Application to Real Samples

Wheat grains were roasted in a pan over a stove under two different roasting conditions until they reached (i) a light pink coloration and (ii) a dark brown to nearly black appearance. Following roasting, 200 g portions from each sample were collected and subjected to acrylamide extraction according to the procedure described in the sample preparation section. The extracts were then analyzed under identical chromatographic conditions using the developed RP-HPLC method.

No detectable acrylamide levels were observed in the lightly roasted wheat sample, indicating that mild roasting conditions were insufficient to induce measurable acrylamide formation. In contrast, acrylamide was detected at a concentration of 5 ppm in the heavily roasted wheat (kavurga) sample, suggesting that prolonged roasting at higher temperatures significantly promotes acrylamide formation.

These findings are consistent with the known temperature-dependent formation mechanism of acrylamide in carbohydrate-rich foods, where higher roasting severity leads to increased Maillard reaction activity. The representative chromatogram obtained from the dark-roasted wheat sample is shown in Figure 3, confirming the successful application of the proposed RP-HPLC method to real food matrices.

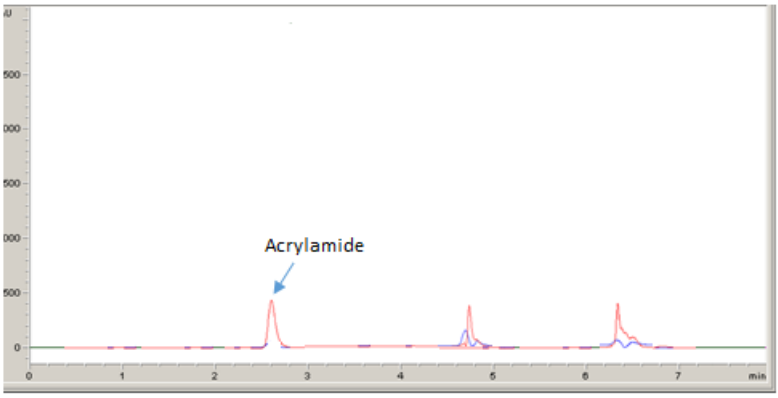

Figure 3. RP-HPLC chromatogram of acrylamide detected in dark-roasted wheat (kavurga) under the optimized analytical conditions.

Conclusion

In this study, a simple, reliable, and cost-effective RP-HPLC method was successfully developed and validated for the determination of acrylamide in roasted wheat (kavurga), a traditional cereal-based food subjected to high-temperature processing. The method demonstrated excellent linearity over the studied concentration range (R^2 = 0.9995), with satisfactory sensitivity, as evidenced by a limit of detection (LOD) of 4 ppm and a limit of quantification (LOQ) of 5 ppm.

Method validation results confirmed good accuracy and precision under both intra-day and inter-day conditions, with relative error and relative standard deviation values well within acceptable limits. The applicability of the method to real food samples was demonstrated by the successful detection of acrylamide in dark-roasted wheat samples, while no detectable levels were observed in lightly roasted samples. This finding highlights the critical role of roasting severity in acrylamide formation and underscores the importance of controlling processing conditions in cereal-based foods.

Overall, the developed RP-HPLC method offers a practical analytical alternative for routine monitoring of acrylamide in roasted wheat and similar cereal products, particularly in laboratories lacking access to advanced mass spectrometric instrumentation. The results of this study contribute to the limited literature on acrylamide occurrence in kavurga and may support

future efforts aimed at improving processing strategies to minimize acrylamide formation while preserving product quality and safety.

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Effect of Glass-Fiber Reinforcement on the Flexural Strength of a Provisional Crown Material

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ABSTRACT

This study aimed to evaluate the effect of different glass-fiber reinforcement ratios on the flexural strength of an autopolymerizing PMMA provisional restoration material and to determine the influence of thermal aging on these mechanical properties. PMMA specimens reinforced with glass fibers at 0.75%, 1.5%, 3%, 6%, and 12% by weight were prepared, along with a non-reinforced control group. Each group was divided into thermocycled and non-thermocycled subgroups (n = 10). A total of 120 specimens were tested. Flexural strength was measured using a three-point bending test after applying 2,500 thermal cycles (5 °C – 55 °C) to relevant subgroups. Before thermal cycling, only the 12% glass-fiber group demonstrated a significant increase in flexural strength compared with the control. After thermal cycling, the 12% group showed significantly higher flexural strength than all other reinforced groups and displayed results comparable to the control group. Thermal aging generally reduced flexural strength, except in the highest fiber-reinforced specimens. Glass-fiber reinforcement - particularly at 12% - enhances the flexural strength of autopolymerizing PMMA, providing improved performance after aging. Higher reinforcement ratios may offer clinical benefits for long-term provisional restorations.

Keywords – Temporary restorations, polymethyl methacrylate, glass fiber, flexural strength

INTRODUCTION

Polymethyl methacrylate (PMMA) has been used in dentistry since 1937 and has become one of the most preferred materials for denture bases (Ladha & Shah, 2011). PMMA offers several clinically desirable characteristics, including durability, dimensional and chemical stability, biocompatibility, cost-effectiveness, and an acceptable taste (Ladha & Shah, 2011). Although PMMA provides advantages such as favorable esthetics and ease of manipulation, it also exhibits certain limitations (Yazdanie & Mahood, 1985). Its relatively low transverse strength, impact resistance, and fatigue resistance may lead to fractures during clinical function (Yazdanie & Mahood, 1985).

Autopolymerizing PMMA resin is widely used for the fabrication of provisional restorations due to its ease of use, low cost, and ability to achieve a polished surface. However, the mechanical properties of PMMA are not ideal. Occlusal forces that exceed the elastic limit of the material can generate stress - particularly in the marginal regions of connectors - resulting in deformation or fracture (Protopapa et al., 2011). Provisional restorations

represent a critical phase in fixed prosthodontic treatment; they must provide pulpal and periodontal protection, exhibit proper marginal integrity and esthetics, and withstand functional masticatory loads (Rosenstiel et al., 2001). A fractured provisional restoration compromises treatment continuity, may lead to unplanned clinical visits, and negatively affects patient comfort (Ireland et al., 1998).

Flexural strength is one of the most important mechanical properties of provisional restorations, particularly in cases involving long-span prostheses with short pontics and connectors (Rosenstiel et al., 2006). This property becomes even more crucial in patients exhibiting parafunctional habits such as bruxism or clenching (Nejatidanesh et al., 2009). Fracture of provisional prostheses creates significant clinical challenges, leading to esthetic and functional problems as well as potential tooth movement. Repair procedures are often time-consuming, technically demanding, and inconvenient for both the clinician and the patient (Ireland et al., 1998). Therefore, patients undergoing long-term provisionalization - such as those receiving full-mouth rehabilitation - require materials with enhanced mechanical performance (Kamble et al., 2012).

Fiber reinforcement of acrylic resins has been shown to improve flexural strength, impact resistance, and fatigue resistance (Alla et al., 2013). Various fiber types, including nylon, polyethylene, polyamide, and especially glass fibers, have been investigated due to their biocompatibility and favorable esthetic and mechanical properties (Tacir et al., 2006). The incorporation of glass fibers has been demonstrated to significantly enhance the flexural strength, impact strength, toughness, and Vickers hardness of acrylic resins (Moreno-Maldonado et al., 2012; Yu et al., 2012; Singh et al., 2016). Owing to these improvements - particularly in flexural behavior and fatigue resistance - glass fibers have become one of the most commonly used reinforcement materials for denture base polymers (Tacir et al., 2006). The aim of the present study was to evaluate the effect of different glass-fiber reinforcement ratios on the flexural strength of an autopolymerizing PMMA provisional restoration material and to investigate how thermal aging influences these properties. Accordingly, the first null hypothesis was that varying glass-fiber ratios would not affect the flexural strength of the provisional PMMA material, and the second null hypothesis was that thermal aging would have no influence on the flexural strength.

MATERIALS AND METHODS

A powder–liquid polymethyl methacrylate (PMMA)-based provisional acrylic resin, Temdent Classic (Schütz Dental GmbH, Friedberg, Germany), was used in this study. Glass fibers (Dost Kimya, Istanbul, Turkey) with a length of 3 mm were incorporated into the resin at five different weight percentages: 0.75%, 1.5%, 3%, 6%, and 12%. Fiber

quantities were measured using a precision balance. Together with an unreinforced control group, six experimental groups were established. Each group was subsequently divided into two subgroups based on whether thermocycling was applied (n = 10), yielding a total of 120 specimens.

Specimen fabrication was performed using metal molds with cavity dimensions of 64 × 10 × 3 mm. The autopolymerizing acrylic resin was mixed at room temperature according to the manufacturer's instructions and packed into the molds, followed by trial closures. A pressure of 2 bar was applied, and after a 20-minute polymerization period, the specimens were retrieved from the molds. Half of the specimens in each group underwent thermocycling consisting of 2,500 cycles between 5 °C and 55 °C using a chewing simulator (MOD Chewing Simulator; MOD Dental, Ankara, Türkiye). Flexural strength was assessed using a three-point bending test performed in a universal testing machine (Model 2519-106; Instron Corp., USA) with a crosshead speed of 1 mm/min. All statistical analyses were carried out using statistical software (IBM SPSS Statistics, Version 27; IBM Corp., Armonk, NY, USA).

RESULTS

Table 1 presents the mean flexural strength values and standard errors of all specimens with and without thermocycling. In the groups without thermocycling, the 12% fiber-reinforced group exhibited significantly higher flexural strength than all other groups ($p < 0.05$). Although the control group showed higher mean values compared with the 3% and 6% fiber-reinforced groups, these differences were not statistically significant. However, the control group demonstrated significantly higher flexural strength than the 1.5% and 0.75% fiber-reinforced groups ($p < 0.05$).

In the thermocycled groups, the 12% fiber-reinforced group showed significantly higher flexural strength values than all groups except the control ($p < 0.05$).

When comparing specimens with and without thermocycling within each group (control and all fiber-reinforced groups), no statistically significant differences were observed between the subgroups ($p > 0.05$). Bar graphs showing distribution of flexural strength values (MPa - megapascals) according to study groups in Figure 1.

Table 1. Flexural strength values of the samples (MPa-megapascals)

Group	Non-TS Mean ±Std. Err.	TS Mean ±Std. Err.
Control	83.70 ±10.72[b]	88.94 ±12.66[a,b]
0.75%	75.39 ±16.96[d]	67.59 ±13.37[c]
1.50%	72.05 ±12.32[cd]	75.35 ±9.27[b,c]
3%	81.98 ±9.19[bc]	82.61 ±7.92[b]
6%	80.64 ±10.70[bc]	84.91 ±14.68[b]
12%	100.72 ±7.37[a]	103.44 ±7.39[a]

Non-TS: Without thermocycling, TS: Thermocycling, Std. Err.: Standard error.
Mean values sharing the same letter are not significantly different ($p < 0.05$).

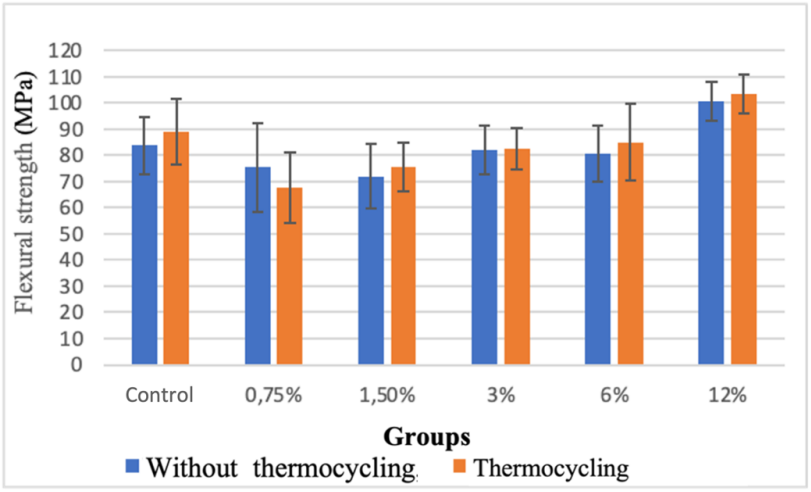

Figure 1. Flexural strength values of the groups (MPa).

DISCUSSION

In the present study, the incorporation of glass fibers into PMMA-based provisional acrylic resin resulted in varying effects on flexural strength depending on the fiber ratio and the application of thermocycling. In the non-thermocycled groups, only the 12% glass fiber–reinforced specimens demonstrated a statistically significant increase in flexural strength compared with the control group, while no significant differences were observed among the other fiber concentrations. Therefore, the first hypothesis - anticipating a significant improvement in all reinforced groups - was rejected. In contrast, within the thermocycled groups, the 12% fiber-

reinforced specimens exhibited significantly higher flexural strength than all groups except the control, supporting the second hypothesis ($p < 0.05$).

Previous research has widely explored reinforcement strategies to enhance the fracture resistance of provisional fixed partial dentures, often through the incorporation of various fibers into acrylic polymers. Studies have examined the effects of polyethylene, carbon (graphite), and glass fibers on acrylic resin performance (Larson et al., 1991). PMMA remains the most commonly used denture base material due to its favorable esthetics, color stability, ease of manipulation, and polishability; however, its mechanical limitations necessitate reinforcement to improve clinical performance (Polyzois et al., 1996). Traditional approaches to strengthening PMMA-based prostheses involve using high-strength resins or embedding reinforcing materials into the polymer matrix, and advancements in fiber-reinforced composites have further stimulated interest in improving the mechanical behavior of dental acrylics (Stipho, 1998).

Among fiber reinforcement types, glass fibers have consistently demonstrated superior performance. Their addition enhances flexural and impact strength and improves fatigue resistance, thereby extending the functional lifespan of provisional prostheses (Alla et al., 2013). Earlier authors reported that compared with nylon, polyethylene, and aramid carbon/graphite fibers—which may suffer from poor esthetics, inadequate bonding to the resin matrix, or impractical laboratory application—glass fibers outperform alternative reinforcement materials (Alla et al., 2013; Gad et al., 2018). Limitations of other fiber systems include polishing difficulties and unaesthetic appearance in carbon or aramid fibers (Kaine et al., 2000), as well as complex surface treatment requirements for polyethylene fibers (Dixon & Breeding, 1992). In contrast, the advantages of glass fibers include excellent esthetics, superior mechanical properties, and biocompatibility, making them the most suitable reinforcement option for PMMA-based materials (Goguta et al., 2006).

Numerous studies corroborate the reinforcing effect of glass fibers. Mosharraf et al. (2019) demonstrated that adding glass fibers significantly improved fracture resistance, highlighting their clinical relevance for FDPs exposed to high occlusal loads. Similarly, PMMA-based FDPs showed satisfactory long-term clinical performance, supporting their continued use as durable provisional restorations (Huettig et al., 2016). Alhotan et al. (2021) further reported that increasing filler content generally enhances surface hardness, and among different reinforcements, glass fibers produced the largest improvement in flexural strength, outperforming ZrO_2 and TiO_2 fillers. Additional evidence indicates that PMMA reinforced with glass and nylon fibers exhibits significantly greater flexural strength than unreinforced denture base resins, particularly when the fibers are treated with coupling agents and incorporated with monomers to optimize bonding (Kannaiyan et al., 2020).

Environmental and intraoral factors also influence the mechanical properties of PMMA. The flexural strength of heat-cured acrylic resin decreases with changes in salivary pH (Alzaid et al., 2023), and although PMMA is widely used for temporary crowns and bridges, its inherent hardness and fracture resistance remain insufficient under complex occlusal loads (Alt et al., 2011). Heat-cured PMMA exhibits superior properties compared with cold-cured PMMA (Frazer et al., 2005). Glass fibers, being inorganic materials with an amorphous structure and randomly oriented tetrahedral silica bonds, differ from organic fibers such as polyethylene. Their lower water absorption and reduced susceptibility to discoloration support their clinical viability (Tuncdemir & Aykent, 2012). Additionally, glass fibers - available in woven or loose forms - offer esthetic and mechanical advantages over other reinforcement types. When silanated, they provide enhanced bonding, thus improving the flexural strength of PMMA, although the magnitude of reinforcement depends on fiber type, surface treatment, and orientation within the matrix (John et al., 2001). Fiber reinforcement distributes stresses more uniformly across the resin, reducing stress concentration and improving the material's resistance to applied loads (Caneppele et al., 2013).

The literature offers mixed findings on the effect of thermocycling. Bergamo et al. (2022) reported that thermocycling increased the flexural strength of conventional PMMA used for interim prostheses, whereas Ribeiro et al. (2023) concluded that thermocycling generally decreases flexural strength of most provisional materials, including acrylic resins, although it does not alter surface roughness. Differences in experimental design, material composition, and fiber pretreatment may explain these discrepancies. Regarding material processing, milled PMMA has been shown to exhibit greater flexural strength and color stability compared with 3D-printed PMMA, although further investigations are needed to confirm these findings (Shenoy et al., 2022). Color stability also varies among provisional materials, with PMMA-based imicryl showing the highest degree of color change in comparative studies (Yesil et al., 2023). Despite these challenges, high-quality provisional restorations are essential for achieving successful long-term definitive prostheses (Mizrahi, 2019).

Previous research found that reinforcing PMMA with low concentrations of glass fibers (0.25%, 0.50%, and 1.0%) significantly increases flexural strength compared with unreinforced specimens, as confirmed by three-point bending tests (Yerliyurt et al., 2023). These findings align with the present results, particularly the substantial enhancement observed in the 12% reinforcement group, suggesting that increased fiber concentration, up to an optimal threshold, may further improve mechanical performance.

The present study supports the evidence that glass-fiber reinforcement improves flexural strength, particularly at higher concentrations, and that this

benefit is largely maintained following thermocycling. However, this in-vitro study does not fully replicate the complexity of intraoral conditions. Only one fiber type and limited concentration ranges were tested, and thermocycling was used as the sole aging method; therefore, the findings may not directly predict long-term clinical performance.

CONCLUSION

Glass-fiber reinforcement improved the flexural strength of the autopolymerizing PMMA provisional material, with the 12% fiber ratio demonstrating the highest performance both before and after thermal aging. Thermal cycling reduced flexural strength in most groups, yet reinforced specimens-particularly those with higher fiber content-retained superior mechanical behavior. These findings suggest that higher concentrations of glass fibers may provide clinically advantageous reinforcement for long-term provisional restorations.

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Traditional Medicinal Uses of Crataegus Taxa Distributed in Türkiye

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ABSTRACT

The genus *Crataegus*, which belongs to the Rosaceae family, is represented in Turkish flora by 34 taxa, 10 of which are endemic. This study aims to compile the traditional medicinal uses of *Crataegus* taxa that are naturally distributed in Turkey, and to evaluate commonly used species in light of existing literature. A comprehensive literature review revealed that 15 taxa of the genus *Crataegus* growing in Turkey are traditionally used by local populations to treat various health conditions. The most frequently cited species are *C. monogyna*, *C. orientalis* and *C. azarolus*. *Crataegus* species are particularly known for their supportive role in the treatment of cardiovascular disorders. These plants are also believed by the public to possess cardioprotective, antihypertensive and digestive properties. The parts of the plant most commonly used are the fruits, leaves and flowers. While the fruits are often eaten fresh, they can also be used to make infusions, decoctions, marmalades, vinegar and jam. The leaves and flowers are generally prepared as infusions or decoctions. In conclusion, *Crataegus* species continue to be valued as traditional remedies, particularly in rural areas of Turkey. However, further scientific research is required to clarify their pharmacological effects, bioavailability and toxicological safety profiles. Such research would support the safe and effective development of functional foods and phytotherapeutic products derived from *Crataegus* species, and could contribute to the discovery of new natural drug candidates.

Keywords – Crataegus, ethnobotany, medicinal plants, Rosaceae, Anatolia

INTRODUCTION

Turkey has an exceptionally rich and diverse flora, making it one of the most botanically significant countries in the world. It is particularly notable for its abundance of wild plant species, exhibiting considerable richness in overall floral diversity and endemic taxa. With an estimated 12,000 plant species,

Turkey is a global biodiversity hotspot (Ekim 2024). This diversity is largely attributed to the country's unique phytogeographical location and the long-standing cultural heritage of Anatolia, establishing Turkey as a key centre for ethnobotanical research (Ertuğ, 2014).

According to Davis (1965-1988), 8,796 species have been documented within the Turkish flora. Furthermore, the Illustrated Flora of Turkey identifies approximately 9,996 species across 1,320 genera and 167 families, amounting to nearly 12,000 taxa (Güner et al., 2012). The rate of endemism is notably high, with approximately 32% of plant species being endemic to the country (özmen, 2025).

The genus *Crataegus*, belongs to the Rosaceae family and comprises thorny, deciduous shrubs or small trees. The leaves are typically lobed with serrated margins, and the flowers are generally white or pink, each with five petals. The fruits are classified as pseudocarps and range in colour from red to dark purple when ripe. *Crataegus* species are predominantly distributed across the temperate regions of the Northern Hemisphere and parts of Central America. Due to frequent hybridisation and apomixis, the genus is considered taxonomically complex (Christensen, 1992; Dönmez and Bani 2008).

Members of this genus are commonly used as ornamental plants and for hedges. They typically inhabit forest edges, woodlands, scrublands, and both low and high mountain zones (Christensen, 1992). Globally, the genus comprises approximately 283–300 taxa, 10 of which are endemic to Turkey, where the genus is represented by 34 taxa in total. The endemism rate within Turkey is around 30% (Güner et al., 2012).

Species of the genus *Crataegus* have long been used in traditional medicines in Europe, Asia and North America. In recent years, this genus has received increasing scientific attention, resulting in a substantial rise in pharmacological research. Numerous studies have demonstrated the therapeutic potential of *Crataegus* species, particularly in the prevention and management of cardiovascular diseases (Baytop, 1999; Mustafa et al., 2024). Notably, investigations focusing on *Crataegus monogyna* and *Crataegus oxyacantha* have

shown that fruit and leaf extracts exhibit significant antioxidant, anti-inflammatory, and cardioprotective activities (Kumar et al., 2012).

They have been employed for ages as herbal remedies against cardiovascular diseases, valued for their broad spectrum of health benefits including anti-inflammatory, anticoagulant, antithrombotic, antioxidant, hypotensive, and hypolipidemic properties (Cui et al., 2024). These applications are also supported by evidence-based research, which has led scientists to label *Crataegus* species as 'the most valuable remedy for the cardiovascular system that can be found in nature' (Martinelli et al., 2021). Products of *Crataegus* extracts are promoted worldwide against angina, arrhythmia, and congestive heart failure (Edwards et al., 2012). Heart failure is a persistent and worsening clinical syndrome resulting from structural or functional impairments of the heart. Mortality rates in patients with heart failure are rather very high (Arrigo et al., 2020).

Clinical studies have further substantiated the vasodilatory and positive inotropic effects of *Crataegus* extracts, particularly in treating conditions such as heart failure and hypertension (Güleç et al., 2023). Furthermore, both in vitro and in vivo experiments have indicated that Crataegus extracts contribute to improved lipid metabolism, modulation of cholesterol levels, and reduction of oxidative stress (Gözcü et al., 2024). The fruits of *Crataegus* species are also recognised for their high flavonoid and phenolic compound content, which has been linked to immunomodulatory effects and enhanced immune system function (Liao et al., 2024).

The primary aim of this review is to provide a comprehensive analysis of the traditional medicinal uses of *Crataegus* species in Turkey. By synthesising existing literature, the review aims to establish a scientific basis for future research into the biological activities of *Crataegus* species and their potential integration into pharmacological studies and evidence-based phytotherapeutic practices.

METHODOLOGY

Ethnobotanical studies carried out in different regions of Turkey from 2005 to 2025 were reviewed and medicinal uses, of *Crataegus* taxa were determined. Relevant studies were searched in detail and were collected from books, doctorate dissertations and master's theses, and scientific literature databases (PubMed, Scopus, Google Scholar, Web of Science, SciFinder, Springer, and Elsevier, National Thesis Center (YÖK Ulusal Tez Merkezi Veritabanı) (YÖK Tez Veri Tabanı, 2025). Key words such as "Ethnobotany Turkey, *Crataegus* ethnobotany, Türkiye'de alıç, *Crataegus* Turkey, Medicinal plants Turkey, Medicinal Plants *Crataegus*" for search were used to reach articles or thesis. The scientific names of plants and plant families were verified using Plants of the World online (Plants of the World Online, 2024).

RESULT AND DISCUSSION

Turkey has a rich and diverse flora, most of which is endemic, and it also has a long-standing ethnobotanical tradition. This study has compiled information on the traditional uses of taxa belonging to the genus *Crataegus* that are native to Turkey and have known medicinal applications among the local population.

Over 100 ethnobotanical and medicinal plant studies were reviewed, among which Crataegus taxa were cited in 68 sources. 15 different taxa of the genus *Crataegus* growing in Türkiye are traditionally used by local populations to treat various health conditions (Table 1). These taxa, which are widely distributed across the Turkish flora, hold significant ethnobotanical value due to their extensive traditional usage and demonstrated medicinal potential. Crataegus monogyna emerged as the most frequently cited species for therapeutic purposes. Notably, *C. monogyna* Jacq. has been

recognized across various cultures as a plant of considerable importance in traditional medicine systems.

The berries of Crataegus are commonly consumed for their reputed therapeutic effects, particularly in the management of heart failure and other age-related conditions (Barros, 2011; Çalışkan, 2015). In addition to the immature fruits, the flowers and leaves are also valued for their pharmacologically active constituents. Research has shown that the leaves generally contain higher levels of flavonoids compared to the fruits (Belabdelli et al., 2022).

Other taxa commonly used in folk medicine in Türkiye include C. orientalis, C. azarolus, C. tanacetifolia and *C. pentagyna* (Fig. 1). The medicinal applications of Crataegus taxa in Turkey have been categorized into ten major therapeutic groups (Table 2)

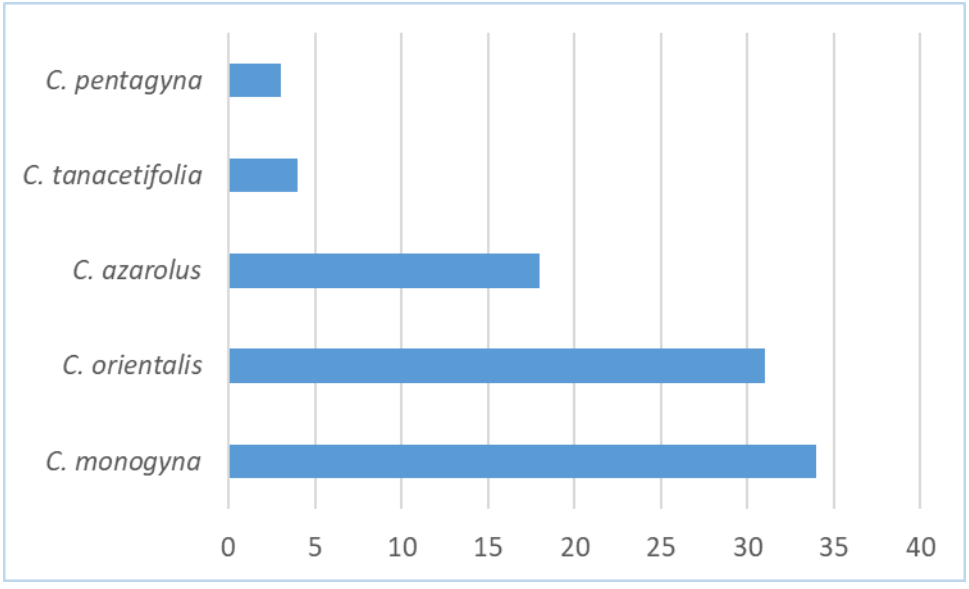

Figure 1. The most used taxa for medicinal porpuses in Türkiye.

Primarily, these taxa are traditionally employed in the treatment of cardiovascular disorders, such as arrhythmias, hypertension, vascular insufficiencies, hypercholesterolemia, and

embolic conditions. The widespread ethnobotanical use of *Crataegus* taxa for cardiovascular disorders clearly reflects a strong traditional association with the circulatory system. This alignment is strongly supported by contemporary pharmacological research, which consistently highlights the cardioprotective, cardiotonic, antihypertensive, hypolipidemic, anti-inflammatory, antithrombotic, antioxidant, and even sedative properties of the genus (Cui et al., 2024; Martinelli et al., 2021; Belabdelli et al., 2022; Taleghani et al., 2024). Specifically, leaf extracts of C. tanacetifolia have demonstrated hypotensive effects on blood pressure and heart rate, particularly through interactions with beta-blockers and atropine (Taleghani et al., 2024).

Table 1. Medicinal uses of *Crataegus* taxa in Türkiye

Scientific name	Local name	Used part	Preparation methods	Application methods	Use purposes	References
Crataegus azarolus L.	Alıç, Aluç, Azrulê, Dukok, Gijok, Guhij, İzaran, Müzmüldek, Sarı alıç	Fruits	Infusion	Internal	Antitussive	Kerar and Akan, 2019
					Tachycardia	İlbaş et al., 2024
			Vineger	Internal	Cardiovascular dissease, shortness of breath	Çorlu, 2023
			Eaten raw	Internal	Edema dispenser, diabetes	Bilgiç, 2023
					Boosting the immune system	Atay, 2025
		Leaves	Infusion	Internal	Kidney dissease	Atay, 2025
					Edema dispenser, diabetes	Bilgiç, 2023
		Flowers	Decoction	Internal (two times a day)	Cholesterol	Çorlu, 2023
				Internal	Cholesterol	Çorlu, 2023
		Fruits, leaves	Decoction	Internal	Pass a kidney stone	Aksu et al., 2024
Crataegus	Ahlat, Alıç	Fruits	Infusion	Internal	Headache,	Gürdal and Kültür,

azarolus var. *aronia* L. (Syn: *Crataegus aronia* L. Bosc.)					cardiovascular problems	2013
			Decoction	Internal	Cardiac dissease	Kerar and Akan, 2019
			Eaten raw	Internal	Antihypertensive	Yeşil and İnal 2021
					Heart disseases	Kılıç et al., 2020
		Leaves, flowers, fruit	Decoction	Internal	Embolism	
Crataegus azarolus v*ar. pontica* (K.Koch) K.I.Chr.	Alıç, Aluç, Datıbabı, Kart, Kırkat	Fruits	Marmelade	Internal	Cardiovascular problems	Kazancı et al., 2020
			Eaten raw with lemon and honey	Internal	Cardiac dissease, antihypertensive	Karakaya et al., 2019
						Karakaya et al., 2020
Crataegus curvisepala Lindm.	Alıç	Roots	Decoction	Internal	Tuberculosis	Demir, 2020
Crataegus meyeri Pojark.	Alıç, Rığok, Roğık	Roots	Decoction	Internal	Tuberculosis	Demir, 2020
		Fruits and roots	Eaten raw, decoction	Internal	Antidiarrheal, diabetes	Yeşil and Akalın, 2009
Crataegus microphylla K.Koch	Kocakarı armutu, Kocakarı elması, Kocakarı	Fruits	Infusion, eaten raw	Internal	Cardiac and tension problems, shortness of breath	Özdemir Nath, 2016

	hurması, Yemişgen					
Crataegus monnogyna Jacq	Alıç, Alıç gülü, Aluç, Arıç, At gözü, Ayoş, Beyaz diken, Bilanok, Deli alıç, Ekşi muşmula, Gîvij, Guhîj, Guviç, Guler, Haliç, İzaran, Keçi alıcı, Kırıntı, Kırmızı alıç, Kız Elması, Koca karı hurması, Öküzgötü, Yağlı alıç, Yemişen, Yemişgen,	Fruits	Eaten raw	Internal	Tonic	Saraç and Özkan, 2013
					Sedative, cardiovascular problems	Uğulu et al., 2009
					Embolism	Yıldırımlı, 2004
			Decoction	Internal	Headache, constipation, amnesia	Akbulut and Karaköse, 2023
					Cholesterol	Gözcü et al., 2024
					Cardiovascular problems	Doğan, 2024
					Rheumatism (on a full stomach, one tea cup)	Çorlu, 2023
				Internal (on an full stomach, one tea cup)	Rheumatism, Cholesterol, shortness of breath	Çorlu, 2023
			Jam, marmelade	Internal	Haemorrhoids,	Kadıoğlu et al., 2021

	Yemişgen çalısı	Dried fruits	Infusion		asthma, bronchitis	
		Fruits	Infusion	Internal	Kidney dissease, cardiac dissease, diabetes	Kazancıet al., 2020
		Fruits, dried fruits	Infusion, ekstrat	Internal	Sedative, cardiovascular problems	Uğulu et al., 2009
		Fruits		Internal	Antihypertensive	Doğan et al., 2024
		Fruits	As fruits juice	Internal	Cardiovascular problems	Demir and Demir, 2022
			Infusion, marmelade	Internal	Respiratory problems, common cold	Arı et al., 2015
			Vineger, pickle	Internal	Asthma, bronchitis, antitussive	Akkan, 2024
			Vineger	Internal	Cardiovascular problems	Çorlu, 2023
		Flowers	Decoction	Internal	Kolesterolü düşürücü, shortness of breath	Çorlu, 2023
					Rheumatism, cholesterol,	Çorlu, 2023

					shortness of breath	
			Infusion	Internal	Cardiac dissease	Baykan et al., 2023
					Embolism, asthma	Yıldırımlı, 2004
		Leaves	Infusion	Internal	Cardiac dissease, embolism, icterus	Aslan et al., 2020
		Stems			Cardiovascular problems	Kocabaş and Gedik, 2016
		Aerial parts	Infusion	Internal	Stomachache, Cardiovascular problems	Emre at al., 2021
		Fruits and leaves	Infusion	Internal	Sedative, cardiovascular problems	Mumcu and Korkmaz, 2018
			Infusion	Internal	Constipation, digestive system problems	Doğan and Durumlu, 2024
			Infusion, jam	Internal	Cardiac and digestive system dissease	Olcay et al., 2022
			Eaten raw	Direct	Cardiac dissease, embolism, icterus	Aslan et al., 2020
		Fruits and flowers	Decoction	Internal	Urinary tract disorders	Bulut and Tuzlacı, 2013

		Branches with flowers, leaves	Decoction, infusion	Internal (three or four times a day)	Sedative, cardiac dissease, antihypertensive	Ecevit Genç and Özhatay, 2006
		Fruits, flowers, leaves	Infusion	Internal	Diuretic, sedative, painkiller	Yalçın et al., 2021
			Infusion, eaten raw	Internal	Cardiovascular problems, painkiller, embolism, ulcer	Özdemir Nath, 2016
			Infusion and decoction	Internal	Constipation, rheumatism, antihypertensive	Olcay et al., 2022
		Fruits, flowers, stems,	Decoction	Internal	Cardiac disease, reducing heart rate, antihypertensive, stomach disorders, headache, diuretic	Olgun, 2019
Crataegus orientalis Pall. ex Bieb.	Ahlat, Alıç, Aloş, Aluç, Ardıç, Civicasur,	Fruits	Infusion	Internal	Cardiac disease, shortness of breath	Kazancı et al., 2020
			Decoction	Internal	Cardiovascular	Alagöz, 2023

	Civica zer, Gaheşik, Gırgat, Guhiş, Gühüj, Haziran, Kırmızı alıç, Kocakarı Fruitssi, Kürdili, Sez, Sev, Sınz, Sönz, Şeze, Şilan, Risok, Roğık, Tatlı baba, Töngel				problems	
					Diabetes	Yiğit and Gözcü, 2024
					Haemorrhoids	Şener at al., 2023
					Diabetes, rheumatism	Arı et al., 2015
					Diuretic, cardiotonic	Yeşil and İnal, 2021
					Constipation	Doğan, 2024
			Juice	Internal	Cardiovascular problems	Demir and Demir, 2022
			Infusion, decoction	Internal	Nervous system diseases, antidiarrheal, antihypertensive	Akbulut and Karaköse, 2023
			Eaten raw	Direct	Cardiac disease, antihypertensive	Karakaya et al., 2019
					Cardiac disease, antihypertensive	Karakaya et al., 2020
					Rheumatism	Geylani, 2024
					Pacemaker	Ulcay and Şener, 2024
		Flowers	Infusion	Internal	Antihypertensive, hyperglycemia	Gürbüz et al., 2021
					Asthma,	Çorlu, 2023

					bronchitis	
		Dried flowers	Infusion	Internal	Cardiac disease	Geylani, 2024
		Leaves	Infusion	Internal	Cardiovascular problems, diabetes	Polatman and Demir, 2024
		Fruits, leaves	Infusion	Internal	Edema dispenser, diabetes	Bilgiç, 2023
		Flowers, fruits, branches	Marmelade, infusion	Internal	Itch relief, asthma - bronchitis, intestinal problems, embolism	Korkmaz and Alparslan, 2014; Korkmaz and Karakuş, 2015
		Flowers, fruits, leaves	Infusion and eaten raw	Internal	Stomach disorders, atherosclerosis, embolism	Ulcay and Şener, 2024
		Flowers, fruits, leaves, roots	Decoction, infusion, pickle	Internal	Nervous system diseases, antidiarrheal, antihypertensive	Akbulut et al., 2023
		Fruits, leaves, dal, roots	Decoction	Internal	Shortness of breath, haemorrhoids	Kadıoğlu et al., 2021

		Flowers, fruits, dal, stems, roots	Decoction	Internal	Antihypertensive, cardiotonic	Olgun, 2019
				Internal	Artheriosclerosis, rheumatism, osteoarthritis	Cansaran et al., 2010
Crataegus orientalis subsp. *szovitsii* (Pojark.) K.I.Chr. (*) (Syn: *Crataegus szovitsii* Pojark.)	Alıç, Kan alıcı, Yemişen	Leaves	Infusion	Internal	Cardiac dissease	Özdemir Nath, 2016
		Fruits	Decoction		Tachycardia, antihypertensive, artheriosclerosis, postnasal drip	Somuncu, 2024
		Fresh branches	Infusion		Cardiovascular problems, diabetes	Somuncu, 2024; Korkmaz and Alparslan, 2014
		Flowers and leaves	Infusion		Blood circulation	Bulut et al., 2017
		Leaves and branches with flowers	Decoction		Cardiovascular problems, prostate, haemorrhoids, kidney dissease, carminative for pregnants	Ecevit Genç and Özhatay, 2006

Crataegus pentagyna Waldst. & Kit. ex Willd.	Siroğ, Karakat, at gözü, Kömüş dikeni, Yabani kırkat	Fruits	Jam with *Mentha* sp.	Internal	Cardiac disorders, antihypertensive	Kazancı et al., 2020
			Eaten raw	Direct	Cardiotonic	Akkavak Zurnacı, 2019
Crataegus pseudoheterophylla Pojark.	Gıvij, Öküzgötü	Fruits	Eaten raw	Direct	Cardiovascular problems	Demir and Demir, 2022
		Fruits, leaves	Infusion	Internal	Edema dispenser, diabetes	Bilgiç, 2023
Crataegus rhipidophylla Gand	Alıç	Fruits	Infusion, decoction, jam	Internal	Cardiovascular problems, urinary tract infection, common cold, flu	Karaköse, 2022
Crataegus tanacetifolia	Alıç, Sarı alıç	Fruits	Eaten raw	Direct	Shortness of breath	Yeşilyurt et al., 2017
		Flowers	Infusion	Internal	Sore throat, antihypertensive, artheriosclerosis	Uzun et al., 2016
		Leaves	Infusion		Shortness of breath	Yeşilyurt et al., 2017
		Fruits, branches, flowers	Infusion, marmelade		Throat infection, Cardiovascular problems, diabetes, antihypertensive, sedative	Korkmaz and Karakurt, 2015

Crataegus × *bornmuelleri* Zabel	Alıç, Cıvica zar, Gıvica zar	Flowers, fruits, leaves	Decoction, infusion	Internal	Antihypertensive, rheumatism	Yeşil and Akalın, 2009
		Roots, bark of roots, secondary roots				
Crataegus × *sinaica* Boiss.	Alıç	Fruits	Infusion	Internal	Antitussive, common cold, flu	Demir, 2020

Secondly, Crataegus taxa are widely used in the treatment of respiratory system disorders, including cough, shortness of breath, bronchitis, and asthma. This is likely attributed to the plant's expectorant and soothing properties on the respiratory tract[77].

Thirdly, these taxa are utilized to alleviate digestive disorders, such as constipation, gastric pain, and bloating. Fourthly, they are traditionally applied in the management of musculoskeletal conditions, especially rheumatism and joint pain, likely due to their anti-inflammatory properties[78].

Furthermore, Crataegus taxa have shown relevance in the treatment of endocrine disorders, particularly for their blood glucose-lowering effects in diabetic conditions. In the context of nervous system disorders, they have been used—albeit to a lesser extent—for issues such as headaches, memory impairments, and anxiety, largely due to their sedative effects. Additionally, some sources recommend Crataegus for alleviating anxiety and insomnia[79].

Phytochemical studies on various parts of C. microphylla (leaves, stem bark, and fruits) collected from Turkey have revealed antioxidant properties (DPPH and FRAP assays), DNA damage protection, and inhibitory activity against α-glucosidase. Enzyme inhibition related to the nervous system, such as acetylcholinesterase and tyrosinase, has also been observed[80].

Moreover, in Crataegus pentagyna, a total of 49, 42, and 33 phenolic compounds have been identified in the fruit, leaves, and roots, respectively. These compounds exhibit strong antioxidant and antibacterial activities[23]. The flowers and leaves of Crataegus taxa native to Turkey have also shown substantial antioxidant capacity, with C. monogyna consistently demonstrating particularly high levels of antioxidant activity[81].

Table 2. The categories and use records of hawthorn in Türkiye.

Categories	Numbers of Use Record
Cardiovascular System Disorders	74
Respiratory System Disorders	31
Digestive System Disorders	20
Musculoskeletal System Disorders	13
Endocrine System Disorders	12
Nervous System Disorders	11
Urinary System Disorders	9
Lymphatic & Fluid Regulation Issues	4
Immune System	3
Reproductive / Other Conditions	1

In Turkey, the parts of *Crataegus* taxa most commonly used in traditional folk medicine are, in order of frequency, the fruits (33 use records), leaves (17 use records), flowers (17 use records), roots (8 use recırds) and branches (5 use records). Additionally infusion is the most commonly used (38 use records) method for the traditional medicinal application of *Crataegus* taxa. This preparation technique is primarily used to treat cardiovascular, digestive and respiratory system disorders. The second most common method is decoction (23 use records), in which the plant's fruits as well as its harder parts such as roots and branches are boiled in water. This method is traditionally used to treat cardiovascular diseases, respiratory tract infections and digestive problems. The third most common method is consuming the raw plant material (13 use records), particularly the fresh fruits. This practice is widespread among the general population for purposes such as boosting immunity, supporting heart health and aiding digestion. These taxa, which are also commonly consumed as food and are believed to have therapeutic value when used in various food forms such as vinegar, pickles, and jam.

The notable alignment between traditional ethnomedical practices and contemporary scientific findings regarding Crataegus highlights not only its longstanding therapeutic value but also affirms its continued relevance in the management and prevention of cardiovascular and systemic disorders.

This intersection of knowledge systems underscores the critical role of ethnobotanical insights as a foundational pillar for phytopharmacological advancements and the evolution of integrative medical approaches.

Crataegus taxa are also traditionally used in the treatment of rheumatism, diabetes, and kidney-related disorders. However, the pharmacological validation of these uses remains limited[82], indicating a need for expanded research in these areas. In particular, the anti-inflammatory properties of *Crataegus* suggest potential therapeutic relevance in rheumatic diseases, while its traditional use in diabetes calls for renewed investigation into its effects on glucose metabolism and insulin regulation.

CONCLUSION

This study demonstrates the significant ethnobotanical relevance of *Crataegus* species in Türkiye, particularly for cardiovascular, respiratory, and digestive disorders. Traditional uses are largely supported by pharmacological studies, especially regarding antioxidant, anti-inflammatory, and cardioprotective effects. However, some applications—such as for rheumatism, diabetes, and kidney disorders—require further scientific validation. Overall, the alignment between traditional knowledge and modern research highlights *Crataegus* as a promising genus for future phytopharmacological and integrative medical research.

Conflict of interest statement

The authors declare that they have no conflicts of interests.

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N-Lactoyl-Phenylalanine as a Chronometabolic Signal: Exercise Timing, Circadian Rhythms, and Appetite Regulation

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ABSTRACT

Exercise is increasingly recognized as a powerful regulator of metabolic health not only through its intensity and volume, but also through its timing relative to endogenous circadian rhythms. N-lactoyl-phenylalanine (Lac-Phe), a recently identified exercise-inducible metabolite formed from lactate and phenylalanine, has emerged as a potential mediator of post-exercise appetite suppression and energy balance regulation. Existing literature has primarily characterized Lac-Phe as an acute, intensity-dependent anorexigenic signal linked to lactate flux; however, its integration within circadian metabolic networks remains largely unexplored.

Circadian clocks coordinate daily rhythms in substrate utilization, hormone secretion, appetite regulation, and feeding behavior across central and peripheral tissues, including skeletal muscle, adipose tissue, liver, gut, and the brain. These temporal programs substantially influence metabolic responses to identical physiological stimuli delivered at different times of day. Exercise itself acts as a non-photic zeitgeber capable of modulating peripheral clocks, suggesting that Lac-Phe production, systemic availability, and downstream signaling may exhibit time-of-day dependence.

This chapter synthesizes current evidence on Lac-Phe biology within the framework of metabolic chronobiology. We review the biochemical origins and physiological actions of Lac-Phe, integrate these findings with established principles of circadian appetite regulation and exercise metabolism, and propose a conceptual model in which Lac-Phe functions as a temporally gated metabolic signal linking exercise timing to appetite control and energy homeostasis. Finally, we discuss methodological considerations and key knowledge gaps that must be addressed to advance chrono-exercise–based metabolic interventions.

Keywords – Lactoyl-phenylalanine (Lac-Phe); circadian rhythm; exercise timing; appetite regulation; energy homeostasis

INTRODUCTION

The global rise in obesity and metabolic disease has intensified interest in interventions that regulate both energy intake and energy expenditure with greater precision and sustainability. While physical exercise is a cornerstone of metabolic health promotion, its physiological effects extend beyond total workload, intensity, or energy expenditure. Increasing evidence indicates that the timing of exercise relative to endogenous circadian rhythms critically shapes metabolic outcomes, influencing substrate utilization, hormonal responses, appetite regulation, and long-term energy balance (Panda, 2016; Zarrinpar et al., 2016; Gabriel & Zierath, 2019).

Circadian rhythms are generated by a hierarchical system of biological clocks, with the suprachiasmatic nucleus (SCN) acting as the central pacemaker and peripheral clocks residing in metabolically active tissues such as skeletal muscle, adipose tissue, liver, and the gastrointestinal tract (Takahashi, 2017). These clocks regulate daily oscillations in glucose metabolism, lipid oxidation, mitochondrial function, and appetite-related hormones, thereby conferring strong time-of-day dependence to metabolic processes (Bass & Takahashi, 2010; Asher & Sassone-Corsi, 2015). Disruption of circadian alignment—through shift work, irregular feeding, or mistimed physical activity—is associated with increased cardiometabolic risk independent of total caloric intake or physical activity volume (Scheer et al., 2009; Vetter et al., 2016).

Circadian timing is also integral to appetite regulation. Hunger perception, meal timing vary across the day and converge on hypothalamic feeding circuits that integrate endocrine and nutrient signals to regulate energy intake (Johnston et al., 2014; Morton et al., 2014). Importantly, exercise itself acts as a potent non-photic zeitgeber, particularly for peripheral clocks, and identical exercise stimuli performed at different times can yield distinct metabolic and endocrine responses (Ezagouri et al., 2019; Gabriel & Zierath, 2019; Wolff & Esser, 2012).

Within this chronobiological framework, the identification of N-lactoyl-phenylalanine (Lac-Phe) as an exercise-inducible metabolite introduces a candidate small-molecule signal linking acute metabolic stress to appetite control. Lac-Phe is formed through the conjugation of lactate and phenylalanine and rises robustly following high-intensity exercise. In rodents, exogenous Lac-Phe suppresses food intake and attenuates weight gain in diet-induced obesity models without evidence of reduced locomotor activity, while in humans circulating Lac-Phe increases with exercise intensity and correlates with reduced ad libitum energy intake after acute exercise (Li et al., 2022). However, whether Lac-Phe exhibits circadian rhythmicity at baseline or time-of-day–dependent responses to standardized exercise stimuli, and whether such differences translate to meaningful effects on appetite-related outcomes, remains unknown.

Mechanistically, circadian control of glycolytic flux, lactate transport and clearance, and amino acid handling may influence Lac-Phe production and systemic exposure, while circadian variation in gut hormone secretion and hypothalamic sensitivity may gate the anorexigenic impact of a given Lac-Phe concentration. Accordingly, this chapter synthesizes current evidence on Lac-Phe biology within a metabolic chronobiology framework, reviews established principles of circadian appetite regulation and exercise metabolism, and proposes testable models in which Lac-Phe functions as a temporally gated signal linking exercise timing to appetite control and energy homeostasis. We conclude by outlining methodological standards and key

knowledge gaps required to advance chrono-exercise–based metabolic interventions.

1. Circadian Regulation Of Metabolism And Appetite, and Exercise

Circadian rhythms are endogenous biological oscillations with an approximately 24-hour periodicity that align physiology and behavior with predictable environmental cycles. In mammals, circadian regulation is organized hierarchically, with the SCN serving as the central pacemaker and synchronizing peripheral clocks located in metabolically active tissues, including skeletal muscle, adipose tissue, liver, pancreas, and the gastrointestinal tract (Bass & Takahashi, 2010; Takahashi, 2017). This multi-clock system ensures temporal coordination of nutrient handling, energy expenditure, and feeding behavior.

At the molecular level, circadian rhythms arise from transcriptional–translational feedback loops involving core clock genes such as CLOCK, BMAL1, PER, and CRY. These oscillators regulate a substantial fraction of the metabolic transcriptome, with approximately 10–20% of genes in peripheral tissues exhibiting circadian rhythmicity (Panda, 2016; Asher & Sassone-Corsi, 2015). Consequently, key metabolic processes—including glucose tolerance, insulin sensitivity, lipid oxidation, and mitochondrial function—display pronounced time-of-day dependence (Bass & Takahashi, 2010). Beyond regulating transcriptional programs, circadian clocks impose temporal structure on metabolite production, clearance, and tissue responsiveness, thereby shaping the biological meaning of identical metabolic signals delivered at different times of day.

Circadian timing also plays a central role in appetite regulation. Hunger perception, meal timing, and macronutrient preference follow robust daily rhythms, while circulating levels of appetite-regulating hormones such as ghrelin, leptin, peptide YY (PYY), and glucagon-like peptide-1 (GLP-1) fluctuate across the day (Johnston et al., 2014; Panda, 2016). These peripheral signals, together with nutrient- and metabolite-derived cues, converge on hypothalamic feeding circuits, particularly within the arcuate nucleus, where orexigenic and anorexigenic neurons integrate hormonal and metabolic cues to regulate energy intake (Morton et al., 2014). Disruption of circadian alignment has adverse metabolic consequences. Experimental circadian misalignment impairs glucose metabolism, alters appetite control, and promotes positive energy balance independently of caloric intake or physical activity levels (Scheer et al., 2009; Vetter et al., 2016). These findings highlight biological timing as a critical, yet often overlooked, determinant of metabolic health. Importantly, the responsiveness of hypothalamic feeding circuits to peripheral signals is itself circadian, resulting in temporal gating whereby identical hormonal or metabolic inputs can elicit divergent feeding responses depending on circadian phase.

Physical exercise acts as a potent non-photic zeitgeber, particularly for peripheral clocks. These observations indicate that exercise-derived metabolic signals are generated and interpreted within a circadian framework rather than in temporal isolation. Exercise can phase-shift clock gene expression and modulate metabolic responses in a time-of-day–dependent manner, such that identical exercise stimuli performed in the morning versus evening elicit distinct metabolic and hormonal outcomes (Wolff & Esser, 2012; Gabriel & Zierath, 2019; Ezagouri et al., 2019). Exercise can phase-shift clock gene expression and modulate metabolic responses in a time-of-day–dependent manner, such that identical exercise stimuli performed in the morning versus evening elicit distinct metabolic and hormonal outcomes (Wolff & Esser, 2012; Gabriel & Zierath, 2019; Ezagouri et al., 2019). These observations indicate that exercise-derived metabolic signals operate within a circadian framework rather than in temporal isolation.

Together, the circadian regulation of metabolism, appetite, and exercise responsiveness provides a critical foundation for understanding metabolite-based signaling in energy homeostasis. Establishing this chronobiological context is essential for interpreting the physiological relevance of exercise-inducible metabolites, such as N-lactoyl-phenylalanine, which are produced and act within a temporally structured metabolic environment.

2. N-lactoyl-phenylalanine (Lac-Phe): Biogenesis and Metabolic Actions

N-lactoyl-phenylalanine (Lac-Phe) is a member of the growing class of N-lactoyl amino acids, metabolites formed through the conjugation of lactate with amino acids during conditions of elevated glycolytic flux. Interest in Lac-Phe has increased substantially following its identification as a robust exercise-inducible metabolite with putative roles in appetite regulation and body-weight control (Li et al., 2022). Unlike classical exercise-responsive hormones or cytokines, Lac-Phe represents a small-molecule metabolic signal directly derived from substrate metabolism, positioning it as a potential link between acute energetic stress and downstream behavioral responses. Biochemically, Lac-Phe is generated through the condensation of lactate and phenylalanine, a process catalyzed by cytosolic nonspecific dipeptidase 2 (CNDP2) under conditions of elevated intracellular lactate availability. CNDP2 has been shown to catalyze the formation of multiple N-lactoyl amino acids during states of high glycolytic flux, such as intense muscular contraction (Contrepois et al., 2020; Li et al., 2022). Accordingly, circulating Lac-Phe concentrations rise sharply following high-intensity exercise, closely tracking lactate accumulation and returning toward baseline during recovery. This kinetic profile supports the interpretation of Lac-Phe as a metabolite that encodes information about acute glycolytic stress rather than sustained energy deficit.

Available evidence indicates that Lac-Phe exhibits rapid, exercise-linked kinetics, with circulating concentrations rising sharply following high-intensity exercise and returning toward baseline during recovery (Contrepois et al., 2020; Li et al., 2022). This temporal profile closely parallels lactate accumulation and clearance, supporting the interpretation of Lac-Phe as a signal of acute glycolytic stress rather than sustained energy deficit (Brooks, 2018; Li et al., 2022). However, key pharmacokinetic parameters—including tissue distribution, half-life, and routes of clearance—remain poorly defined, as these aspects have not been systematically characterized in either animal or human models (Li et al., 2022). These unknowns are particularly relevant for interpreting Lac-Phe's physiological role and for designing studies aimed at resolving potential time-of-day effects.

Experimental evidence from animal models indicates that Lac-Phe exerts biologically meaningful effects on feeding behavior. In mice, exogenous administration of Lac-Phe suppresses food intake and attenuates weight gain in diet-induced obesity models without affecting locomotor activity or energy expenditure, suggesting a primary action on appetite rather than generalized sickness behavior or reduced physical capacity (Li et al., 2022). Importantly, these effects appear to be selective for feeding, distinguishing Lac-Phe from stress hormones or inflammatory mediators that broadly impair behavior.

Human data, while more limited, support a role for Lac-Phe as a correlate of post-exercise appetite modulation. Targeted metabolomic analyses demonstrate that Lac-Phe concentrations increase in proportion to exercise intensity and are associated with reduced ad libitum energy intake following acute exercise bouts (Li et al., 2022). These observations align with broader evidence that intense exercise transiently suppresses hunger and energy intake, although causality between Lac-Phe and appetite regulation in humans has not yet been established. Notably, Lac-Phe responses appear to vary substantially between individuals, suggesting modulation by factors such as fitness status, substrate availability, and metabolic phenotype.

Despite these advances, the physiological mechanisms through which Lac-Phe influences feeding remain incompletely understood. The molecular targets of Lac-Phe have not been conclusively identified, and it remains unclear whether its effects are mediated through direct central nervous system actions, peripheral sensory pathways, or indirect modulation of established appetite hormones. Given that lactate itself can act as a signaling molecule in the brain and periphery, Lac-Phe may represent a chemically modified extension of lactate signaling with altered stability, tissue distribution, or receptor interactions (Brooks, 2018). However, definitive receptor identification and tissue-specific signaling pathways remain key knowledge gaps. Accordingly, Lac-Phe is best conceptualized at present as a metabolite-level signal whose biological effects are inferred from physiological

associations and preclinical intervention studies, rather than as a defined ligand–receptor system (Contrepois et al., 2020; Li et al., 2022).

Importantly, Lac-Phe biology has thus far been examined largely without consideration of circadian timing. Lactate production and clearance, amino acid metabolism, appetite hormone secretion, and hypothalamic feeding circuitry all exhibit pronounced diurnal rhythmicity (Johnston et al., 2014; Panda, 2016; Takahashi, 2017). Exercise performed at different times of day elicits distinct metabolic and endocrine responses, including differences in glycolytic flux, lactate accumulation, and appetite-related outcomes (Ezagouri et al., 2019; Gabriel & Zierath, 2019). Consequently, both the generation and physiological impact of Lac-Phe are likely to be influenced by circadian phase. Exercise performed at different times of day may therefore yield distinct Lac-Phe exposure profiles and downstream effects, not only in magnitude but also in biological relevance for appetite regulation. Recognizing Lac-Phe as a metabolite embedded within circadian metabolic architecture provides the conceptual foundation for examining how exercise timing shapes its signaling dynamics.

3. Exercise Timing, Circadian Phase, and Lac-Phe Dynamics

The metabolic and endocrine responses to exercise are not uniform across the day but are strongly influenced by circadian phase. Identical exercise stimuli performed at different times of day elicit distinct physiological outcomes, reflecting the temporal organization of substrate availability, hormonal milieu, and peripheral clock activity (Gabriel & Zierath, 2019; Ezagouri et al., 2019). Within this framework, the production and signaling of exercise-induced metabolites such as N-lactoyl-phenylalanine (Lac-Phe) are likely shaped not only by exercise intensity and duration but also by the timing of exercise relative to endogenous circadian rhythms. Although many studies operationalize exercise timing using external clock time (e.g., morning vs evening), the underlying determinant of metabolic responsiveness is circadian phase, which may differ between individuals depending on chronotype, sleep timing, and light exposure (Panda, 2016; Vetter et al., 2016).

Lactate metabolism exhibits pronounced diurnal variation. Skeletal muscle glycolytic flux, lactate production, and lactate clearance capacity differ between the biologically active and rest phases, influenced by circadian regulation of glycogen availability, mitochondrial oxidative capacity, and monocarboxylate transporter expression (Panda, 2016; Brooks, 2018). Human studies demonstrate that high-intensity exercise performed later in the day is often associated with greater peak lactate concentrations and enhanced exercise performance compared with morning exercise, suggesting time-of-day differences in glycolytic engagement and buffering capacity (Ezagouri et al., 2019). Because Lac-Phe formation is closely coupled to lactate availability, diurnal variation in lactate kinetics provides a biologically plausible

mechanism by which Lac-Phe generation could differ according to circadian phase, although this has not yet been directly tested.

Exercise timing also modulates the hormonal environment in which Lac-Phe is produced and acts. Cortisol, catecholamines, insulin sensitivity, and growth hormone secretion all display circadian rhythmicity and interact with exercise responses in a time-dependent manner (Gabriel & Zierath, 2019). Morning exercise is typically performed under higher cortisol concentrations and lower insulin sensitivity, whereas afternoon or evening exercise occurs during a metabolic state more permissive to glucose uptake and glycolytic flux. These hormonal differences may influence both the magnitude of Lac-Phe production and the responsiveness of downstream appetite-regulating pathways.

Emerging evidence supports the concept that exercise acts as a non-photic zeitgeber capable of phase-shifting peripheral clocks, particularly in skeletal muscle and adipose tissue (Wolff & Esser, 2012; Dyar et al., 2018). The timing of exercise determines the direction and extent of these phase shifts, thereby altering the temporal alignment of metabolic gene expression. Repeated exercise at consistent times of day can entrain peripheral clocks, potentially shaping the rhythmicity of metabolite production across days or weeks. In this context, Lac-Phe may function not only as an acute signal of energetic stress but also as part of a temporally structured metabolic program influenced by habitual exercise timing.

Importantly, appetite regulation itself is a circadian phenomenon. Hunger perception, gut hormone secretion, and hypothalamic sensitivity to orexigenic and anorexigenic signals vary across the day (Johnston et al., 2014; Morton et al., 2014). Consequently, the physiological impact of a given Lac-Phe concentration may be temporally gated, such that identical circulating levels elicit different appetite-related responses depending on circadian phase and central feeding circuit sensitivity (Johnston et al., 2014; Morton et al., 2014). For example, Lac-Phe produced during periods of heightened central appetite drive may exert more pronounced anorexigenic effects than identical concentrations generated during phases of reduced feeding propensity. This temporal gating could partially explain interindividual variability in post-exercise appetite responses and inconsistencies across human studies.

Despite these mechanistic considerations, direct investigations of circadian modulation of Lac-Phe dynamics are currently lacking. Most human studies have assessed Lac-Phe responses to exercise without controlling for or systematically varying time of day. As a result, potential interactions between circadian phase, lactate metabolism, and Lac-Phe signaling remain unresolved. Addressing this gap will require study designs that incorporate standardized exercise protocols performed at different circadian phases, alongside high-temporal-resolution metabolomic sampling and careful assessment of appetite-related outcomes.

Taken together, these considerations generate several testable predictions. First, identical exercise bouts performed at different circadian phases should yield distinct Lac-Phe kinetic profiles, including differences in peak concentration and exposure duration. Second, the relationship between Lac-Phe and post-exercise appetite suppression may vary by time of day, independent of exercise intensity. Third, interindividual variability in Lac-Phe responses may be partially explained by circadian phenotype rather than fitness or metabolic status alone. Addressing these predictions will require chrono-controlled exercise studies with high-temporal-resolution metabolomic sampling.

4. Lac-Phe And The Integration Of Central And Peripheral Appetite Signaling

Appetite regulation emerges from the coordinated integration of central neural circuits and peripheral hormonal and metabolite-derived signals, enabling organisms to align energy intake with energetic demand. Central control is centered in the hypothalamus, particularly within the arcuate nucleus, where orexigenic neuropeptide Y/agouti-related peptide (NPY/AgRP) neurons and anorexigenic pro-opiomelanocortin (POMC) neurons integrate hormonal, nutrient, and neural inputs to regulate feeding behavior (Morton et al., 2014). These circuits are modulated by peripheral signals originating from the gastrointestinal tract, adipose tissue, pancreas, and skeletal muscle, including hormones, nutrients, and exercise-induced metabolites.

Exercise acutely alters this signaling landscape by modifying circulating concentrations of appetite-regulating hormones and metabolites. Intense exercise transiently suppresses hunger and reduces subsequent energy intake, an effect commonly attributed to reductions in acylated ghrelin and elevations in anorexigenic peptides such as PYY and GLP-1 (King et al., 2013; McCarthy et al., 2024). However, hormonal changes alone do not fully account for the magnitude or variability of post-exercise appetite suppression, suggesting that additional metabolic signals contribute to central appetite control.

Within this context, Lac-Phe has emerged as a candidate mediator linking exercise-induced metabolic stress to feeding behavior. Preclinical evidence demonstrates that Lac-Phe administration suppresses food intake in mice without affecting locomotor activity, implicating central or neuroendocrine mechanisms rather than nonspecific malaise (Li et al., 2022). Although the molecular targets of Lac-Phe have not yet been identified, its behavioral specificity is consistent with engagement of appetite-regulatory pathways, although the underlying mechanisms remain undefined.

Whether Lac-Phe acts directly on the central nervous system or indirectly through peripheral signaling pathways remains unresolved. Small metabolites can influence brain function either by crossing the blood–brain barrier or by modulating peripheral afferent pathways, such as vagal sensory

signaling from the gut (Berthoud et al., 2017). Lactate itself has been shown to act as a signaling molecule in the brain, influencing neuronal activity and energy sensing, raising the possibility that Lac-Phe represents a chemically modified extension of lactate signaling with distinct stability or receptor interactions (Brooks, 2018). Alternatively, Lac-Phe may modulate the secretion or sensitivity of established appetite hormones, thereby indirectly shaping hypothalamic activity.

Circadian regulation adds an additional layer of complexity to this integration. Hypothalamic feeding circuits exhibit time-of-day–dependent sensitivity to orexigenic and anorexigenic signals, influenced by circadian input from the suprachiasmatic nucleus and peripheral clocks (Panda, 2016). Consequently, the central response to a given peripheral signal may vary across the day. In this framework, Lac-Phe generated at different circadian phases may exert differential effects on appetite regulation, even if circulating concentrations are comparable. Such circadian modulation implies that the same peripheral signal may be differentially interpreted by central feeding circuits depending on circadian phase (Panda, 2016; Morton et al., 2014).

Peripheral tissues involved in appetite regulation are also under circadian control. The gut exhibits rhythmic secretion of GLP-1 and PYY, adipose tissue displays diurnal variation in leptin release, and skeletal muscle metabolism follows circadian patterns that influence metabolite production (Johnston et al., 2014; Asher & Sassone-Corsi, 2015). Lac-Phe therefore operates within a temporally structured signaling environment in which both its production and its targets fluctuate across the day. This raises the possibility that Lac-Phe contributes to a broader gut–muscle–brain communication axis that integrates exercise timing with feeding behavior.

Collectively, Lac-Phe represents a novel exercise-induced metabolic signal that may interface with appetite regulation through multiple central and peripheral pathways. While preclinical evidence supports a role in feeding suppression, the sites and mechanisms of action remain unresolved, and their modulation by circadian timing has not been directly tested. Viewing Lac-Phe within a temporally structured gut–muscle–brain communication axis provides a coherent framework for future studies aimed at determining when, as well as how, this metabolite influences appetite regulation. These considerations have important implications for translating Lac-Phe biology into chrono-informed exercise and metabolic interventions.

5. Clinical And Translational Implications Of Chrono-Exercise And Lac-Phe Signaling

Recognition that exercise timing modulates metabolic responses has important implications for interpreting exercise-induced signals, including Lac-Phe, within clinical and lifestyle interventions. Traditional exercise prescriptions emphasize frequency, intensity, and duration, yet growing evidence indicates that when exercise is performed meaningfully influences

glucose handling, insulin sensitivity, substrate utilization, and appetite regulation (Gabriel & Zierath, 2019; Savikj et al., 2019). Positioning Lac-Phe within a chronobiological framework therefore offers an opportunity to refine exercise-based strategies for metabolic health by aligning metabolite signaling with circadian physiology.

From a clinical perspective, appetite regulation represents a particularly relevant target. Acute exercise is known to transiently suppress hunger and reduce subsequent energy intake, but the magnitude and duration of this effect vary substantially between individuals (King et al., 2013; McCarthy et al., 2024). If Lac-Phe contributes to post-exercise appetite suppression, as suggested by preclinical and associative human data (Li et al., 2022), optimizing exercise timing to maximize Lac-Phe signaling during periods of heightened feeding drive could enhance the practical effectiveness of exercise for weight management. For example, exercise performed prior to habitual meal times or during circadian phases characterized by elevated appetite signaling may yield greater reductions in subsequent energy intake than identical exercise performed at other times of day. At present, these considerations remain hypothesis-generating, as no studies have directly tested whether manipulating exercise timing alters Lac-Phe-mediated appetite responses in humans.

These considerations may be particularly relevant in populations with circadian disruption, such as shift workers, individuals with irregular sleep–wake patterns, or patients with metabolic disease. Circadian misalignment is associated with increased hunger, impaired glucose tolerance, and elevated cardiometabolic risk independent of total physical activity (Scheer et al., 2009; Vetter et al., 2016). In such contexts, strategically timed exercise could serve as a behavioral countermeasure, potentially restoring aspects of metabolic rhythmicity while simultaneously engaging Lac-Phe–mediated appetite pathways. Although direct evidence linking Lac-Phe responses to circadian misalignment is currently lacking, the convergence of chrono-exercise and metabolite signaling provides a plausible translational avenue. Whether Lac-Phe signaling is preserved, blunted, or dysregulated under conditions of circadian misalignment remains unknown.

Beyond lifestyle interventions, Lac-Phe biology may inform future metabolite-based strategies aimed at appetite regulation, although translational application remains speculative at present. Metabolite-based therapeutics that mimic or potentiate endogenous exercise signals have attracted increasing interest, particularly in the context of obesity and type 2 diabetes. However, translating Lac-Phe into a therapeutic target requires careful consideration of timing, dosing, and physiological context. Given the circadian regulation of appetite circuits and hormone sensitivity, any Lac-Phe–based intervention would likely exhibit time-of-day–dependent efficacy, emphasizing the need for chronopharmacological approaches rather than static dosing paradigms (Panda, 2016). Importantly, any such approach would

require rigorous demonstration of mechanism, safety, and time-of-day–dependent efficacy before clinical consideration.

Importantly, expectations regarding the magnitude of Lac-Phe–mediated effects must remain realistic. Exercise-induced appetite suppression is transient, and compensatory behaviors such as delayed hyperphagia or reduced non-exercise activity can offset short-term energy deficits (King et al., 2013). Lac-Phe signaling, even if causally involved, is unlikely to produce large or sustained energy deficits in isolation. Instead, its value may lie in fine-tuning appetite control and enhancing adherence to broader lifestyle interventions when integrated with nutrition, sleep, and behavioral strategies. Accordingly, Lac-Phe signaling should be viewed as a fine-tuning mechanism within a broader behavioral and metabolic system, rather than as a driver of sustained negative energy balance.

Finally, interindividual variability represents a central translational challenge. Lac-Phe responses to exercise vary widely, likely reflecting differences in fitness status, metabolic phenotype, substrate availability, and circadian characteristics such as chronotype. Chrono-informed exercise prescriptions that account for biological timing and individual responsiveness may therefore be required to meaningfully engage Lac-Phe signaling. As with other exercise-induced mediators, Lac-Phe should be understood as one component of a complex, temporally organized signaling network rather than as an independent determinant of metabolic health.

6. Methodological Considerations And Future Research Directions

Progress in understanding N-lactoyl-phenylalanine (Lac-Phe) as a chronometabolic signal will depend on methodological rigor that explicitly incorporates biological timing into study design. To date, most human investigations of exercise-induced metabolites have relied on single time-point sampling and have not standardized or systematically varied the time of day of exercise, limiting interpretability when circadian regulation is likely to be operative (Panda, 2016; Gabriel & Zierath, 2019). Future studies should therefore integrate circadian principles at every stage, from participant selection to sampling strategies and outcome assessment. Where feasible, exercise timing should be referenced to circadian phase rather than external clock time, as individuals performing exercise at the same clock hour may differ substantially in biological timing due to chronotype, sleep history, and light exposure (Panda, 2016; Vetter et al., 2016).

Exercise timing must be treated as an independent experimental variable rather than a logistical convenience, with pre-specified hypotheses regarding time-of-day or circadian-phase effects. Controlled crossover designs in which identical exercise protocols are performed at distinct circadian phases (e.g., morning versus evening) are particularly well suited to isolating time-of-day effects on Lac-Phe production and kinetics (Ezagouri et al., 2019). Such studies should be accompanied by dense temporal sampling

to capture peak concentrations, area-under-the-curve responses, and clearance dynamics, as single post-exercise measurements may miss critical temporal features of Lac-Phe signaling. Parallel assessment of lactate, glucose, amino acids, and appetite-regulating hormones would permit integrative modeling of metabolic and endocrine interactions. Because Lac-Phe appears to rise and fall rapidly following intense exercise, sparse post-exercise sampling risks missing peak exposure and obscuring differences attributable to circadian phase.

Accurate quantification of Lac-Phe requires validated targeted metabolomic approaches. Liquid chromatography–mass spectrometry (LC-MS) methods with appropriate internal standards are essential to distinguish Lac-Phe from structurally related N-lactoyl amino acids and to ensure reproducibility across laboratories (Contrepois et al., 2020; Li et al., 2022). Preanalytical variables—including fasting duration, recent physical activity, and sample handling—should be tightly controlled, as these factors can influence lactate availability and downstream metabolite formation. Where feasible, repeated measures across multiple days would allow assessment of within-individual stability and circadian rhythmicity. Standardization of analytical platforms and reporting units will be essential for cross-study comparability, as modest differences in Lac-Phe concentration may carry distinct physiological implications depending on timing and context.

Characterizing central versus peripheral mechanisms represents another critical research priority. The molecular targets of Lac-Phe remain unidentified, and it is unclear whether its anorexigenic effects are mediated through direct actions on the central nervous system, modulation of gut–brain signaling, or interaction with established appetite hormones. Experimental approaches combining metabolite administration with neural circuit mapping, vagal manipulation, or hormone receptor blockade will be necessary to resolve these pathways (Berthoud et al., 2017; Morton et al., 2014). In humans, indirect evidence may be obtained by examining whether Lac-Phe responses predict changes in appetite independently of ghrelin, GLP-1, or PYY dynamics. Resolving these pathways is essential for distinguishing whether Lac-Phe acts as a primary afferent signal, a permissive modulator, or a downstream correlate of appetite-regulatory processes.

Circadian phenotype should also be considered. Chronotype, sleep duration, and circadian alignment influence metabolic responses to both exercise and feeding, yet these variables are rarely assessed in metabolomic studies (Vetter et al., 2016). Stratifying participants by chronotype or incorporating objective measures of circadian phase, such as dim-light melatonin onset, could clarify interindividual variability in Lac-Phe responses. Similarly, populations characterized by circadian disruption—such as shift workers or individuals with sleep disorders—offer a valuable translational context in which to examine whether Lac-Phe signaling is altered or blunted. Failure to account for circadian phenotype may therefore contribute to

unexplained variability and apparent null effects in human studies of exercise-induced appetite regulation.

Finally, long-term and translational studies are needed to determine whether repeated engagement of Lac-Phe signaling through timed exercise produces sustained adaptations. Acute appetite suppression does not necessarily translate into long-term energy balance, given the potential for compensatory behaviors (King et al., 2013). Longitudinal interventions combining standardized exercise timing with careful monitoring of energy intake, non-exercise activity, and body composition will be required to evaluate the cumulative metabolic impact of Lac-Phe within real-world contexts.

Addressing these methodological challenges will allow Lac-Phe research to progress from descriptive association toward mechanistic and translational insight. Embedding metabolite signaling within a circadian framework necessitates study designs that are time-aware, kinetically informed, and physiologically grounded. By adopting chrono-controlled exercise protocols, high-resolution metabolomic sampling, and rigorous assessment of appetite-related outcomes, future research can determine whether Lac-Phe functions as a temporally gated signal linking exercise timing to appetite regulation and energy homeostasis.

7. Conclusion

The identification of N-lactoyl-phenylalanine (Lac-Phe) as an exercise-inducible metabolite has expanded current understanding of how acute metabolic stress is communicated to systems governing appetite and energy balance. Rather than functioning as an isolated anorexigenic factor, Lac-Phe appears embedded within a temporally organized metabolic network shaped by circadian regulation, exercise timing, and the integration of peripheral and central signaling pathways. This perspective reframes Lac-Phe not merely as a marker of exercise intensity, but as a candidate time-sensitive signal whose physiological relevance depends on when it is generated and interpreted.

Across this chapter, evidence from circadian biology, exercise physiology, and metabolomics converges on a central principle: metabolic signals operate within a dynamically regulated physiological environment. Fluctuations in substrate availability, hormonal milieu, neural sensitivity, and behavior across the circadian cycle confer time-of-day specificity to exercise responses. Accordingly, Lac-Phe production and its downstream effects on appetite regulation are unlikely to be uniform across the day, but instead may be gated by circadian phase, peripheral clock alignment, and central feeding circuit responsiveness.

Recognizing Lac-Phe within a chronometabolic framework carries important conceptual and practical implications. Conceptually, it cautions against reductionist interpretations of exercise-induced appetite suppression

that overlook biological timing. Practically, it highlights the limitations of exercise prescriptions that focus exclusively on intensity, duration, or volume while neglecting temporal context—an omission that may be particularly consequential in populations characterized by circadian disruption or metabolic disease. Importantly, the modest magnitude and transient nature of Lac-Phe–associated effects support a modulatory role, contributing to fine regulation of appetite rather than serving as a primary determinant of long-term energy balance.

Advancing this field will require integrative approaches spanning chronobiology, metabolomics, neuroendocrinology, and exercise science. Future research must adopt time-aware study designs capable of resolving whether Lac-Phe functions as a temporally gated signal linking exercise timing to appetite regulation and energy homeostasis. By situating Lac-Phe within the temporal architecture of metabolism, this chapter provides a framework for moving beyond static models toward a more dynamic understanding of exercise–metabolite–appetite interactions.

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Proteomic Technologies and Biomarker Discovery: LC–MS/MS Approaches and Applications in Clinical Diagnosis

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ABSTRACT

Omics technologies, by integrating the genomic, transcriptomic, proteomic, and metabolomic layers, have enabled a multidimensional understanding of biological systems and have assumed a central role in elucidating disease mechanisms, biomarker discovery, and the advancement of personalized medicine. Although the genome and transcriptome define genetic information and gene expression patterns, the primary determinants of cellular phenotype most often emerge at the protein level. Protein abundance is dynamically regulated by translational efficiency, proteolytic degradation, protein–protein interactions, and post-translational modifications (PTMs), resulting in a limited correlation between mRNA and protein levels. Consequently, proteomics directly interrogates the functional layer of biology and complements the gaps inherent in genomic and transcriptomic data. The backbone of modern proteomic analysis is liquid chromatography–tandem mass spectrometry (LC–MS/MS), where discovery-based and targeted strategies are employed in a complementary manner. In discovery proteomics, data acquisition schemes such as data-dependent acquisition (DDA) and data-independent acquisition (DIA/SWATH-MS), combined with label-free quantification (LFQ) or isotopic labeling approaches (e.g., TMT, iTRAQ), enable large-scale and unbiased protein profiling. In contrast, targeted proteomics utilizes selected reaction monitoring/multiple reaction monitoring (SRM/MRM) or parallel reaction monitoring (PRM) on triple quadrupole or high-resolution mass spectrometers to validate selected peptides with high sensitivity, specificity, and reproducibility. In clinical settings, proteomic approaches contribute to the identification of disease-specific protein signatures for early diagnosis and risk stratification. Nevertheless, several challenges persist, including the wide dynamic range of plasma and serum proteomes, the need for standardized sample preparation, effective detergent removal strategies (e.g., FASP, S-Trap), appropriate protease selection, and the management and interpretation of large-scale datasets. Overall, proteomics, supported by standardized workflows and advanced bioinformatics and artificial intelligence tools, represents a key component of translational research and the development of robust and clinically applicable biomarkers.

Keywords – Omics technologies, proteomics, LC–MS/MS, mass spectrometry, biomarker discovery, and clinical validation

INTRODUCTION

Advances in molecular biology and genetics have led to the acquisition of novel insights at both the cellular and organismal levels (Hasin, Seldin, & Lusis, 2017). Omics technologies including genomics, transcriptomics, proteomics, and metabolomics play a crucial role in elucidating the functional organization of biological systems (Aebersold & Mann, 2016; Liu, Beyer, & Aebersold, 2016). These approaches extend beyond the analysis of genetic information alone, enabling comprehensive investigation of metabolic processes such as protein expression patterns, metabolite profiles, and complex cellular interactions (Geyer, Kulak, Pichler, Holdt, Teupser, & Mann, 2017). By complementing and surpassing traditional molecular biology techniques, omics technologies facilitate systems-level analyses and contribute substantially to the understanding of disease mechanisms, the discovery of novel biomarkers, and the development of personalized medical strategies (Rifai, Gillette, & Carr, 2006; Mertins et al., 2016).

1.1. The Rise of Omics Technologies

Omics technologies have become a cornerstone of modern biomedical research by enabling the investigation of biological systems at the molecular level, from the genome to the metabolome. While genomics reveals the genetic information content of cells, transcriptomics explains how this information is regulated in response to environmental and physiological conditions. However, it has been demonstrated that genomic or transcriptomic analyses alone are insufficient to fully explain cellular phenotypes and disease pathophysiology. This limitation arises because biological functions are regulated not only at the level of gene expression but also through protein modifications, protein–protein interactions, and metabolic processes (Hasin et al., 2017). As a result, the importance of functional omics disciplines such as proteomics and metabolomics has increased. Proteomics provides functional insight into genetic information by analyzing the abundance, structural alterations, and post-translational modifications of proteins actively present within the cell (Aebersold & Mann, 2016). Metabolomics, on the other hand, examines cellular metabolite profiles to identify the end products of biochemical reactions, thereby offering a holistic view of the physiological state of the organism (Johnson et al., 2016). In this respect, metabolomics is considered an omics layer that directly reflects the interplay between genetic background and environmental influences. Rapid advances in next-generation sequencing (NGS) technologies, together with the expansion of mass spectrometry–based analytical platforms, have enabled the development of single-cell omics approaches and facilitated the acquisition of previously inaccessible

biological data. The analysis of such complex datasets has required methodologies beyond conventional statistical approaches, leading to the integration of bioinformatics, machine learning, and artificial intelligence–based algorithms into omics research (Libbrecht & Noble, 2015). In particular, deep learning methods offer substantial advantages in pattern recognition, disease classification, and functional prediction from omics datasets (Topol, 2019). One of the most prominent current applications of omics technologies is the integration of multiple omics layers through multi-omics approaches. Multi-omics strategies allow simultaneous analysis of the effects of genomic variation on the transcriptome, proteome, and metabolome, enabling system-level modeling of biological processes. These approaches are especially valuable for defining molecular subtypes of complex and multifactorial diseases (Karczewski & Snyder, 2018). Multi-omics analyses have been widely applied to heterogeneous conditions such as cancer, neurodegenerative disorders, metabolic syndromes, and fibrotic diseases. The integration of omics technologies into clinical practice has accelerated the development of translational medicine and personalized therapeutic strategies. Comprehensive evaluation of an individual's genetic profile, epigenetic regulation, protein expression patterns, and metabolic status is critical for disease risk prediction, early diagnosis, prognostic assessment, and prediction of treatment response (Subramanian et al., 2020).

1.2. Scope of Proteomic Science

Proteomics is a scientific discipline that enables the systematic analysis of the complete set of proteins (the proteome) expressed in a cell, tissue, or organism at a given time point. Unlike the genome, the proteome is highly dynamic and exhibits substantial variability depending on physiological conditions, environmental stimuli, disease states, and temporal regulation. Consequently, proteomics extends beyond simple protein identification and aims to elucidate protein abundance, functional interactions, subcellular localization, and post-translational modifications (PTMs) that critically regulate protein function (Aebersold & Mann, 2016). Modern proteomic strategies are predominantly based on mass spectrometry (MS) technologies, which offer high sensitivity and a broad dynamic range, allowing the identification and quantitative measurement of thousands of proteins within a single experiment. These capabilities have positioned proteomics as a central tool not only in basic biological research but also in translational and clinical applications, including disease biomarker discovery, monitoring of therapeutic responses, and support of clinical decision-making processes (Zhang et al., 2014; Aebersold & Mann, 2016). Proteomic research encompasses a wide spectrum of subdisciplines and methodological approaches designed to address diverse biological questions. Global proteome analysis aims to characterize overall protein expression

profiles within a sample, thereby enabling comparative assessments between different cellular states or conditions. In contrast, sub-proteomic approaches focus on specific protein subsets to achieve more detailed and targeted insights. For instance, membrane proteomics plays a crucial role in elucidating receptor-mediated signaling pathways and cell–environment interactions, whereas phosphoproteomics and other PTM-focused analyses investigate the regulation of intracellular signaling networks (Olsen & Mann, 2013).

Post-translational modifications constitute a fundamental component of proteomic studies, as they are key regulators of protein function. PTMs such as phosphorylation, acetylation, ubiquitination, and glycosylation influence protein stability, enzymatic activity, and subcellular distribution. Comprehensive PTM analyses enable the molecular-level characterization of activation or suppression events within cellular signaling pathways, providing critical insights into physiological and pathological mechanisms (Choudhary et al., 2014). In addition, proteomics addresses the analysis of protein–protein interactions and the organization of protein complexes. Techniques such as affinity purification coupled with mass spectrometry (AP-MS) and cross-linking approaches facilitate the investigation of the structural and functional architecture of protein assemblies, thereby contributing to a deeper understanding of cellular processes, including signal transduction and transcriptional regulation (Hein et al., 2015). The methodological breadth of proteomics is further expanded through the integration of fractionation strategies and quantitative analysis techniques. Protein- or peptide-level fractionation enhances the detection of low-abundance proteins in complex biological samples. Quantitative proteomic approaches based on isotopic labeling methods (e.g., SILAC, iTRAQ, TMT) as well as label-free strategies enable reliable and reproducible measurement of changes in protein expression levels (Bantscheff et al., 2012). Moreover, targeted proteomic techniques such as selected/multiple reaction monitoring (SRM/MRM) and parallel reaction monitoring (PRM) allow precise and sensitive quantification of predefined proteins or peptides, thereby strengthening the integration of proteomic data into clinical and translational research frameworks.

1.3. Why Is Proteomics Critical in the DNA–RNA–Protein Relationship?

While genomic and transcriptomic analyses define the genetic architecture of the cell and its gene expression profiles, the proteome represents the molecular layer that most directly reflects the real-time functional state of the cell. The flow of information from DNA to RNA constitutes only the initial stages of cellular regulation; in contrast, protein

abundance and function are governed by multiple regulatory mechanisms, including mRNA stability, translational efficiency, post-translational modifications (such as phosphorylation, acetylation, and ubiquitination), protein–protein interactions, and proteolytic degradation pathways. Owing to these complex regulatory networks, numerous studies have demonstrated that the correlation between mRNA levels and protein abundance is often limited and highly context dependent (Vogel & Marcotte, 2012; Liu et al., 2016). As a result, reliance solely on genomic and transcriptomic data is frequently insufficient to fully explain cellular functions or disease-related mechanisms. Proteomic approaches address this limitation by directly measuring protein abundance, activation states, and functional modifications, thereby filling critical gaps in biological interpretation and enabling a more mechanistic understanding of cellular processes. In particular, proteomic analyses focused on post-translational modifications provide insights into whether signaling pathways are activated or suppressed, facilitating the identification of key regulatory molecules involved in disease pathogenesis (Olsen & Mann, 2013; Choudhary et al., 2014). Furthermore, the investigation of protein complexes and interaction networks reveals the network-based organization of cellular functions, offering system-level insights that cannot be obtained from genomic or transcriptomic data alone. By integrating quantitative and functional protein-level information, proteomics serves as an essential bridge between genetic information and cellular phenotype, substantially enhancing our ability to interpret biological systems in both physiological and pathological contexts (Aebersold & Mann, 2016).

1.4. The Value of Proteomics in Clinical Diagnosis

Clinical proteomics is an approach that goes beyond conventional diagnostic methods by aiming to elucidate the molecular basis of diseases at the protein level. Unlike genomic and transcriptomic data, proteomic analyses directly examine functionally active proteins and their post-translational modifications, thereby providing immediate and biologically relevant information about ongoing cellular processes (Aebersold & Mann, 2016). From this perspective, proteomics contributes to a more accurate understanding of disease pathophysiology and facilitates the identification of highly specific and clinically meaningful biomarkers.

Proteomic technologies such as liquid chromatography–tandem mass spectrometry (LC–MS/MS) enable sensitive and accurate detection of protein diversity and quantitative differences in clinical samples. These techniques allow the identification of molecular alterations that arise at early stages of disease, often preceding the appearance of clinical symptoms, thereby supporting earlier and more precise diagnosis (Geyer et al., 2017). In

particular, proteomic profiling has proven valuable in oncology through the characterization of tumor microenvironment–specific protein signatures, in cardiovascular diseases through the identification of risk-associated biomarkers, and in neurodegenerative disorders through monitoring disease progression and molecular changes (Rifai et al., 2006).

Targeted proteomic approaches, which are increasingly applicable in routine clinical laboratories, enable the precise and reproducible quantification of selected proteins. Methods such as selected/multiple reaction monitoring (SRM/MRM) and parallel reaction monitoring (PRM) are widely used in biomarker validation studies and provide robust quantitative data for clinical diagnostic workflows (Zhang et al., 2013). These strategies offer important advantages over traditional immunoassay-based analyses by improving analytical specificity and allowing multiplexed measurement of multiple protein targets within a single assay.

Moreover, molecular insights derived from proteomic data contribute to the understanding of interindividual biological variability and strongly support the advancement of personalized medicine. The determination of patient-specific protein expression patterns is critical for predicting therapeutic response, stratifying patients, and developing individualized treatment strategies, thereby enhancing the effectiveness and precision of modern clinical care (Geyer et al., 2017).

1.5. Modern Requirements for Biomarker Discovery

Today, biomarker development requires not only identifying candidate molecules but also validating them for clinical use. Biomarker discovery encompasses key evaluation criteria such as analytical accuracy, biological specificity, clinical sensitivity, and reproducibility (Diamandis, 2012). Multi-omics data mining supported by artificial intelligence enables proteomic datasets to be used more reliably in biomarker research. In addition, throughout the process from discovery to clinical implementation, infrastructures such as biobanks, prospective cohorts, and standardized sample-processing protocols play a central role. This integrated approach reduces the long-standing problem of “biomarkers that are discovered but fail to perform successfully in the clinic” and enhances the effectiveness of translational research (Diamandis, 2012).

1.6. Major Challenges in Proteomics

Although proteomic analyses have strong potential to interrogate biological systems in depth, they involve substantial biological and technical challenges. Unlike the genome, the proteome is highly dynamic and

continuously changes depending on cell type, environmental influences, and physiological conditions. This dynamic nature increases the diversity of protein expression levels and post-translational modifications (PTMs), thereby complicating analysis (Aebersold & Mann, 2016). In clinical specimens such as human plasma and serum, the very wide dynamic range of protein abundance (on the order of 10–12) makes it difficult to detect low-abundance proteins as potential biomarkers. Signals from high-abundance proteins (e.g., albumin and immunoglobulins) can suppress detection, causing clinically meaningful low-abundance proteins to be underrepresented (Anderson & Anderson, 2002). Consequently, additional steps such as sample fractionation and depletion of high-abundance proteins become necessary; however, these procedures can also increase the risk of sample loss. Sample preparation is a critical stage that directly influences the accuracy and reproducibility of proteomic workflows. Losses during protein extraction, chemical factors affecting enzymatic digestion (particularly tryptic digestion), and technical variability during peptide cleanup can substantially shape results. Moreover, differences in peptide ionization efficiency in mass spectrometry may introduce bias into quantitative measurements (Ting et al., 2015). Another major challenge arises after data acquisition: the analysis and biological interpretation of large-scale proteomic datasets. High-resolution mass spectrometry generates complex outputs that require advanced bioinformatics tools, robust statistical modeling, and substantial computational capacity. Preventing false positives, ensuring confidence in protein identifications, and controlling statistical errors arising from multiple testing represent persistent issues in proteomic data analysis (Nesvizhskii, 2014).

In clinical proteomics, additional barriers include standardization and validation. Variations across laboratories in sampling protocols, instrument parameters, and data-analysis strategies reduce comparability. Furthermore, translating biomarker candidates into clinical use requires long-term, costly, multicenter validation studies. Regulatory validation requirements demanded by agencies such as the FDA and EMA for diagnostic tests also slow the integration of proteomics-based assays into routine clinical practice (Rifai et al., 2006). Therefore, future proteomic research should prioritize optimized sample-preparation protocols, standardized analytical methods, and improved bioinformatics tools. Making data-analysis pipelines more accessible, transparent, and reproducible will further strengthen the reliability of proteomic technologies in clinical and translational research (Ting et al., 2015).

2. Proteomic Approaches

Proteomic analyses are strategies aimed at comprehensively investigating proteins within biological systems and are broadly categorized into discovery-based and targeted proteomics. These two approaches are used in a complementary manner depending on the research question and clinical objectives. The primary goal of discovery proteomics is to identify as many proteins as possible in a biological sample and to determine their relative or absolute abundances. This is typically achieved using high-resolution mass spectrometry and LC–MS/MS-based workflows, enabling the discovery of previously undetected proteins, isoforms, and post-translational modifications (Aebersold & Mann, 2016). In particular, labeled quantitative methods (SILAC, TMT, iTRAQ) and label-free quantification are widely applied to compare protein-expression changes across biological conditions or disease states.

Targeted proteomics, in contrast, focuses on measuring selected proteins or peptides with high specificity and sensitivity. Techniques such as Selected Reaction Monitoring (SRM/MRM) and Parallel Reaction Monitoring (PRM) are essential for verifying candidate biomarkers and evaluating them quantitatively for clinical use (Zhang et al., 2013). Compared with immunoassay-based methods, these approaches generally provide higher specificity and permit multiplexed measurement of numerous target proteins in the same run.

In recent years, cell-based and single-cell proteomics have emerged as rapidly developing fields due to their improved resolution and data quality. Defining cell-specific protein-expression profiles contributes to understanding cellular heterogeneity and elucidating disease mechanisms in greater detail (Budnik et al., 2018). These strategies are particularly valuable in cancer biology and immunology by enabling the identification of molecules specific to distinct cellular subpopulations. The reliability and reproducibility of proteomic measurements are closely tied to the biochemical properties of sample types and to appropriate sample-preparation methods. Even minor variations in protein extraction, digestion protocols, or peptide-cleanup steps can affect outcomes. In clinical matrices (e.g., blood, tissue, urine), the wide dynamic range of protein concentrations challenges analytical sensitivity and increases the need for standardized protocols. Therefore, successful implementation of proteomic approaches requires optimization of both analytical methods and sample-preparation workflows (Michalski et al., 2011).

2.1. Discovery Proteomics

Discovery proteomics is an omics-based, hypothesis-free approach that aims to analyze the protein composition of biological samples in detail. Its primary objective is to identify proteins present in a given sample and to determine their relative or absolute abundances. By not being restricted to predefined targets, discovery proteomics enables the identification of novel proteins, isoforms, and disease-associated molecular alterations (Aebersold & Mann, 2016). High-resolution liquid chromatography and mass spectrometry (LC–MS/MS) serve as the central analytical tools in this approach. Orbitrap and time-of-flight (TOF)-based mass spectrometers, in particular, provide high mass accuracy and resolution, supporting deep coverage of complex proteomes.

To increase proteome depth, fractionation and enrichment strategies at the protein or peptide level are commonly applied prior to LC–MS/MS analysis (Michalski et al., 2011). Quantitative discovery proteomics may be performed using label-free approaches or isotopic labeling methods. Labeling strategies such as SILAC, TMT, and iTRAQ facilitate comparisons across biological conditions, whereas label-free approaches are often better suited for analyzing larger numbers of samples and for adapting workflows to clinical research settings (Zhang et al., 2013). These techniques are central to detecting differences in protein profiles between diseased and healthy states. Discovery proteomics is widely used in biomarker discovery, mechanistic studies, and reconstruction of biological pathways. In complex diseases such as cancer, neurodegenerative disorders, and cardiovascular conditions, identifying disease-specific proteins and potential prognostic markers represents a major application domain (Rifai et al., 2006). However, translating discovery findings into clinical practice requires verification using targeted proteomic methods. Overall, discovery proteomics is a foundational approach for translational research, and its scope and reliability continue to increase with advances in mass spectrometry, data-analysis software, and bioinformatics methods (Aebersold & Mann, 2016).

2.1.1. Data-Dependent Acquisition (DDA)

Data-Dependent Acquisition (DDA) is one of the most widely used data-collection strategies in discovery proteomics. In this approach, the mass spectrometer first detects ions present in the sample during an MS1 survey scan and then selects the most intense ions within a given time window for MS/MS (MS2) fragmentation and analysis. This selection typically follows a “top-N” principle, in which the N most abundant precursor ions from each MS1 scan are fragmented (Michalski et al., 2011). One advantage of DDA is that it generates high-quality, information-rich fragmentation spectra for

selected ions, supporting accurate peptide sequencing and protein identification. When combined with high-resolution instruments, DDA enables identification of proteins in complex biological samples (Aebersold & Mann, 2016). For this reason, DDA remains a standard approach in discovery proteomics and in the identification of candidate biomarkers. Nevertheless, DDA has important limitations. Because it prioritizes the most intense ions, low-abundance peptides can be systematically overlooked. In samples such as human plasma, peptides derived from high-abundance proteins dominate the analysis and hinder detection of clinically relevant low-abundance proteins (Michalski et al., 2011). Moreover, repeated analyses of the same sample may result in different ions being selected for fragmentation, reducing inter-run reproducibility and limiting quantitative comparisons. Another constraint is limited MS/MS sampling capacity in highly complex mixtures: short chromatographic peak widths and the finite number of ions that can be fragmented per unit time prevent comprehensive capture of the entire proteome. To address these issues, researchers use fractionation strategies, faster-scanning mass spectrometers, and improved data-analysis algorithms (Michalski et al., 2011). Despite these limitations, DDA remains one of the most frequently applied acquisition methods for deep proteome profiling, protein identification, and discovery-focused studies. Today, DDA is often used alongside Data-Independent Acquisition (DIA) and targeted proteomic strategies to achieve more comprehensive and reliable proteomic outcomes (Aebersold & Mann, 2016).

2.1.2. Data-Independent Acquisition (DIA, SWATH-MS)

Data-Independent Acquisition (DIA) is an increasingly important, hypothesis-free acquisition strategy in proteomics. Unlike DDA, which focuses on high-intensity ions, DIA systematically fragments all ions within predefined m/z windows across the entire sample. As a result, low-abundance peptides and proteins are more likely to be captured than with DDA (Gillet et al., 2012). SWATH-MS (Sequential Window Acquisition of All Theoretical Mass Spectra) is a widely used DIA implementation. In SWATH-MS, the instrument sequentially scans defined m/z ranges, fragments all ions within each range, and generates MS/MS spectra. This systematic acquisition yields fragmentation data for virtually all peptides, minimizing the risk of missing ions and improving overall proteome coverage (Gillet et al., 2012). A key advantage of DIA is its high reproducibility. Compared with DDA, DIA reduces between-run variability and can provide more reliable quantitative results. In addition, DIA improves detection of low-abundance proteins that may be clinically important. These features make DIA particularly suitable for biomarker verification, quantitative analysis of clinical samples, and translational research (Gillet et al., 2012). A critical consideration in DIA workflows is the use of spectral

libraries. Reliable peptide identification and quantification often require reference libraries composed of previously characterized peptide spectra. Thus, appropriate libraries should be planned and generated prior to analysis. Moreover, DIA datasets require advanced bioinformatics tools and computational resources for effective processing and interpretation (Gillet et al., 2012).

2.1.3. Label-Free Quantification

Label-Free Quantification (LFQ) is a quantitative proteomics approach that estimates protein or peptide abundances directly from chromatographic peak areas or MS signal intensities. Because it does not require chemical or isotopic labeling, LFQ is cost-effective and facilitates analysis of large sample cohorts. It enables high-throughput assessment of many biological samples, reduces the need for additional reagents, and can simplify sample-preparation workflows in clinical studies. However, LFQ can be sensitive to small variations in instrument performance and differences in ionization efficiency, which may influence quantitative accuracy. Therefore, robust normalization strategies and advanced data-processing algorithms are essential to ensure reliable LFQ-based comparisons. Detection of low-abundance peptides also depends on sample complexity and instrument sensitivity. LFQ is widely used in discovery proteomics and biomarker-screening studies, and improvements in bioinformatics tools have strengthened its ability to compare proteomic changes with appropriate statistical rigor. Overall, LFQ is recognized as a practical proteomic strategy offering speed, cost-efficiency, and clinical compatibility (Cox et al., 2014; Aebersold & Mann, 2016).

2.1.4. Isotope Labeling Approaches (TMT, iTRAQ)

Isotope labeling strategies—particularly Tandem Mass Tag (TMT) and Isobaric Tags for Relative and Absolute Quantitation (iTRAQ)—enable multiplexed comparison of multiple samples within a single LC–MS/MS run. By allowing simultaneous quantitative analysis of several samples, these techniques increase experimental throughput. For example, TMTpro labels can support comparison of up to 16 samples in a single experiment, whereas iTRAQ is commonly applied for 4- or 8-plex designs. A major advantage of these approaches is improved quantitative precision under highly consistent analytical conditions, which minimizes technical variation between runs. In addition, multiplexing shortens overall analysis time and improves efficiency. Although isobaric tags have identical mass at the MS1 level, they generate sample-specific reporter ions upon fragmentation at the MS2 or MS3 level, enabling quantification. Despite these advantages, isotope labeling approaches also have limitations, including “ratio compression,”

where signals from high-abundance peptides can suppress signals from low-abundance peptides, potentially biasing quantitative ratios—an issue that is especially relevant in complex samples. Overall, TMT and iTRAQ are considered reliable and reproducible quantitative methods in biomarker discovery, clinical proteomics, and comparative proteomic analyses (Thompson et al., 2003).

2.2. Targeted Proteomics

Targeted proteomics is a quantitative strategy that measures selected proteins or peptides with high sensitivity and accuracy. It is typically used to validate and quantify targets identified in discovery-based studies. After candidate proteins with clinical or biomarker potential are selected from discovery datasets, targeted proteomic methods quantify these targets in a systematic and reproducible manner. This step is particularly important for clinical translation because it provides both reproducibility and quantitative accuracy, enabling reliable measurement of biomarkers in patient-derived samples (Picotti & Aebersold, 2012). Among targeted approaches, Selected Reaction Monitoring (SRM/MRM) and Parallel Reaction Monitoring (PRM) are widely used. SRM/MRM is performed on triple quadrupole instruments by monitoring predefined precursor-to-fragment ion transitions, whereas PRM is typically conducted on high-resolution Orbitrap-based systems that provide improved mass accuracy and selectivity (Picotti & Aebersold, 2012; Gallien et al., 2011). These methods enable robust quantification even when proteins are present at very low abundance (Peterson et al., 2012). Because targeted proteomics delivers high accuracy and reproducibility across diverse clinical specimens, it is increasingly recognized as a key enabling platform for personalized medicine and biomarker-guided therapeutic strategies (Picotti & Aebersold, 2012). When integrated with modern bioinformatics, targeted proteomics supports high-throughput, reliable, and reproducible outputs in both research and clinical applications (Gillet et al., 2012).

2.2.1. Multiple Reaction Monitoring (MRM)

Multiple Reaction Monitoring (MRM) is a targeted mass spectrometry method implemented on triple quadrupole instruments. In MRM, a specific precursor ion (peptide) and its characteristic fragment ions are selectively monitored to perform quantitative analysis (Picotti & Aebersold, 2012). A major advantage of MRM is its high sensitivity and specificity, enabling reliable detection of low-abundance proteins. In addition, MRM protocols are generally compatible with clinical laboratory environments (Anderson et al., 2004), which has facilitated their adoption in biomarker validation and clinical applications. MRM is widely used to target

proteins linked to cardiovascular biomarkers, oncologic markers, and metabolic diseases (Addona et al., 2009). Its reproducibility and quantitative accuracy provide reliable data for translational studies. Moreover, MRM supports high sample throughput and rapid analysis, making it valuable in both research and clinical contexts. In modern proteomic pipelines, MRM is often compared with PRM: while PRM offers broader ion monitoring and high-resolution measurement, MRM remains attractive due to lower cost and strong sensitivity (Gallien et al., 2012).

2.2.2. Parallel Reaction Monitoring (PRM)

Parallel Reaction Monitoring (PRM) is a targeted proteomic method performed on high-resolution mass spectrometers, particularly Orbitrap or Q-TOF platforms. Unlike MRM, PRM acquires all fragment ions for a selected precursor simultaneously at high resolution (Peterson et al., 2012). This capability provides improved selectivity and reduces interference in complex biological matrices. As a result, PRM enables more reliable quantitative assessment of peptide signals and can improve accuracy when targeting low-abundance proteins. Another important advantage of PRM is that full MS/MS spectra are recorded, allowing re-analysis of data and verification of additional peptides if needed. Therefore, PRM has become prominent in biomarker validation, clinical proteomics, and studies targeting low-abundance proteins. It is increasingly used in cardiovascular, oncologic, and neurological biomarker research due to its reliable and reproducible performance (Peterson et al., 2012; Gallien et al., 2012).

2.2.3. Importance in Clinical Validation

Candidate proteins and biomarkers identified through discovery proteomics must be validated using targeted proteomic approaches before they can be applied in clinical diagnosis, prognostic evaluation, or monitoring of treatment response. This validation is carried out using high-sensitivity, high-accuracy methods such as MRM and PRM. Targeted proteomics is particularly strong in reproducibility and standardization, enabling comparisons across laboratories and supporting generation of robust, consistent data (Picotti & Aebersold, 2012; Addona et al., 2009). These methods also support both absolute and relative quantification, allowing protein levels to be measured with molecular precision. Accurate quantification of low-abundance proteins in clinical samples is crucial for evaluating biomarkers in cardiovascular, oncologic, and neurological diseases. For these reasons, targeted proteomics is widely regarded as one of the “gold standard” analytical strategies for determining clinical utility and validating biomarker candidates for translation into clinical practice (Addona et al., 2009; Gallien et al., 2012).

2.3. Single-Cell Proteomics

Single-cell proteomics is a proteomic strategy that enables the investigation of tissue and cellular heterogeneity by profiling the proteome at the level of individual cells. Unlike conventional bulk proteomics, it can reveal molecular differences between distinct cell types and subpopulations, thereby providing valuable insights into tissue heterogeneity, disease subtypes, and the identification of therapy-responsive versus therapy-resistant cells (Budnik et al., 2018). Single-cell proteomics plays a crucial role in cancer biology, immunology, and developmental biology by resolving functional differences among cells. Recent advances in high-sensitivity LC–MS/MS technologies have enabled protein quantification from a single cell at picogram levels, supporting reliable analysis of low-abundance proteins. Key objectives of single-cell proteomic studies include mapping cellular heterogeneity, distinguishing disease types at the molecular level, comparing responder and non-responder cell populations, and elucidating microenvironmental interactions between cells. Consequently, this approach has gained prominence in biomarker discovery, clinical diagnostics, and the development of personalized medicine applications (Addona et al., 2009; Budnik et al., 2018). The success of clinical proteomic studies depends strongly on the structural characteristics of biological specimens and the optimization of sample-preparation procedures. Blood, plasma, serum, urine, cerebrospinal fluid, and tissue biopsies are commonly used clinical sample types in proteomics, each with distinct advantages and limitations. One determinant of proteomic compatibility is the sample-collection process: variables such as hemolysis, clotting time, and storage temperature can directly affect protein stability and therefore analytical accuracy. In addition, pre-analytical variation—including protease activity, protein degradation, and repeated freeze–thaw cycles—can distort protein profiles. The wide dynamic range of proteins in clinical matrices is another major challenge; for example, plasma proteins can span a concentration range of approximately 10^12, which hampers detection of low-abundance proteins. Lipid and salt content can also affect LC–MS/MS performance by altering ionization efficiency, thereby limiting reliable detection of low-abundance targets. Collectively, these factors necessitate standardized protocols and rigorous quality-control procedures to ensure reproducibility and reliability of proteomic data for biomarker discovery and clinical applications (Geyer et al., 2017).

3. LC–MS/MS Proteomic Technology

LC–MS/MS-based proteomics is a leading analytical platform used in biological and clinical research. This technology consists of sequential stages including sample preparation, liquid chromatography (LC), mass

spectrometric measurement (MS/MS), and data analysis. During sample preparation, extraction, cleanup, digestion, and preparation of proteins are critical—particularly for detection of low-abundance proteins and for ensuring measurement accuracy. Liquid chromatography separates complex protein/peptide mixtures before entering the MS instrument, reducing sample complexity and improving ionization efficiency (Aebersold & Mann, 2016). In the MS/MS stage, peptides are ionized and detected according to their mass-to-charge (m/z) ratios; subsequent fragmentation (MS/MS) enables identification and quantification. Finally, bioinformatic and statistical analyses support accurate and reliable interpretation of the resulting data. The strength of LC–MS/MS lies in its capacity for high resolution, sensitivity, and broad dynamic range, enabling detailed profiling of protein landscapes and supporting biomarker discovery, mechanistic research, and translation to clinical applications (Aebersold & Mann, 2016).

3.1. Sample Preparation

Sample preparation is considered the most critical step in proteomic workflows, because the efficiency of protein extraction, purification, and enzymatic conversion into peptides directly determines the accuracy and reliability of LC–MS/MS outputs. Clinical samples—especially plasma, serum, and tissue biopsies—have heterogeneous composition and broad dynamic ranges that demand tailored preparation protocols. For example, in plasma, high-abundance proteins (albumin, immunoglobulins) can obscure low-abundance biomarkers; thus, fractionation strategies may be used to reduce complexity. Adding protease inhibitors prevents proteolytic degradation, and appropriate storage conditions are essential. For tissue specimens, homogenization and lysate preparation must be optimized to minimize protein loss. During peptide generation, enzymatic digestion—typically using trypsin—plays a decisive role: digestion efficiency influences peptide detectability and quantitative accuracy. Therefore, optimized and standardized sample-preparation workflows for each sample type are essential to achieve reliability and reproducibility in proteomics (Geyer et al., 2017).

3.1.1. Preparation of Tissue, Serum, Plasma, and Cells

Tissue specimens are commonly solubilized using mechanical homogenization and detergent-containing lysis buffers. Given the complexity of tissue proteomes, addition of protease and phosphatase inhibitors helps prevent degradation (Wiśniewski et al., 2009). Serum and plasma exhibit wide dynamic ranges due to abundant proteins such as albumin and immunoglobulins; accordingly, stability measures and immunodepletion strategies are often applied (Geyer et al., 2017). In cell

culture systems, proteomic analysis can be more controlled; following lysis, rapid denaturation and inhibitor supplementation can yield high-quality protein extracts. Subcellular fractionation (cytoplasmic, membrane, nuclear fractions) enables separation of sub-proteomes for more targeted interrogation (Geyer et al., 2017).

3.1.2. Protein Extraction and Buffers

Protein extraction is a core component of proteomics and refers to solubilizing proteins from the sample, stabilizing them, and preparing them for downstream peptide analysis. Extraction buffers typically include denaturants such as urea, detergents such as SDS, buffering agents such as Tris-HCl, and reducing agents that help preserve protein stability. Buffer composition is optimized based on sample type and the targeted protein sub-fractions; for instance, stronger detergents and higher concentrations of denaturants may be required to extract membrane proteins effectively (Wiśniewski et al., 2009). Reduction (commonly using DTT or TCEP) and alkylation (IAA or CAA) disrupt disulfide bonds, improving digestion efficiency and increasing peptide recovery. These steps enhance accurate identification and quantitative measurement by LC–MS/MS, particularly in complex samples. Buffer pH and ionic strength can further influence protease performance and protein solubility. Modern proteomic protocols therefore use sample-type–optimized extraction and buffer systems to achieve higher recovery, lower technical variability, and improved reproducibility (Wiśniewski et al., 2009).

3.1.3. Detergent Removal (FASP, S-Trap)

Because detergents interfere with MS analysis, their removal is essential. Filter-Aided Sample Preparation (FASP) is based on retaining proteins on a filter while detergents are removed by washing, enabling efficient peptide recovery (Wiśniewski et al., 2009). S-Trap systems also work effectively with SDS-containing buffers by binding proteins to a filter matrix and removing detergents. These methods are particularly useful for complex clinical samples where detergent removal can otherwise be challenging (Zougman et al., 2014).

3.1.4. Trypsin and Alternative Proteases

Trypsin is the most commonly used protease in proteomics; it cleaves at the carboxyl side of lysine (K) and arginine (R), producing peptides of suitable length and ionization properties for LC–MS/MS. This specificity supports reliable protein identification and quantification. However, alternative proteases can be used to increase proteome coverage

and reveal regions not accessible by trypsin alone. For instance, LysC is resistant to high urea concentrations and remains effective under harsh denaturing conditions; GluC cleaves at glutamate residues and can reveal complementary peptide sets; chymotrypsin cleaves at aromatic residues, generating distinct peptide profiles (Giansanti et al., 2016; van der Weerden et al., 2018). Using alternative or sequential proteases expands peptide diversity, facilitates detection of low-abundance proteins, and improves protein-identification efficiency. Many contemporary studies apply multi-protease strategies to broaden LC–MS/MS proteome coverage (Wang et al., 2020).

3.2. Liquid Chromatography (LC)

Liquid chromatography is a key step in proteomics that separates peptide mixtures before MS analysis, increasing analytical sensitivity in complex samples (Sandra et al., 2013; Shen et al., 2009). The most widely used approach is reversed-phase chromatography, which separates peptides based on hydrophobic interactions (Sandra et al., 2013). Nano-flow and micro-flow LC systems improve detection of low-abundance proteins by using low flow rates that enhance ionization efficiency and increase proteome depth (Beyerlein et al., 2019). Extending column length, reducing particle size, and optimizing gradient duration are fundamental parameters that directly improve MS signal quality, peak capacity, and resolution (Shen et al., 2009; Smith et al., 2020). Multidimensional LC techniques further enhance fractionation, increasing the number of proteins identified in complex samples. Collectively, these optimizations improve reproducibility, quantitative accuracy, and reliable measurement of low-abundance proteins in clinical specimens (Beyerlein et al., 2019). Through such advances, LC innovations contribute substantially to making biomarker discovery and mechanistic investigations more robust and reliable (Shen et al., 2009).

3.3. Mass Spectrometry (MS, MS/MS)

Mass spectrometry is a core technology in proteomics that measures the mass-to-charge (m/z) ratios of peptides, enabling protein identification and quantitative analysis. When coupled with tandem mass spectrometry (MS/MS), fragmentation patterns reveal peptide sequences and support confident protein identification (Aebersold & Mann, 2016). Three major instrument types are widely used in LC–MS/MS proteomics: triple quadrupole (QqQ), Orbitrap, and Q-TOF (Quadrupole Time-of-Flight) systems. Triple quadrupoles are favored for targeted proteomics (MRM/PRM-style quantification) due to high sensitivity and quantitative precision. Orbitrap instruments provide high resolution and mass accuracy, enhancing identification capacity in discovery proteomics. Q-TOF systems

offer rapid acquisition and broad mass-range coverage, supporting comprehensive proteome profiling. Selecting the appropriate platform and protocol directly influences proteome depth, reproducibility, and analytical accuracy, providing major advantages for biomarker discovery and clinical proteomics (Michalski et al., 2011; Aebersold & Mann, 2016).

3.4. Data Analysis

Processing MS data constitutes the computational stage of proteomics. Raw MS/MS outputs do not directly provide biological insight; therefore, data processing is essential to ensure accuracy and reliability. The data-analysis workflow includes spectrum matching, quantification, statistical validation, biological interpretation, and visualization. In modern proteomics, datasets produced by high-resolution LC–MS/MS platforms are processed using advanced bioinformatics tools and algorithms. These systems support quality control, reduce inter-run variation, and improve confidence in protein identifications. Thus, data analysis not only identifies proteins but also enables extraction of biologically and clinically meaningful information for downstream applications (Cox & Mann, 2008).

CONCLUSION

In conclusion, considering proteomics within the context of omics technologies emphasizes its indispensable role in understanding biological systems at the functional level. Although genomic and transcriptomic analyses provide fundamental information about genetic content and gene expression, it is evident that protein-level regulation is the principal determinant of cellular phenotype and disease pathophysiology. Because protein abundance, activity, and post-translational modifications directly influence signaling pathways, cellular adaptation mechanisms, and disease progression, proteomic analyses complement molecular biology by capturing its functional dimensions. LC–MS/MS-based technologies have enabled both discovery and targeted proteomic strategies to analyze biological samples at high resolution and to validate candidate biomarkers with strong reliability. The combined use of data-acquisition strategies such as DDA and DIA, together with isotope-labeled and label-free quantitative approaches, expands proteome coverage while improving reproducibility. Nevertheless, major limitations persist, including the broad dynamic range of proteins in clinical samples, challenges in sample preparation, complexity of data analysis, and the need for standardization. Overcoming these barriers will depend on optimized sample-preparation protocols, advanced mass spectrometry infrastructure, and integration of bioinformatics and artificial intelligence–based analytical approaches. Overall, proteomics emerges as a strategic discipline for biomarker discovery, elucidating disease

mechanisms, and advancing personalized medicine, further strengthening its status as a core omics field shaping the future of systems biology and translational medical research.

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Pneumovagina in Mares

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ABSTRACT

Pneumovagina is an important gynecological condition in mares, characterized by the aspiration of air into the vagina due to inadequate anatomical and functional closure of the vulvar lips, resulting in negative effects on reproductive performance. With the transition of horse breeding toward sport- and race-oriented production systems, achieving optimal fertility within a limited official breeding season has become increasingly critical, particularly for broodmares. In this context, pneumovagina represents not only a clinical disorder but also a significant reproductive management problem affecting breeding efficiency.

The etiology of pneumovagina is multifactorial and is mainly associated with vulvar and perineal conformational defects, age-related loss of muscle tone, multiparity, decreased body condition score, and anatomical changes following parturition. Clinically, the condition is often first recognized through breeders' complaints, including audible air aspiration from the vulva during movement or tail elevation, restlessness during exercise, repeated estrous cycles, infertility, or failure to conceive. Diagnosis is based on clinical and gynecological examination, speculum inspection of the vagina, and supportive diagnostic methods such as uterine cytology and bacteriological culture to identify concurrent uterine infections.

Treatment strategies depend on the severity of the condition and associated pathologies. While conservative management may provide temporary improvement in mild cases, definitive resolution is generally achieved through surgical intervention. Caslick's operation and its modified techniques are commonly applied, and when appropriate case selection and postoperative management are ensured, the prognosis is generally favorable with significant improvement in fertility and reproductive performance.

Keywords – Pneumovagina, Mare, Reproductive performance, Vulvar conformation, Caslick's operation

INTRODUCTION

Horse breeding, influenced by increasing industrialization and technological advancements, has gradually shifted away from its historical functional roles in agriculture, transportation, and warfare, and has evolved into a production activity predominantly focused on sport and racing purposes. This transformation has led to a noticeable decline in the global horse population compared to previous periods. Today, as in many countries worldwide, horse breeding in Türkiye is largely maintained at a professional level, primarily centered on flat racehorse breeding (Matyar & Ergün, 2010; Öcal, 2014).

Horses are physiologically seasonally polyestrous mammals, and in mares, the most important environmental factor initiating and regulating sexual cycles is photoperiod, in other words, the duration of daylight (Ekici, 2015). Increasing day length stimulates ovarian activity through its effects on the hypothalamic–pituitary–gonadal axis. Therefore, reproductive activity in mares exhibits a distinct seasonal pattern (McCue, 2014; Öcal, 2014).

One of the fundamental principles of horse breeding is the achievement of one foal per year, which is of great biological and economic importance for breeders. Considering the high costs associated with horse management and nutrition, along with increasing fixed expenses such as feed, forage, and labor, the primary objective of breeders is to obtain a healthy foal during each breeding season. This goal becomes even more critical for enterprises engaged in racehorse breeding (Seyrek İntaş & Çetin, 2015).

The official breeding season and the corresponding official foaling season, determined by international racing authorities, restrict reproductive activities in racehorse breeding to a specific time period (Ekici, 2015). For countries located in the Northern Hemisphere, the official breeding season is generally accepted to occur between February 15 and June 30. Within this limited timeframe, breeders are required to successfully impregnate their broodmares (Usman et al., 2023). Consequently, thorough and proper preparation of mares prior to the breeding season is a critical factor directly affecting pregnancy success (Hagstrom, 2003; Öcal, 2014; Usman et al., 2023).

Before the breeding season, detailed gynecological examinations of barren mares are meticulously performed, aiming to ensure an uneventful entry into the season through careful evaluation of the uterus, cervix, vagina, and vulvar anatomy (McCue, 2014). In mares that were pregnant during the previous season, the postpartum period is closely monitored, with particular attention given to uterine involution and the identification of potential pathological conditions (Matyar & Ergün, 2010; Öcal, 2014).

One of the most significant clinical problems adversely affecting reproductive performance in mares is pneumovagina, which is characterized by the aspiration of air into the vagina as a result of incomplete anatomical and functional closure of the vulvar lips (Kang et al., 2007; Farage & Maibach, 2016). In cases of pneumovagina, not only air but also environmentally derived microbial agents may enter the vagina, initially leading to vaginitis and subsequently progressing to infections involving the endometrium (Seyrek İntaş & Çetin, 2015). As a result, serious reproductive disorders such as chronic endometritis, infertility, and early embryonic loss

may occur (Ball, 1988; Pycock, 2003; Kang et al., 2007; Matyar et al., 2010).

Pneumovagina is of clinical importance not only in broodmares but also in mares actively participating in racing activities. During racing and training, increased intra-abdominal pressure facilitates vaginal air aspiration, which may lead to discomfort, behavioral abnormalities, and indirectly to a decline in athletic performance (Matyar & Ergün, 2010; Öcal, 2014). In this respect, pneumovagina should be considered a significant clinical condition affecting both reproductive efficiency and athletic performance (van Ittersum & van Buitten, 1999; Pycock, 2003).

ETIOLOGY

Pneumovagina is a multifactorial clinical condition in mares that generally develops as a result of the complex interaction of multiple predisposing factors rather than a single etiological cause (Thornbury, 1975; Seyrek İntaş & Çetin, 2015). Among these factors, vulvar conformational defects represent the most prominent and consistently reported cause. Under normal conditions, the vulvar lips are positioned vertically and form a tight anatomical seal that prevents the aspiration of air and external contaminants into the vaginal canal. However, anatomical alterations affecting the perineal region may compromise this functional integrity, leading to inadequate vulvar closure and facilitating the development of pneumovagina (Thornbury, 1975).

One of the most critical conformational changes involves disruption of the caudodorsal inclination of the perineal angle. When this anatomical alignment is altered, the dorsal commissure of the vulva is displaced ventrally, creating a funnel-like configuration that predisposes the vagina to air aspiration during movement or physical activity (van Ittersum & van Buitten, 1999; Pycock, 2003; Karam et al., 2020). This abnormal conformation not only allows air entry but also promotes the ingress of environmental contaminants, thereby increasing the risk of secondary reproductive tract infections.

Several physiological and management-related factors contribute to the deterioration of vulvar closure mechanisms. Prolonged or traumatic parturition may result in relaxation and stretching of the perineal musculature, leading to permanent structural weakening (Pycock, 2003). Similarly, age-related loss of muscle tone and repeated pregnancies (multiparity) progressively impair the supportive structures of the vulva, further exacerbating conformational defects (Matyar et al., 2010). A reduced body condition score also plays a significant role, as loss of adipose and

muscular support in the pelvic and perineal regions leads to collapse of these structures and insufficient apposition of the vulvar lips (Pycock, 2003; Matyar et al., 2010; Karam et al., 2020).

In addition, perineal lacerations sustained during parturition, traumatic injuries, or the development of scar tissue and fibrosis following surgical interventions may permanently disrupt normal vulvar anatomy. Such pathological alterations reduce tissue elasticity and further compromise the effectiveness of the vulvar barrier. Beyond anatomical factors, functional demands associated with performance disciplines also contribute to the etiology of pneumovagina. In mares engaged in racing, galloping, or jumping, increased intra-abdominal pressure during exercise facilitates the forced entry of air through inadequately closed vulvar lips, thereby accelerating the onset and progression of pneumovagina (Seyrek İntaş & Çetin, 2015).

Taken together, these findings indicate that pneumovagina develops through the cumulative effects of anatomical, physiological, and functional factors, emphasizing the importance of comprehensive clinical evaluation and individualized management strategies in affected mares pneumovagina (Seyrek İntaş & Çetin, 2015).

CLINICAL FINDINGS AND DIAGNOSIS

The clinical findings of pneumovagina are often first recognized through breeders' observations and complaints, and these field-based reports provide valuable preliminary information during the diagnostic process (Seyrek İntaş & Çetin, 2015). Breeders frequently describe audible sounds associated with the passage of air from the vulva, commonly referred to as "windsucking," particularly when the mare elevates her tail, walks, or moves at speed. Such signs are especially noticeable during exercise, training, or racing, when increased physical activity exacerbates air aspiration. In addition to these characteristic sounds, breeders often report behavioral changes including restlessness, repeated tail swishing, and frequent licking of the perineal or genital region, all of which may reflect discomfort associated with vaginal air entry (Hemberg et al., 2005).

From a reproductive standpoint, pneumovagina is frequently suspected in broodmares exhibiting repeated estrous cycles, failure to conceive, early embryonic loss, or prolonged periods of infertility despite appropriate breeding management. These complaints are particularly significant in intensively managed breeding programs, where reproductive inefficiency has direct economic consequences (Hemberg et al., 2005).

Clinical examination plays a central role in diagnosis and typically reveals inadequate closure of the vulvar lips, deviation of the vulva from the vertical axis, and disruption of the normal caudodorsal inclination of the perineal region (Sarath et al., 2025). These conformational abnormalities may be evident on visual inspection and palpation and are often more pronounced in older or multiparous mares. Speculum examination of the vagina is considered a key diagnostic step, as the presence of air bubbles within the vaginal lumen represents a characteristic and highly suggestive finding of pneumovagina. In addition, varying degrees of hyperemia, irritation, or inflammation of the vaginal mucosa are commonly observed, reflecting chronic mechanical irritation caused by repeated air movement.

In more advanced cases, contamination of the cervical region and the presence of uterine discharge may be detected, indicating ascending infection and the development of chronic endometritis (Hemberg et al., 2005). To confirm the diagnosis and to identify concurrent reproductive tract pathology, ancillary diagnostic techniques such as uterine cytology and bacteriological culture are routinely employed. Studies have demonstrated that opportunistic microorganisms, particularly Escherichia coli and Streptococcus equi subsp. zooepidemicus, are isolated at higher frequencies in mares affected by pneumovagina, supporting the association between vaginal air aspiration and uterine contamination (Pasolini et al., 2016).

Based on the combined evaluation of breeders' complaints, clinical findings, and laboratory diagnostic results, pneumovagina is defined as a significant clinical condition that directly compromises reproductive performance and requires targeted diagnostic and therapeutic intervention (Seyrek İntaş & Çetin, 2015).

TREATMENT APPROACHES AND PROGNOSIS

The therapeutic approach to pneumovagina in mares should be determined based on several factors, including the severity of the condition, the extent of vulvar and perineal conformational defects, the presence of concurrent uterine or vaginal infections, and the intended reproductive or athletic use of the mare (Seyrek İntaş & Çetin, 2015). In mild cases, conservative management strategies may initially be considered, aiming to alleviate clinical signs and prevent further progression of the condition. These approaches focus on improving general management conditions and maintaining appropriate perineal hygiene. However, conservative measures generally provide only temporary symptomatic relief and do not address the underlying anatomical abnormalities responsible for vaginal air aspiration. Consequently, their effectiveness as a sole treatment option remains limited

in mares with evident conformational defects (van Ittersum & van Buitten, 1999; Papa et al., 2014; Sarath et al., 2025).

For this reason, permanent and effective treatment of pneumovagina is achieved primarily through surgical intervention in the majority of affected mares. The most widely applied surgical technique is the classical Caslick operation, which involves surgical apposition of the dorsal portion of the vulvar lips to prevent the entry of air into the vagina. By restoring the functional integrity of the vulvar seal, this procedure reduces vaginal air aspiration, limits microbial contamination of the reproductive tract, and supports the maintenance of reproductive health (Kalkan & Alaçam, 2002). Due to its relative simplicity, short operative time, and low incidence of complications, the classical Caslick procedure is frequently preferred, particularly in broodmares managed under intensive breeding conditions (Papa et al., 2014).

Nevertheless, the classical Caslick operation may be insufficient in cases where severe perineal deformities or extensive tissue loss are present. In such situations, modified Caslick techniques are recommended to enhance surgical outcomes. These modifications aim to reposition weakened perineal support tissues, reinforce the vestibulovaginal barrier, and achieve a more effective and durable anatomical closure (Kalkan & Alaçam, 2002; Papa et al., 2014). In mares with advanced perineal defects, a history of traumatic parturition, or pronounced fibrosis, reconstructive perineal surgical techniques may be required. These procedures seek to restore normal vulvar and vestibular anatomy and improve long-term functional outcomes (Kalkan & Alaçam, 2002).

The prognosis following treatment of pneumovagina is generally favorable. When appropriate surgical techniques are applied with correct indications, significant improvements in reproductive performance are commonly observed in affected mares (Kalkan & Alaçam, 2002). Several studies have reported a marked increase in pregnancy rates following surgical correction, highlighting the effectiveness of these interventions in restoring fertility (Trotter et al., 1988). However, in cases where uterine infections coexist with pneumovagina, adjunct medical therapy targeting the infectious process is essential to optimize treatment success and improve prognosis (Seyrek İntaş & Çetin, 2015). With proper case selection, appropriate surgical technique, and adequate postoperative care, recurrence rates are reported to be low, and long-term clinical success can be achieved in both broodmares and performance mares (Trotter et al., 1988).

CONCLUSION

Pneumovagina is a gynecological condition frequently encountered in mares that is often overlooked; however, it has a decisive impact on reproductive performance and overall clinical success (Seyrek İntaş & Çetin, 2015). Developing under the influence of predisposing factors such as vulvar and perineal conformational defects, aging, decreased body condition score, and anatomical changes occurring after parturition, pneumovagina creates a predisposition for vaginal air aspiration. This condition, in turn, facilitates microbial contamination, leading to serious reproductive disorders including chronic endometritis, infertility, and early embryonic losses (Ball, 1988; Hagstrom, 2003; Matyar & Ergün, 2010).

Although clinical examination supported by breeders' complaints is often guiding during the diagnostic process, cytological and bacteriological evaluations are of great importance for identifying concurrent uterine infections (Seyrek İntaş & Çetin, 2015). In terms of treatment strategies, conservative methods provide limited benefit depending on the severity of the condition, whereas permanent and effective outcomes are predominantly achieved through surgical interventions (Seyrek İntaş & Çetin, 2015). In particular, the appropriate application of Caslick's operation and modified surgical techniques allows restoration of the vulvar barrier function and results in marked improvements in reproductive performance in affected mares (Pycock, 2003; Matyar & Ergün, 2010).

In this context, pneumovagina should be considered not only a clinical disorder but also an important management problem that may lead to economic losses and decreased performance in both broodmares and sport mares. Therefore, early recognition and timely intervention by veterinarians involved in reproductive management, in collaboration with breeders, are essential for achieving optimal reproductive outcomes (Matyar & Ergün, 2010).

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Genomic Approaches to Myocardial Infarction Cases

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ABSTRACT

A heart attack (myocardial infarction) is a serious health problem caused by the sudden blockage of the coronary arteries that supply the heart with blood. Although lifestyle factors (smoking, diet, inactivity) play an important role, genetic predisposition is also one of the key factors affecting the risk of heart attack. A family history of heart attack at an early age suggests that an individual's risk may be increased.

A family history of heart attacks, especially those occurring before age 55 in men and age 65 in women, indicates an increased genetic risk. Certain genes can affect cholesterol and fat metabolism, leading to elevated LDL cholesterol. This accelerates hardening of the arteries (atherosclerosis) and increases the risk of heart attack. Genes related to the clotting system and the structure of the inner surface of blood vessels also play a role in susceptibility to heart attacks.

Genomic diseases in humans are diseases that arise due to changes (mutations) in DNA. Genomic diagnostic technologies developed for the accurate and early diagnosis of these diseases are one of the most important areas of modern medicine. One of the most fundamental methods is DNA sequencing technology. Sanger sequencing is used for detailed examination of a specific gene. Next-Generation Sequencing (NGS), on the other hand, allows for the analysis of thousands of genes or even the entire genome at the same time. This method is widely used in the diagnosis of other diseases,.

In conclusion, genomic diagnostic technologies enable a better understanding of heart attack cases, early diagnosis, and personalized treatment approaches.

Keywords: Heart Attack, Gene Mutations, Genetic Polymorphism, Risk Factors and Genomic Technologi

1. INTRODUCTION

1.1. **Structure of the HeartGeneral Anatomy**

The heart is an organ composed of muscle tissue located in the middle mediastinum. Its average weight is 275±75 grams in women and 325±75 grams in men. It is adjacent to the mediastinal surfaces of the lungs on the right and left sides, the diaphragm below, the sternum and cartilaginous ribs in front, remnants of the thymus, part of the lungs, and the esophagus behind. The heart has an apex (Apex cordis), a base (Basis cordis), four sides, and four surfaces. The apex extends forward and to the left, while the base extends backward, to the right, and slightly upward. In the mediastinum, it runs obliquely to the left from the 2nd intercostal space to the 5th intercostal space. The heart has its own unique axis of orientation that does not align with any orthogonal plane of the body. The body can be examined in three standard anatomical planes that are perpendicular to each other: transverse, horizontal, and sagittal. Although these planes are perpendicular to each other, the heart also has three planes: transverse (short axis), horizontal long axis (four-chamber view), and vertical long axis (two-chamber view) (Sinan & Unlu, 2017). The heart's own planes do not lie in the same plane as the body's standard planes. They intersect obliquely. Two conventional methods are used to learn cardiac anatomy. The first is the inlet-outlet method, and the other is the tomographic ventricular slicing method (Bulut, 2016). The inlet-outlet method is not consistent with the images obtained by tomographic imaging methods used in the clinic, except for cavitary angiography. While this method can easily show cardiac cavity and valve diseases, it cannot simultaneously show the effects of the

disease on nearby structures. In the ventricular slicing method, the ventricle is divided into slices perpendicular to the ventricular septum, like slices of bread.

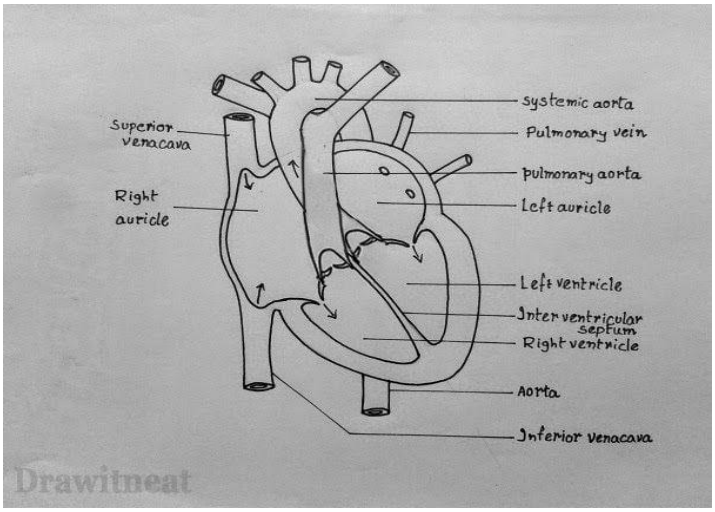

Figure 1: Structure of the Heart

1.2. What is a Heart Attack?

A heart attack (myocardial infarction) is defined as irreversible myocardial damage and necrosis resulting from severe and prolonged ischemia (Feng & Li, 2022). The most common cause of a heart attack is a thrombus sitting on an unstable or ruptured atherosclerotic plaque (Li et al., 2021). Autoregulatory mechanisms within the coronary arteries usually maintain adequate oxygen delivery to the myocardium even in the presence of atherosclerotic plaques. However, when these protective mechanisms are disrupted, prolonged ischemia or myocardial infarction may occur (Ekmekçi et al., 2008). Myocardial infarction, a segmental disease, develops with the total occlusion of only one of the three major coronary arteries or branches. The contractility disorder that occurs after a heart attack develops within seconds and is initially limited to the affected segment. A heart attack caused by occlusion in the left anterior descending artery develops in the apical and anterior parts of the left ventricle, the interventricular septum, the papillary muscles in the anterolateral wall, and the inferoapical wall of the left ventricle. According to the definition by the World Health Organization (WHO), a heart attack is the presence of at least two of the following criteria.

1-Typical chest pain 2-Elevated CK-MB concentration in serum 3-Typical ECG findings including pathological Q waves The lack of sensitivity of CK-MB for myocardial necrosis has led to incomplete and delayed diagnosis in many patients in clinical practice. With the increasing importance of Troponin T and I markers, which are highly specific for myocardial damage, and new imaging techniques, the European Society of Cardiology (ESC) and the American College of Cardiology (ACC) introduced a new definition in 2000 to increase the sensitivity and specificity for heart attack.

1.3.STEMI (ST-Elevation Myocardial Infarction)

Patients with STEMI require urgent intervention to restore coronary blood flow and minimize the risk of death. This can be achieved with percutaneous coronary intervention (PCI) or fibrinolytic therapy. Patient selection, ischemic and bleeding risks, especially in patients with significant comorbidities (additional diseases) or a short life expectancy, should be carefully evaluated.

In the absence of a life-limiting disease and contraindications, emergency reperfusion is required in patients presenting within 12 hours of the onset of chest pain. Primary percutaneous intervention is preferred if it can be performed within 90 minutes of the first medical contact. For patients who cannot reach an appropriate facility within this time frame, fibrinolytic therapy is a life-saving option and should be administered

immediately. Primary percutaneous intervention is reasonable within 24 hours, but if fibrinolytic therapy fails, urgent primary percutaneous intervention is critical for rescue. The success of treatment is determined 60-90 minutes after fibrinolysis. Within minutes, an ECG taken from the patient can confirm a decrease of 50% or less in ST elevation, hemodynamic instability (consistent with stable blood pressure and blood flow), or persistent chest pain (Winkelmann & Hager, 2000).

1.4. Non-Stemı (Non-St-Segment Elevatıon Myocardıal Infarctıon)

Compared to STEMI, diagnosing non-STEMI is more complex. Here, the diagnosis should be based not only on the patient's elevated troponin levels but also on the interpretation of the entire clinical presentation within the context of the universal definition of myocardial infarction. Once a non-STEMI diagnosis is confirmed, initial treatment includes antiplatelet therapy and anticoagulation.

1.5. Heart Attack Diagnostic Methods

According to current consensus, a myocardial infarction is defined by elevated cardiac biomarkers with an upward or downward trend and at least one of the following:

Symptoms related to ischemia Changes in an electrocardiogram, such as ST segment changes, new left bundle branch block, or pathological Q waves Changes in heart wall motion on imaging Demonstration of thrombus on angiogram or autopsy Myocardial infarction is generally classified based on ST elevation, which is a portion of the heartbeat recorded graphically on an ECG (Rauch et al., 2001). Wave intervals on the electrocardiogram STEMI (ST-elevation myocardial infarction) indicates complete occlusion and accounts for 25-40% of myocardial infarctions. NSTEMI (non-ST elevation myocardial infarction) indicates partial occlusion (Solak, 2023).

2. HEART ATTACK (MYOCARDIAL INFARCTION) AND GENETIC AND ENVIRONMENTAL INTERACTION

The development of myocardial infarction (MI) may be influenced by genetic factors, but the condition is typically considered a multifactorial disease involving both genetic and environmental contributions. A family history of myocardial infarction is a known risk factor for the condition. Individuals with a first-degree relative (parent, sibling, or child) who had a heart attack at an early age (usually before age 55 for men and age 65 for women) have a higher risk of developing MI. This suggests a genetic component contributing to this risk (Li et al., 2021) ..

2.1. Environmental Variations

Various genetic variations or mutations that may increase susceptibility to myocardial infarction have been identified. These variations can affect various biological processes related to cardiovascular health, including lipid metabolism, inflammation, blood clotting, and arterial function.

Lipid Metabolism: Some genetic variants are associated with abnormalities in lipid metabolism, such as high low-density lipoprotein cholesterol (LDL-C) levels or low high-density lipoprotein cholesterol (HDL-C) levels, both of which are risk factors

Inflammation: Genetic variations can affect the production and regulation of inflammatory molecules. Chronic inflammation is associated with the development and progression of atherosclerosis, the underlying condition that leads to most cases of MI.

Blood Clotting: Genetic factors can influence the clotting process, including the formation and dissolution of blood clots. Specific genetic variations can increase the risk of abnormal blood clotting and potentially lead to blockages in the coronary arteries. Genetic factors can interact with environmental factors, such as lifestyle choices, to influence the risk of myocardial infarction. For example, certain genetic variants can increase susceptibility to MI, particularly when combined with unhealthy behaviors such as smoking, poor nutrition, or physical inactivity. It is important to understand that genetic factors alone do not determine the occurrence of myocardial infarction. Additionally, individual genetic variations have relatively small effects on overall myocardial infarction risk, and the interaction between genetics and environment remains complex (Feng & Li, 2022). If you have concerns about the genetic aspects of myocardial infarction, it is recommended that you consult a healthcare professional or genetic counselor. They can provide personalized information and guidance based on your specific situation and family history.

Furthermore, researchers have developed genetic risk scores that combine information from multiple genetic variants associated with myocardial infarction. These scores aim to estimate an individual's overall genetic risk based on their specific genetic makeup. However, it is important to note that genetic risk scores are still in the research phase and currently have limited clinical utility (Škrlec et al., 2023).

3. HEART ATTACK AND GENETIC FACTORS

Genetic disorders can be grouped into three main categories: chromosomal abnormalities, single-gene (monogenic) disorders, and multi-gene disorders (polygenic).

3.1. Chromosomal Abnormalities

Chromosomal abnormalities typically arise from mutations occurring during meiosis when chromosomes separate. Since the phenotypes caused by gene mutations are easily diagnosed during physical examination, most chromosomal abnormalities can be detected in newborns or infants. Chromosomal abnormalities in newborns often lead to structural heart defects and are found in 5 to 13% of children born alive with congenital heart defects. Research has shown that some important gene regions affecting heart attack risk are located at specific positions on human chromosomes: The chromosome 9p21 region is the genetic region most strongly associated with heart attack and coronary artery disease. This region is associated with the proliferation of blood vessel wall cells and hardening of the arteries (atherosclerosis). Chromosomes 1, 2, and 6: Certain genes located on these chromosomes contribute to heart attack risk by affecting cholesterol metabolism, inflammation, and immune response. Chromosome 19 (LDLR gene) This gene, which is responsible for removing LDL cholesterol from the blood, causes cholesterol to rise and increases the risk of vascular blockage when it is defective. Chromosome 11 (APOA and APOC genes) is related to proteins (Allison & Beldüz, 2014; Yılmazer et al., 2018).

3.2. Single Gene Disorders

A single gene disorder is a hereditary disease caused by a mutation in a single gene. Single gene disorders follow the Mendelian inheritance model; they can be classified as autosomal dominant, autosomal recessive, or X-linked (dominant or recessive) inheritance [4]. In Mendelian inheritance, the defect in a single gene is the cause of the disease; other genes and environmental factors only have a positive or negative effect on the age of onset and course of the disease (Ekmekçi et al., 2008).

3.3. Polygenic Disorders

Polygenic disorders arise from the interaction of genetic mutations and non-genetic factors (such as environmental factors). In this case, the presence of the mutated gene is not sufficient to cause the disease, while its absence does not prevent the disease from developing. However, this disorder can increase the risk of disease development. Multigenic disorders are a contributing factor in the majority of cardiovascular diseases such as atherosclerosis, hypertension, obesity, and diabetes mellitus.

3.4. Genes Associated with Myocardial Infarction

A family history of ischemic heart disease or MI, especially a first-degree male relative (father, brother) who has had a myocardial infarction before the age of 55 or a first-degree female relative (mother, sister) younger than 65, increases a person's risk of MI.

Genome-wide association studies have identified 27 genetic variants associated with an increased risk of myocardial infarction. The strongest association with MI was found on chromosome 9, in locus 21 on the short arm p, which includes the CDKN2A and CDKN2B genes; however, it is located within a region that does not encode the included single nucleotide polymorphisms. It is located on chromosome 9.

Other genes associated with heart attack include: PCSK9, SORT1, MIA3, WDR12, MRAS, PHACTR1, LPA, TCF21, ZC3HC1, APOA5, COL4A1, SMAD3, ADAMTS7, SMG6, LDLR, SLC5A3, MRPS6, KCNE2.

PCSK9: Known as “Proprotein Convertase Subtilisin/Kexin Type 9,” it is a protein encoded by the PCSK9 gene and plays an important role in cholesterol metabolism. PCSK9 is particularly effective in regulating low-density lipoprotein (LDL) cholesterol levels.

The main function of PCSK9 is to break down LDL receptors found in the liver. LDL receptors transport LDL cholesterol from the blood into cells, allowing cholesterol to be taken up by the cells. However, PCSK9's function causes LDL receptors to be degraded, leading to their removal from the cell surface. This can result in the accumulation of LDL cholesterol in the blood and elevated levels.

Certain mutations in the PCSK9 gene can cause high cholesterol levels and an increased risk of heart disease. Conversely, inhibiting or blocking PCSK9 allows LDL receptors to remain active for longer, which facilitates the more effective uptake of LDL cholesterol into cells. Therefore, PCSK9 inhibitors represent a class of drugs used in the treatment of high cholesterol.

SORT1: SORT1 belongs to a gene family also known as “sortilin 1.” This gene, the SORT1 protein gene, is associated with lipoprotein metabolism. In particular, the SORT1 gene and the protein it produces are thought to affect the circulation of cholesterol-carrying lipoproteins. Studies on the SORT1 gene suggest that this gene may be associated with cardiovascular diseases, particularly coronary artery disease risk.

MIA3: The MIA3 gene is known to play a role in cell adhesion and migration. Adhesion is the ability of cells to stick to each other or their surroundings, while migration is the ability of cells to move from one place to another. Such genes can play an important role in many biological processes, such as cell development, tissue regeneration, and the immune system.

WDR12: WDR12 is a protein involved in intracellular protein synthesis processes and affects the formation of ribosomal subunits. Protein synthesis is an important process for cells to sustain life, and ribosomes are one of the fundamental building blocks of this process.

MRAS: MRAS refers to a gene located in the human genome. The MRAS gene encodes a protein called “Muscle RAS Oncogene Homolog.” This gene is a member of the RAS family. The RAS family contains a group of proteins that play an important role in cell growth, division, and cellular signaling.

Research on the MRAS gene suggests that it may play a role in cellular signaling and various biological processes within the cell. It is known that the RAS gene family in general may carry genetic mutations that can contribute to the development of cancer and other diseases.

PHACTR1: The PHACTR1 gene may contribute to important cardiovascular system functions, such as coronary artery development and the regulation of cellular processes in the artery wall. Furthermore, genetic factors associated with the PHACTR1 gene due to genetic variations or mutations are thought to influence an individual's risk of cardiovascular disease.

LPA: The LPA gene encodes a lipoprotein called Lipoprotein(a) or Lp(a) in humans. Lipoprotein(a) is a type of lipoprotein composed of low-density lipoprotein (LDL) and a protein called apolipoprotein(a). The LPA gene regulates Lp(a) levels in the blood.

Lp(a) has a structure similar to LDL, a cholesterol-carrying lipoprotein, but it has a unique subunit to which a unique protein called apolipoprotein(a) is attached. Although the structural properties and function of this unique protein are not fully understood, high Lp(a) levels increase the risk of cardiovascular disease.

TCF21: The TCF21 gene is known to be particularly effective during embryonic development and tissue formation. Its functions are related to the differentiation and development of mesodermal cells found in the cardiovascular system, kidneys, lungs, and other tissues.

APOA5: APOA5 is the name of a gene that encodes apolipoprotein A-V. Apolipoprotein A-V (apoA-V) is a protein that plays an important role in the metabolism of lipoproteins and lipid (fat) transport in the blood. This gene produces a protein that is effective in regulating triglyceride levels in the blood.

LDLR: The LDLR gene encodes receptors that transport low-density lipoproteins (LDL) to cells in the body. LDL is a type of lipoprotein commonly known as “bad” cholesterol. These receptors help cells obtain the lipids they need by taking LDL from the blood into the cells.

MRPS6: The MRPS6 gene is the name of a gene that encodes mitochondrial ribosomal protein S6. This gene encodes a protein that is part of the mitochondrial ribosomal subunits and plays a role in protein synthesis processes. Mitochondria are organelles responsible for energy production within the cell and play a role in various important cellular processes, including protein synthesis.

SMAD3: The SMAD3 gene is the name of a gene that plays an important role in cellular signaling. This gene belongs to the SMAD family and encodes the SMAD3 protein. SMAD3 is a transcription factor involved in cellular signal transduction and the regulation of gene expression.

COL4A1: COL4A1 is the name of a gene found in humans. This gene is responsible for the production of a protein called type IV collagen in the body. Collagen is a family of proteins that support and structure tissues in the body. Type IV collagen is found particularly in an intercellular structure called the basement membrane, thereby ensuring the strength and integrity of various tissues.

KCN2: The KCNE2 gene encodes a protein that is a subunit regulating a potassium channel called K_VLQT1, particularly in heart cells. This channel plays an important role in maintaining the heart's normal rhythm. Mutations in the KCNE2 gene or disorders related to this gene can lead to heart rhythm disorders and contribute to heart diseases such as Long QT syndrome.

ADAMTS7: The ADAMTS7 gene can contribute to various biological processes by acting on the cellular matrix. This gene has been observed to be expressed in many tissues and organ systems. In particular, ADAMTS7 is thought to play a role in the extracellular matrix of joint cartilage and blood vessel walls (Lee, 2018; Wu et al., 2020).

4.MYOCARDIAL INFARCTION AND GENETIC POLYMORPHISM

4.1. Genetic Polymorphism

In addition to these, Single Nucleotide Polymorphisms (SNPs) and Copy Number Variations (CNVs) are discussed, which have enabled significant advances in genetic identification. SNPs are single nucleotide (A, T, C, G) changes in the genome sequence. For example, the conversion of an adenine base to a thymine at a specific position. SNPs largely explain why some individuals are healthier than others under similar conditions, why the same disease progresses differently among different individuals, and why some individuals respond positively to treatment while others do not. Just as our outward appearance differs from one another, our response to the above events is also that different. This difference stems from a 0.1% structural variation in our DNA, with SNPs accounting for the majority of this 0.1% difference.

CNVs, defined in recent years, have added a new dimension to human genetic diversity. Although a cell has two identical copies of DNA, one from the mother and one from the father, it has been understood that there are differences in the number of copies of certain regions of DNA. Unlike SNPs, which consist of a single nucleotide change, CNVs, due to their size, cover more nucleotides in the genome structure and are therefore at least as influential as SNPs in disease formation. CNV-type mutations are increasingly seen today in both Mendelian and complex diseases. They cause disease either alone or in combination with other genetic and environmental factors (Ekmekçi et al., 2008).

4.2. Types of Polymorphisms:

Polymorphisms within a population can be grouped into three categories based on their cause: single nucleotide polymorphism (SNP), insertion, and deletion. Single Nucleotide Polymorphism (SNP): A single base difference is observed in the DNA sequence every 2000-2500 bases. Transition and transversion are subgroups of single nucleotide polymorphism.

Transition: This refers to the conversion of a purine base (A, G) in the sequence to another purine base or a pyrimidine base (T, C) to another pyrimidine base. A, G→ G, A C, T → T, C

Transversion: This refers to the change of a purine base (A, G) in the sequence to a pyrimidine base (T, C) or a pyrimidine base to a purine base. A, G → T, C T, C → G, A Insertion: This is the addition of a single nucleotide or more nucleotides into the DNA. The addition of nucleotides causes the gene to be longer than its normal length. Even if a single nucleotide is inserted, a frameshift can occur, altering the amino acid sequence of the entire protein. As a result, the structure of the synthesized protein may be completely disrupted.

Deletions: The breakage and separation of a single nucleotide or nucleotides from the DNA sequence is defined as a deletion. A deletion on DNA causes the gene to be shorter than its normal length (Hosseini et al., 2022; Wu et al., 2020).

4.3. Effects of SNPs on Protein Formation Single nucleotide polymorphism

Depending on its location on the DNA and how it occurs, it can cause changes in the structure and function of the protein: A silent SNP occurring in the coding region will not cause any changes in the structure and function of the synthesized protein if it does not create an amino acid difference. If an SNP occurring on DNA coincidentally forms a "Stop" codon (TAA, TAG, TGA), protein synthesis will stop from the point where the SNP is located, and the incomplete protein will not be able to perform its functions fully. If the SNP occurs in the promoter region, which contains the regulatory elements involved in gene synthesis, it will cause changes in the level and stability of the mRNA produced by transcription and in the level of gene expression (Ekmekçi et al., 2008).

4.4. SNPs and related diseases

• In epidemiological and biomedical research, SNP identification and comparison are performed in patient and healthy control groups from different populations. These diseases include various types of cancer, cardiovascular diseases, chronic diseases, Alzheimer's, and migraine. Genetic polymorphisms enable the determination of individual differences in susceptibility to certain diseases in medicine. Some polymorphisms increase the risk of a disease, while others may reduce it (Feng & Li, 2022).

• Identifying genes and polymorphisms associated with disease development or susceptibility through future studies will be useful in the early diagnosis and treatment of many diseases.

5. GENOMIC APPLICATIONS IN THE DIAGNOSIS OF MYOCARDIAL INFARCTION

5.1. PCR-RFLP (Polymerase Chain Reaction -- Restriction Fragment Length Polymorphism)

PCR-RFLP is a traditional technique used to identify polymorphisms. DNA samples amplified by PCR are cut using specific restriction enzymes, and the resulting fragments are then separated by agarose gel electrophoresis. Different polymorphic variants respond to changes in DNA sequences by restriction enzymes, resulting in different fragment lengths**.**

5.2. SNP: (Single Nucleotide Polymorphism)

In genotyping and DNA sequencing, SNPs are the most common genetic polymorphisms. SNPs are used in genotyping to identify single nucleotide changes between individuals (Barth & Tomaselli, 2016).

5.3. Massarray

Genotyping is performed using methods such as Taq Man allelic separation and ILLUMINA BEAD ARRAY. Genetic polymorphisms can also be determined using techniques such as Sanger sequencing and next-generation sequencing (NGS). NGS, in particular, provides high-resolution and comprehensive data for genetic analysis (Yılmazer et al., 2018). Furthermore, whole genome scans or exome scans are widely used today**.**

5.4. Gel Electrophoresis of SNPs

Agarose or polyacrylamide gel electrophoresis is used to separate DNA fragments and visualize DNA bands for use in polymorphism analysis. It is particularly effective in the analysis of certain types of polymorphisms, such as RFLP or microsatellite polymorphisms. Each technique may be preferred depending on the specific application and laboratory requirements. Genetic polymorphism analysis plays an important role in many areas, such as research into genetic-based diseases**,** population genetics studies, and personalized medicine applications (Allison & Beldüz, 2014; Yılmazer et al., 2018).

6. CONCLUSION

As you know, the cardiovascular system is affected by various diseases. Fortunately, thanks to proper care and preventive measures, there is research aimed at slowing the progression of heart attacks, improving them, or reducing the risk of their development. As with many diseases, the causes of heart attacks are varied and can vary from person to person. Some common risk factors include poor nutrition, obesity (excessive weight gain), smoking, stress, and a sedentary lifestyle.

Today, genome scans are gaining importance among the methods that enable the understanding of many previously classified heart attack cases through genetic research. Identifying mutations associated with myocardial infarction cases and the environmental factors that interact with these mutations will enable early diagnosis and treatment, allowing for the modification of specific lifestyle factors to reduce serious mortality rates associated with cardiac genetic disorders. Although progress has been made in this area, the current level is still not at the desired level. With genomic screening rapidly advancing towards integration into clinical practice, moving from being a rare event to becoming a diagnostic and prognostic tool, some developments in clinical practice within the field of expertise of healthcare professionals are of vital importance.

We believe that genomic studies will be helpful in diagnosis to effectively reduce your risk of developing cardiovascular disease. In this sense, genomic research is expected to support clinical research. Genomic methods that can be used in these multifactorial diseases include pedigree analysis, family studies, twin studies, direct or indirect mutation studies, and disease gene polymorphism susceptibility analyses. In conclusion, genomic research will be a guide in terms of preventive measures, appropriate treatment selection, drug treatment efficacy, drug interactions, and ensuring patient compliance with treatment.

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Extracellular Vesicles (Exosomes): Molecular Mechanisms and Their Diagnostic and Therapeutic Applications

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ABSTRACT

Extracellular vesicles (EVs), particularly exosomes, have gained significant attention as essential mediators of intercellular communication due to their ability to transfer biologically active molecules between cells. This book chapter aims to provide a comprehensive and integrative overview of the molecular mechanisms governing exosome biogenesis, cargo selection, and functional activity, as well as their emerging diagnostic and therapeutic relevance. The approach of this chapter is based on a critical synthesis of current experimental and clinical literature, focusing on endosomal membrane dynamics, ESCRT-dependent and ESCRT-independent pathways, lipid-driven vesicle formation, and selective molecular packaging mechanisms. Key findings from the literature demonstrate that exosomes carry a highly regulated cargo of proteins, lipids, microRNAs, long non-coding RNAs, and other nucleic acids, enabling them to modulate gene expression, intracellular signaling pathways, immune responses, and disease progression in recipient cells. Moreover, the remarkable molecular stability of exosomes in biological fluids such as blood, urine, and cerebrospinal fluid highlights their strong potential as non-invasive diagnostic biomarkers. Recent advances in exosome engineering and loading strategies further support their application as biocompatible nanocarriers for targeted drug and gene delivery. In conclusion, this chapter emphasizes that exosomes represent a powerful biological platform at the intersection of molecular biology, diagnostics, and therapeutics, while also addressing current limitations, standardization challenges, and future perspectives required for their successful clinical translation.

Keywords – Exosomes, Intercellular communication, Molecular mechanisms, Diagnostic biomarkers, Therapeutic applications, Drug delivery system

1. INTRODUCTION

Intercellular communication is essential for maintaining physiological homeostasis in multicellular organisms. Traditionally, this communication was thought to be mediated primarily by soluble molecules such as hormones, growth factors, and cytokines. However, accumulating evidence has demonstrated that EVs play an active and sequential role in biochemical communication between cells (Théry et al., 2018). Extracellular vesicles are membrane-bound biological carriers secreted by cells, characterized by a lipid bilayer structure and nanoscale size, and are responsible for the transfer of molecular information. Based on their biogenesis and size, EVs are broadly classified into exosomes, microvesicles, and apoptotic bodies (Yáñez-Mó et al., 2015). Exosomes originate from the endosomal system, whereas microvesicles are generated through outward budding of the plasma membrane. These vesicles carry a diverse cargo of biomolecules, including proteins, lipids, DNA, messenger RNA, and microRNAs, enabling them to directly modulate gene expression and intracellular signaling pathways in recipient cells (Raposo & Stoorvogel, 2013). A key biochemical feature of extracellular vesicles is the functional nature of their molecular cargo. Vesicle-mediated transfer of microRNAs contributes to post-transcriptional gene regulation in target cells, while vesicle-associated proteins can activate specific signaling cascades (Valadi et al., 2007). This evidence supports the concept that EVs function not as passive cellular debris but as active biological messengers. Extracellular vesicle–mediated communication plays critical roles in processes such as angiogenesis, tissue regeneration, immune regulation, and neural signaling. Notably, studies in cancer biology have shown that tumor-derived extracellular vesicles actively remodel the tumor microenvironment, promote metastatic niche formation, and contribute to immune evasion and therapy resistance (Kalluri & LeBleu, 2020). Consequently, EVs have become a major focus in studies of disease pathogenesis. Furthermore, their remarkable stability in biological fluids highlights their potential as non-invasive biomarkers for early disease diagnosis, while their natural carrier properties make them promising platforms for drug and gene delivery in therapeutic applications (El Andaloussi et al., 2013).

2. Exosome Biogenesis: Biochemical Mechanisms

Exosomes represent a well-characterized subgroup of extracellular vesicles and are generated through biogenesis pathways originating from the endosomal system. This process involves a coordinated series of intracellular membrane remodeling events, protein–protein interactions, and lipid-mediated regulatory mechanisms. Exosome biogenesis plays a critical role in determining the specificity, efficiency, and functional outcomes of

intercellular communication (Théry et al., 2018). The formation of multivesicular bodies (MVBs) is initiated by inward invaginations of the endosomal membrane, enabling the selective encapsulation of proteins, lipids, and nucleic acids into intraluminal vesicles (ILVs) (Raposo & Stoorvogel, 2013). During this process, key molecular regulators including tetraspanins, ESCRT (Endosomal Sorting Complex Required for Transport) machinery, and chaperone proteins contribute to cargo selection and influence the targeting specificity of exosomes toward recipient cells (Hurley, 2015; Yáñez-Mó et al., 2015). In addition, lipid composition and membrane curvature play a fundamental mechanical role in vesicle formation, facilitating intracellular trafficking and effective delivery of exosomes to target cells (Trajkovic et al., 2008). Collectively, these biochemical and molecular mechanisms establish exosomes not merely as passive carriers, but as active signal transducers capable of modulating gene expression, intracellular signaling pathways, and cellular function in recipient cells. Consequently, exosome biogenesis has emerged as a major research focus in cell biology, with important implications for understanding pathological processes and developing therapeutic strategies (Valadi et al., 2007).

2.1. Endosomal Membrane Dynamics

Exosome biogenesis begins with the maturation of early endosomes into late endosomes and subsequently into MVBs. During this maturation process, inward budding of the endosomal membrane leads to the formation of ILVs. Upon fusion of MVBs with the plasma membrane, these ILVs are released into the extracellular space and are subsequently referred to as exosomes (Raposo & Stoorvogel, 2013). Endosomal membrane dynamics are tightly regulated by Rab family GTPases, which control vesicular trafficking and membrane fusion events. In particular, Rab27, Rab11, and Rab35 play essential roles in directing MVB transport toward the plasma membrane and facilitating exosome secretion. This regulatory network allows exosome release to be dynamically modulated in response to cellular and environmental conditions (Kalluri & LeBleu, 2020). Furthermore, ESCRT machinery and tetraspanin-enriched membrane microdomains contribute to ILV formation and ensure the selective incorporation of specific molecular cargo into exosomes (Hurley, 2015; Yáñez-Mó et al., 2015). Lipid composition, including the presence of curvature-inducing lipids, mechanically facilitates membrane budding and enhances the efficiency of vesicle release (Trajkovic et al., 2008). Importantly, the molecular cargo packaged within exosomes comprising proteins, nucleic acids, and lipids does not merely represent cellular byproducts but actively participates in the regulation of gene expression, signal transduction, and immune responses in recipient cells (Valadi et al., 2007; Raposo & Stoorvogel, 2013).

Accordingly, exosomes function as key mediators of intercellular communication and represent promising therapeutic carriers. Ongoing research into the molecular mechanisms governing exosome biogenesis remains essential for advancing our understanding of cellular regulation and disease pathogenesis (Théry et al., 2018).

2.2. ESCRT Complexes and Protein–Protein Interactions

One of the most well-characterized mechanisms underlying exosome biogenesis involves the ESCRT system. The ESCRT machinery consists of four sequential complexes ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III which act coordinately on the endosomal membrane to facilitate cargo recognition, membrane deformation, and vesicle scission (Henne et al., 2011). ESCRT-0 is responsible for the initial recognition and clustering of ubiquitinated proteins, whereas ESCRT-I and ESCRT-II induce membrane curvature and initiate inward budding. In the final stage, ESCRT-III mediates membrane constriction and scission, thereby completing the formation of ILVs. The disassembly and recycling of ESCRT components are subsequently driven by the ATPase VPS4, ensuring the reversibility and regulation of this process. Specific protein–protein interactions among ESCRT components play a critical role in the selective packaging of proteins and nucleic acids into exosomes, thereby contributing to cargo specificity and functional heterogeneity (Hurley, 2015). In addition to ESCRT-dependent pathways, alternative ESCRT-independent mechanisms have also been identified in certain cell types. These pathways involve lipid reorganization and the action of membrane-associated molecules such as tetraspanins and ceramides, which promote membrane budding and ILV formation independently of the canonical ESCRT machinery (Trajkovic et al., 2008; Yáñez-Mó et al., 2015). The coexistence of ESCRT-dependent and ESCRT-independent routes enables cell type–specific regulation of exosome composition and responsiveness to environmental cues. Collectively, the ESCRT system and its associated molecular interactions are fundamental to ILV formation and the functional regulation of exosomes in intercellular communication (Raposo & Stoorvogel, 2013).

2.3. Lipid Rearrangement and Membrane Curvature

Lipid-mediated mechanisms constitute a critical component of exosome biogenesis, acting either in coordination with or independently of the ESCRT machinery. Alterations in lipid composition and the generation of membrane curvature are key determinants of vesicle formation. In particular, the localized accumulation of curvature-inducing lipids such as ceramides promotes inward bending of the endosomal membrane, thereby facilitating ILV formation (Trajkovic et al., 2008). In parallel, members of the

tetraspanin family including CD9, CD63, and CD81 organize specialized membrane microdomains that regulate membrane architecture and selectively recruit cargo proteins.

These lipid–protein interactions not only influence vesicle budding but also play a decisive role in shaping the biochemical composition and target cell specificity of exosomes. Through the coordinated action of lipid rearrangement, membrane curvature generation, and protein scaffolding, exosome biogenesis emerges as a tightly regulated process integrating endosomal membrane dynamics, ESCRT-dependent mechanisms, and lipid-driven pathways. A comprehensive understanding of these processes is essential for elucidating the roles of exosomes in cellular communication and for advancing their diagnostic and therapeutic applications in clinical settings (Yáñez-Mó et al., 2015).

3. Molecular Composition and Functional Cargo of Exosomes

Exosomes are not merely vesicular structures involved in the disposal of cellular waste; rather, they are highly specialized biological carriers that mediate intercellular communication through their distinct molecular composition. The proteins, nucleic acids, and lipids encapsulated within exosomes induce a wide range of biochemical and functional alterations in recipient cells, thereby playing critical roles in the regulation of both physiological and pathological processes (Kalluri & LeBleu, 2020). Exosomes exhibit selective cargo packaging mechanisms that allow precise modulation of gene expression and intracellular signaling pathways in target cells. For instance, exosomal microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) contribute to translational regulation and transcriptional control, ultimately shaping cellular responses in recipient cells (Valadi et al., 2007; Yáñez-Mó et al., 2015). Among their protein components, tetraspanins, chaperone proteins, and enzymes not only facilitate exosome binding and internalization by target cells but also actively participate in signal transduction processes (Raposo & Stoorvogel, 2013). In parallel, the lipid composition of exosomal membranes enhances vesicle stability and fusion efficiency, ensuring the effective and targeted delivery of molecular cargo (Trajkovic et al., 2008). Collectively, these features underscore the role of exosomes not only as waste disposal entities but also as valuable biomarkers and versatile therapeutic delivery platforms (Théry et al., 2018).

3.1. Protein Cargo: Tetraspanins, Chaperones, and Enzymes

Although the protein composition of exosomes varies depending on their cellular origin and physiological state, several protein groups are consistently enriched across exosome populations. Members of the

tetraspanin family, including CD9, CD63, and CD81, are widely recognized as canonical exosomal markers and play key roles in vesicle membrane organization and stability (Yáñez-Mó et al., 2015). These proteins facilitate exosome–cell interactions and promote efficient uptake by recipient cells. Chaperone proteins, particularly heat shock proteins such as Hsp70 and Hsp90, are also abundantly present in exosomes and contribute to the stabilization and functional integrity of transported proteins. In addition, exosomal cargo frequently includes metabolic enzymes and signaling proteins capable of activating specific biochemical pathways in target cells. Through the transfer of these proteins, exosomes participate in diverse biological processes, including cellular stress responses, immune regulation, signal transduction, and cell differentiation (Raposo & Stoorvogel, 2013). Importantly, the protein composition of exosomes is highly dynamic and can be modulated by environmental and cellular conditions such as hypoxia, inflammation, and metabolic stress. For example, tumor-derived exosomes have been shown to carry distinct integrin profiles and oncoproteins that contribute to metastatic dissemination and remodeling of the tumor microenvironment (Hoshino et al., 2015). Furthermore, post-translational modifications of exosomal proteins such as phosphorylation and ubiquitination can enhance cargo functionality and increase target cell specificity. Consequently, exosomes serve as sophisticated communication vehicles that play pivotal roles in both physiological homeostasis and disease progression (Kalluri & LeBleu, 2020).

3.2. Nucleic Acids: miRNA, lncRNA, and mRNA

One of the defining features of exosomes is their capacity to transport functional nucleic acids, a concept first demonstrated by Valadi et al. (2007). Exosomes are known to carry mRNA and miRNA, which can actively participate in translational and post-transcriptional regulation within recipient cells. Through exosome-mediated transfer, miRNAs can suppress target gene expression, thereby modulating cellular behavior and signaling responses. In contrast, lncRNAs are involved in chromatin remodeling, transcriptional regulation, and the modulation of diverse signaling pathways. Notably, the ability of exosomal mRNAs to be translated into functional proteins in recipient cells underscores the role of exosomes in horizontal genetic information transfer between cells (El Andaloussi et al., 2013). Beyond passive transport, exosomes exhibit selective nucleic acid packaging mechanisms that are tightly regulated by cellular and environmental conditions. Factors such as cellular stress, hypoxia, and inflammatory stimuli can significantly alter exosomal miRNA and lncRNA profiles, leading to context-dependent responses in target cells (Mateescu et al., 2017; Villarroya-Beltri et al., 2013). Moreover, exosomal nucleic acids display disease-specific expression patterns, making them attractive molecular

biomarkers in pathological conditions including cancer, neurodegenerative disorders, and cardiovascular diseases. Collectively, these properties position exosomes as critical mediators of intercellular communication and as promising tools for clinical diagnostics and therapeutic applications (Kalluri & LeBleu, 2020).

3.3. Lipid Composition and Signal Transduction

The lipid composition of exosomes is distinct from that of the parent cell membrane and plays a fundamental role in determining their functional properties. Exosomal membranes are enriched in lipids such as cholesterol, sphingomyelin, and ceramide, which enhance membrane rigidity, structural stability, and resistance to enzymatic degradation (Trajkovic et al., 2008). These lipids also participate directly in signal transduction by interacting with lipid microdomains in the membranes of recipient cells, thereby facilitating the activation of intracellular signaling cascades. In addition, lipid composition is a key determinant of exosome uptake mechanisms, intracellular trafficking, and biodistribution. Variations in lipid content can influence whether exosomes are internalized via endocytosis, membrane fusion, or receptor-mediated pathways. Consequently, exosomes regulate biochemical communication not only through their protein and nucleic acid cargo but also through their lipid architecture. The coordinated interplay between proteins, nucleic acids, and lipids ultimately defines the molecular identity of exosomes and enables them to elicit specific functional responses in target cells. Through these integrated mechanisms, exosomes act as highly effective regulators of intercellular biochemical communication with significant implications for physiology and disease (Théry et al., 2018).

4. Cargo Selectivity and Cellular Regulation

Cargo selectivity and cellular regulation are fundamental concepts in exosome biology. The molecular contents loaded into exosomes are not randomly incorporated; rather, they are determined through highly selective processes governed by the physiological state of the donor cell, environmental cues, and cell type–specific regulatory mechanisms. This selective packaging not only defines exosomal cargo composition but also enables exosomes to induce precise and functionally relevant responses in recipient cells (Théry, Zitvogel, & Amigorena, 2018). At the molecular level, proteins, lipids, and various classes of RNA including mRNA, miRNA, and lncRNA are actively sorted into intraluminal vesicles through specific signaling motifs and adaptor proteins. ESCRT complexes preferentially recognize ubiquitinated proteins and facilitate their incorporation into exosomes, whereas tetraspanins contribute to cargo diversity by supporting ESCRT-independent sorting pathways (Möller &

Lobb, 2020). In parallel, lipid-enriched membrane microdomains promote ESCRT-independent mechanisms by assembling specific protein–lipid complexes that influence cargo selection (Eitan et al., 2016). Cargo selectivity is further regulated at the cellular level and can dynamically change in response to metabolic alterations or stress conditions. For instance, cellular stress has been shown to reshape exosomal cargo profiles, thereby eliciting distinct biological responses in recipient cells. Consequently, exosome loading represents not only a targeted biochemical packaging mechanism but also an extension of intracellular regulatory signaling networks. Understanding these selective processes is critical for elucidating exosome function and for advancing their diagnostic and therapeutic applications (Sun et al., 2022).

4.1. RNA-Binding Proteins and Selective Packaging

The incorporation of RNA into exosomes is predominantly mediated by RNA-binding proteins (RBPs), which play a central role in selective RNA sorting. Several RBPs, including heterogeneous nuclear ribonucleoproteins (hnRNPs), Argonaute 2 (AGO2), and Y-box binding protein 1 (YBX1), have been shown to direct miRNAs and mRNAs into exosomes by recognizing specific RNA sequence motifs (Villarroya-Beltri et al., 2013). These proteins stabilize RNA molecules within cytoplasmic ribonucleoprotein complexes, guide them toward endosomal compartments, and facilitate their selective incorporation into exosomes. Through this tightly regulated process, donor cells can precisely control the genetic information transferred to recipient cells, underscoring the role of exosomes in intercellular gene expression regulation (Mateescu et al., 2017). In addition to sequence recognition, post-transcriptional RNA modifications also influence exosomal RNA loading. For example, nucleotide additions or methylation events at the 3′ ends of RNA molecules can alter RBP affinity, thereby promoting the selective enrichment of specific RNA species within exosomes (Shurtleff et al., 2016). Cellular stress conditions can further modulate these sorting mechanisms, leading to dynamic changes in exosomal RNA profiles that enable cells to convey context-dependent signals to their environment. Such highly specific RNA transport via exosomes is particularly relevant in pathological settings, including cancer, neurodegenerative disorders, and immune-related diseases. Consequently, these mechanisms significantly enhance the potential of exosomes as disease biomarkers and as vehicles for therapeutic RNA delivery (Kowal et al., 2014).

4.2. The Effect of Cellular Stress and Metabolic Status

Cellular stress conditions induce both quantitative and qualitative alterations in exosomal cargo composition. Stressors such as hypoxia, oxidative stress, and inflammation have been shown to increase exosome secretion while simultaneously reshaping the molecular profile of their cargo (King et al., 2012). For instance, exosomes released under hypoxic conditions are enriched in microRNAs that promote angiogenesis, metabolic adaptation, and cell survival. In parallel, cellular metabolic status serves as a key determinant of cargo selectivity. The increased presence of enzymes involved in energy metabolism, along with metabolism-associated RNAs within exosomes, reflects adaptive cellular responses to environmental and energetic demands. These observations indicate that exosomes function not only as mediators of intercellular communication but also as integral components of cellular homeostasis and stress adaptation mechanisms (Kalluri & LeBleu, 2020).

4.3. Cell Type–Specific Biochemical Signatures

The molecular composition of exosomes exhibits distinct biochemical signatures that are specific to their cell of origin. Exosomes derived from immune cells are typically enriched in immunoregulatory proteins, cytokines, and antigen-presenting molecules, whereas neuronal cell–derived exosomes contain synaptic proteins and neuron-specific microRNAs (Yáñez-Mó et al., 2015). This cell type–specific cargo facilitates selective recognition by recipient cells and enables the generation of precise biological responses. Importantly, these biochemical signatures underpin the diagnostic utility of exosomes as biomarkers. The detection and analysis of exosomal cargo in biological fluids such as blood, urine, and cerebrospinal fluid provide valuable insights into cellular origin, tissue status, and disease progression. Owing to these characteristics, exosomes have emerged as promising tools in personalized medicine, disease monitoring, and non-invasive diagnostic strategies (El Andaloussi et al., 2013).

5. Interaction of Exosomes with Target Cells

The biological effects of exosomes extend beyond the molecular cargo they transport and are critically dependent on the mechanisms through which they interact with recipient cells. These interactions involve a series of tightly regulated biochemical events, including receptor–ligand recognition, cellular uptake, and activation of intracellular signaling pathways. Exosome target cell interactions therefore represent a key determinant of the specificity and efficiency of intercellular communication (Mulcahy et al., 2014). Upon reaching target cells, exosomes can deliver their contents through multiple

pathways, including direct membrane fusion, endocytic uptake, or phagocytosis. The selection of these pathways is governed by interactions between exosomal surface proteins and specific receptors on the plasma membrane of recipient cells, thereby ensuring cell type specific delivery. Beyond cargo transfer, exosomes actively modulate intracellular signaling networks, influencing gene expression, cellular proliferation, differentiation, and immune responses within recipient cells. Through these multifaceted mechanisms, exosomes function as critical mediators of intercellular communication in both physiological and pathological contexts, including cancer progression, immune regulation, and tissue remodeling (Costa-Silva et al., 2015).

5.1. Receptor–Ligand Interactions

Surface proteins expressed on the exosomal membrane play a pivotal role in the recognition and targeting of recipient cells. Molecules such as tetraspanins, integrins, and members of the immunoglobulin superfamily mediate exosome binding by interacting with specific receptors on the surface of target cells (Yáñez-Mó et al., 2015). These receptor–ligand interactions confer target cell specificity and initiate downstream biological responses. In particular, integrin expression profiles have been identified as key determinants of exosomal tissue tropism. Distinct integrin patterns on tumor-derived exosomes have been shown to direct vesicles toward specific organs, thereby contributing to the formation and modulation of organ-specific microenvironments (Hoshino et al., 2015). Beyond initial binding, exosomal surface proteins also influence the internalization of vesicles and the subsequent delivery of their molecular cargo. Receptor–ligand interactions can dictate the preferred uptake pathway, including clathrin-mediated endocytosis, macropinocytosis, or direct membrane fusion. Through these mechanisms, exosomes deliver cell type–specific molecular signals, regulate gene expression, and induce context-dependent cellular responses. Moreover, in pathological conditions such as cancer and chronic inflammation, alterations in exosomal surface molecule composition may enhance target cell selectivity, thereby promoting disease progression. Collectively, these properties underscore the significance of receptor–ligand interactions in exosome biology and highlight their potential utility in therapeutic targeting and biomarker development (Costa-Silva et al., 2015; Mulcahy, Pink, & Carter, 2014).

5.2. Endocytosis and Membrane Fusion

Following binding to recipient cells, exosomes are internalized through multiple cellular uptake mechanisms. These include clathrin-mediated endocytosis, caveolin-dependent endocytosis, macropinocytosis, and

phagocytosis, with the specific pathway employed depending on both the molecular composition of the exosome and the phenotype of the target cell (Mulcahy et al., 2014). In certain contexts, exosomes can also deliver their cargo directly into the cytoplasm through membrane fusion with the plasma membrane. This fusion process is strongly influenced by lipid membrane fluidity and composition, allowing for rapid and efficient cytosolic delivery of exosomal contents (Théry et al., 2018). Exosomes internalized via endocytic pathways are typically trafficked to early endosomes, where their cargo may be partially released to exert functional effects within the recipient cell. Alternatively, some internalized vesicles are directed toward lysosomal compartments for degradation. The balance between cargo release and degradation critically determines the intracellular fate of exosomes and the biological efficacy of the signals they convey (Hessvik & Llorente, 2018). Through these tightly regulated uptake and processing mechanisms, exosomes exert precise control over intercellular communication in both physiological and pathological settings.

5.3. Activation of Intracellular Signaling Pathways

Following their delivery to recipient cells, exosomes exert their biological effects through the activation of intracellular signaling pathways mediated by their protein, nucleic acid, and lipid cargo. Exosomal proteins have been shown to modulate key signaling cascades, including the MAPK/ERK, PI3K/Akt, and NF-κB pathways, thereby influencing fundamental cellular processes such as proliferation, survival, differentiation, and inflammatory responses (Raposo & Stoorvogel, 2013). In addition to direct intracellular effects, receptors and ligands presented on the exosomal surface can interact with corresponding molecules on target cell membranes, initiating signal transduction events at the plasma membrane and amplifying downstream signaling cascades. Exosome-mediated transfer of miRNAs contributes to long-term and sustained biological effects by post-transcriptionally suppressing or fine-tuning gene expression in recipient cells. These miRNAs frequently target genes involved in cell cycle regulation, apoptotic signaling, immune modulation, and metabolic control, ultimately leading to the reprogramming of cellular behavior. Furthermore, exosomal lipid components have been shown to influence intracellular signaling by altering membrane architecture, lipid raft organization, and the activity of membrane-associated signaling proteins. Collectively, these multifaceted signaling mechanisms demonstrate that exosomes function not merely as transient messengers but as potent regulators capable of reshaping the functional properties and phenotypic states of target cells. Through their ability to integrate protein, RNA, and lipid-mediated signaling, exosomes play critical roles in diverse physiological processes, including tissue homeostasis and immune regulation, as well as in pathological conditions

such as cancer progression and chronic inflammation (Kalluri & LeBleu, 2020).

6. Biochemical Basis of Exosomes as Biomarkers

Biomarkers play a critical role in disease diagnosis, prognosis, and the monitoring of therapeutic responses. In recent years, exosomes have emerged as promising non-invasive biomarker candidates owing to their rich and disease-relevant molecular cargo. Their remarkable stability in diverse biological fluids including blood, urine, saliva, and cerebrospinal fluid renders exosomes particularly suitable for biochemical and molecular analyses (Théry et al., 2018). Exosomes transport a wide spectrum of biomolecules, such as proteins, lipids, DNA, and nucleic acids including miRNAs and mRNAs, which collectively reflect the physiological state of their cells of origin. For instance, tumor-derived exosomes are enriched in oncogenic miRNAs and cancer-specific proteomic signatures, whereas neuron-derived exosomes isolated from patients with neurodegenerative disorders contain proteins and RNAs characteristic of neural cells (Kalluri & LeBleu, 2020). These disease-associated molecular profiles enhance the capacity of exosomes to mirror pathological status, supporting their use in early diagnosis, disease progression monitoring, and evaluation of therapeutic efficacy. An additional advantage of exosome-based biomarkers lies in the protection of their cargo from enzymatic degradation and other destabilizing factors, conferred by the lipid bilayer membrane. This intrinsic stability enables reproducible and reliable biomarker measurements. Moreover, exosomal membrane architecture, surface proteins, and lipid composition contribute to selective isolation and precise molecular characterization, thereby improving biomarker specificity. A variety of analytical techniques including nanoparticle tracking analysis, nanoproteomics, RNA sequencing, and ELISA-based assays are currently employed to assess the biomarker potential of exosomes. Collectively, these approaches underscore the growing clinical relevance of exosomes in cancer, cardiovascular disorders, neurodegenerative diseases, and immune-related conditions (Zhang et al., 2019).

6.1. Protein and miRNA-Based Biomarkers

The protein cargo of exosomes contains distinctive molecular signatures that reflect the biochemical and functional status of their cells of origin. In addition to commonly used exosomal markers such as the tetraspanins CD9, CD63, and CD81, exosomes may carry disease-specific oncoproteins, immunoregulatory molecules, and metabolic enzymes that are highly informative for diagnostic purposes (Yáñez-Mó et al., 2015). Elevated levels

of particular proteins in cancer cell–derived exosomes, for example, support their application in early disease detection and longitudinal monitoring.

MicroRNAs represent another major class of exosome-associated biomarkers. Compared with free-circulating RNAs, exosomal miRNAs exhibit enhanced stability due to encapsulation within the lipid bilayer membrane. The identification of disease-specific miRNA expression profiles in exosomes has therefore attracted considerable interest, as these molecules offer high sensitivity and specificity for biomarker development (Valadi et al., 2007; El Andaloussi et al., 2013).

6.2. Disease-Specific Molecular Profiles

The biomarker potential of exosomes is primarily determined by their disease-specific molecular profiles. Numerous studies have demonstrated that exosomal cargo undergoes substantial alterations in a wide range of pathological conditions, including cancer, neurodegenerative disorders, cardiovascular diseases, and inflammatory conditions. For instance, tumor-derived exosomes are enriched in proteins and microRNAs that are closely associated with tumor progression, metastatic dissemination, and modulation of the tumor microenvironment (Kalluri & LeBleu, 2020). Similarly, in neurodegenerative diseases, neuron-derived exosomes have been shown to carry disease-relevant proteins and RNA species, with molecular profiles that correlate with disease stage and severity. These findings indicate that exosomes can serve not only as diagnostic biomarkers but also as indicators of disease progression and therapeutic response. Collectively, the disease-specific protein and miRNA signatures contained within exosomes provide valuable biochemical information that enhances diagnostic accuracy. Owing to these characteristics, exosomes have become integral components of liquid biopsy approaches and are increasingly recognized as key tools in translational medicine and precision diagnostics (Raposo & Stoorvogel, 2013).

7. Biochemical Approaches in Therapeutic Applications

Exosomes have emerged as highly promising platforms for therapeutic applications due to their inherent biocompatibility and their ability to interact efficiently with target cells. Their cellular origin, low immunogenicity, and capacity to traverse biological barriers confer distinct advantages over synthetic delivery systems. Consequently, strategies aimed at loading exosomes with therapeutic agents while preserving their biochemical stability have become a central focus of translational research (El Andaloussi et al., 2013). Exosomes can be engineered to carry a broad range of therapeutic cargos, including small interfering RNAs (siRNAs), microRNAs,

proteins, and small-molecule drugs. Such approaches have demonstrated enhanced therapeutic efficacy, particularly in preclinical models of cancer and autoimmune diseases (Zhang et al., 2021). Key properties of exosomes such as prolonged circulation stability, high biocompatibility, and minimal immune activation further support their application as drug delivery vehicles (El Andaloussi, Mäger, Breakefield, & Wood, 2013; Sun, Grange, & Ali, 2021). Therapeutic loading strategies generally fall into two categories: post-isolation loading of purified exosomes and endogenous loading during exosome biogenesis within donor cells. Both approaches have shown success in delivering diverse therapeutic cargos, with method selection largely dependent on the physicochemical properties of the cargo and the characteristics of the target tissue (Sun et al., 2021). For example, electroporation-based loading of miRNAs into exosomes has demonstrated effective gene silencing in cancer models (Kusuma et al., 2022). Moreover, the targeting efficiency of exosomes can be further enhanced through surface engineering and biochemical modification. The functionalization of exosomal membranes with targeting ligands, integrins, or tetraspanins enables selective delivery to specific cell types and tissues (Sun et al., 2025). In addition, localized delivery strategies, such as microneedle-based systems, have been explored to improve exosome stability and therapeutic efficacy in clinical applications (Tian et al., 2024).

7.1. Loaded Exosomes and Molecular Stability

Exosomes employed in therapeutic applications are engineered to carry drug molecules, proteins, enzymes, or nucleic acids and to deliver these cargos selectively to target cells. The lipid bilayer membrane of exosomes provides effective protection against enzymatic degradation, thereby preserving cargo stability during circulation. This property is particularly advantageous for the therapeutic delivery of labile molecules such as miRNAs and siRNAs (Kalluri & LeBleu, 2020). Molecular stability is closely linked to the biochemical architecture of exosomes, as membranes enriched in cholesterol and sphingolipids enhance vesicle integrity while enabling controlled and sustained cargo release. As a result, loaded exosomes are capable of inducing prolonged biological effects within target tissues (Théry et al., 2018). The maintenance of molecular stability depends not only on membrane composition but also on loading strategies and storage conditions. Techniques such as electroporation, passive incubation, and sonication can differentially affect membrane integrity, potentially leading to adverse outcomes such as cargo aggregation or vesicle destabilization (El Andaloussi et al., 2013). Therefore, achieving an optimal balance between loading efficiency and structural stability represents a critical parameter in the clinical development of exosome-based therapeutics. In addition, exosomal surface proteins and glycosylation patterns play decisive roles in determining

circulation time and interactions with recipient cells. Tetraspanins, including CD9, CD63, and CD81, contribute to enhanced biological stability and facilitate cellular uptake mechanisms (Théry et al., 2018). Moreover, circulation persistence can be further modulated through surface engineering and biochemical modifications designed to reduce rapid clearance by the immune system (Wiklander et al., 2015). Storage and transportation conditions also exert a significant influence on the stability of loaded exosomes. Repeated freeze thaw cycles have been shown to compromise membrane integrity and result in cargo loss. Consequently, biochemical strategies such as the use of cryoprotectants, lyophilization, and optimized buffer systems are recommended to preserve the long-term stability of therapeutic exosomes (Lener et al., 2015).

7.2. Enzymatic and Genetic Loading Strategies

Therapeutic loading of exosomes is primarily achieved through enzymatic (physical) and genetic approaches. Enzymatic loading methods are typically applied after exosome isolation and include techniques such as electroporation, sonication, and chemical permeabilization, which transiently increase membrane permeability to allow the incorporation of therapeutic cargos. While these methods offer practical and rapid loading, optimization is required to maximize loading efficiency while preserving membrane integrity (Luan et al., 2017). Genetic loading strategies, by contrast, involve the genetic modification of donor cells to promote endogenous packaging of therapeutic molecules into exosomes during biogenesis. This approach provides a more physiological and tightly regulated loading process, particularly suitable for therapeutic proteins and RNA molecules. Genetically engineered exosomes can exert targeted therapeutic effects by modulating specific intracellular signaling pathways in recipient cells (El Andaloussi et al., 2013). However, both loading strategies present inherent advantages and limitations. Physical and enzymatic methods may induce structural damage to exosomal membranes and lead to heterogeneous cargo distribution, with electroporation in particular associated with nucleic acid aggregation and alterations in vesicle size distribution (Lamichhane et al., 2015). In contrast, genetic loading approaches offer improved homogeneity and cargo packaging but require careful evaluation of the effects of genetic manipulation on donor cell physiology (Yim et al., 2016). In recent years, hybrid strategies that combine enzymatic and genetic approaches have gained increasing attention. For example, exosomes derived from genetically modified donor cells can be further loaded with additional therapeutic agents post-isolation, thereby enhancing cargo capacity and therapeutic efficacy (Luan et al., 2017). Furthermore, bioengineering strategies such as the incorporation of targeting peptides or antibody fragments onto the exosomal surface have been shown to significantly improve tissue specificity and

cellular targeting efficiency, reinforcing the potential of exosomes as precision therapeutic delivery platforms (Kooijmans et al., 2016).

8. Conclusion and Future Perspectives

Exosomes have emerged as biologically significant entities that have garnered substantial attention in recent years for their roles in mediating intercellular biochemical communication. Accumulating evidence from the literature demonstrates that exosomes are not merely cellular waste products, but rather functional vesicles that actively regulate both physiological and pathological processes through their selectively enriched molecular cargo composed of proteins, nucleic acids, and lipids (Raposo & Stoorvogel, 2013; Théry et al., 2018). The studies reviewed in this chapter highlight that exosome biogenesis is tightly regulated by coordinated mechanisms involving endosomal membrane dynamics, ESCRT machinery, and lipid-driven pathways. These interconnected biochemical processes enable exosomal cargo composition to be dynamically shaped by cellular state and environmental cues (Hurley, 2015; Yáñez-Mó et al., 2015). The functional delivery of proteins, microRNAs, and other nucleic acids via exosomes can elicit sustained biological effects in recipient cells by modulating gene expression and intracellular signaling networks (Valadi et al., 2007). The biomarker potential of exosomes represents one of the most rapidly expanding areas of exosome research. Their remarkable stability in biological fluids and their cell type–specific molecular signatures render exosomes highly attractive tools for non-invasive diagnostics and disease monitoring. In particular, protein- and miRNA-based exosomal profiles have been shown to exhibit disease-specific patterns in cancer, neurodegenerative disorders, and inflammatory conditions (Kalluri & LeBleu, 2020; El Andaloussi et al., 2013). Nevertheless, variability in isolation protocols and analytical methodologies remains a major challenge, limiting the reproducibility and cross-study comparability of findings. From a therapeutic perspective, exosomes offer considerable promise as natural delivery vehicles for targeted drug and gene therapies. Their intrinsic biocompatibility and molecular stability facilitate the efficient transport of therapeutic agents to specific tissues (Luan et al., 2017). However, successful clinical translation will require further optimization of dosing strategies, targeting specificity, large-scale production, and quality control. Looking ahead, the integration of advanced omics technologies, single-cell analyses, and bioinformatic approaches is expected to provide deeper insights into the biochemical heterogeneity and functional diversity of exosomes. Moreover, broader implementation of international guidelines such as the Minimal Information for Studies of Extracellular Vesicles (MISEV) will promote methodological standardization and enhance the

reliability of exosome-based diagnostic and therapeutic applications in clinical practice (Théry et al., 2018).

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The Impact of Health Information Systems (HIS) and Artificial Intelligence (AI) on the Professional Roles and Competencies of Medical Secretaries

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ABSTRACT

Healthcare systems have gone digital exceptionally and are almost halfway through the journey to digitization with the innovations of Health Information Systems (HIS) and Artificial Intelligence (AI) quickly becoming the most important/central activities. This chapter introduces the key innovations such as these which are influencing the configuration of the infrastructure, the workflow, and even the duties of the medical secretaries which are usually -the- ones not being in focus in digital health issues. Formerly, medical secretaries who were mainly assigned with daily administrative tasks have now discovered a totally different side where they even work with data ecosystems, keeping health records, and finding out ways means of information flow between both clinical and administrative units.

With the growing presence of AI—ranging from automated documentation to intelligent scheduling—repetitive tasks are being gradually replaced by machines, thus pushing secretaries into positions with more analytical and supervisory responsibilities. Naturally, such progress raises a variety of questions regarding ethical issues, such as data protection, and accountability, as well as the vagueness of the borders between mankind and machinery work.

The chapter besides this also raises the concern over the possibility of the existing training programs providing the necessary support for this transformation. In Türkiye, to quote, despite the adoption of digital infrastructure, the development of human capital has sometimes not been satisfactory. Citing examples from both domestic and foreign articles, the present conversation proposes that the instrument is not the thief of the office, but the shaper. Digital transformation is considered a universal theme as such a book provides an indirect suggestion for other sectors and organizations to reflect on their own paths. This shift calls on the attention of teachers, decision-makers as well as healthcare organizations). They should ensure that the professional roles, training structures, and organizational systems grow hand in hand with the technologies that they now utilize.

Keywords – Health Information Systems; Artificial Intelligence; Medical Secretaries; Professional Roles and Competencies; Digital Health Transformation

INTRODUCTION

Throughout the past twenty years, the digital shift in the healthcare sector has been profound and is now complete almost in all corners of the world. This change is primarily caused by the remarkable development of information and communication technologies that have changed the delivery, documentation, and management modes of health services. A process that initially appeared to be just a technical upgrade—like changing from paper files to digital ones—has changed into a much broader end of the spectrum: a paradigm shift that is now affecting everything such as decision-making processes and sectors' professional roles (Bertelsen & Nøhr, 2005; Knudsen & Bertelsen, 2021).

The most notable of these is prevalent use of Health Information Systems (HIS), especially in recent years, the integration of Artificial Intelligence (AI) tools have been crucial. The employment of these technologies leads to improvements in efficiency, service quality, and safety across the sector (Santavirta et al., 2021; Stanfill & Marc, 2019). Their bearing is not only on systems nor infrastructure-they also alter the manner of people's work in those structures. Amid the numerous medical professions that have been impacted, one that usually tends to be overlooked is medical secretaries.

Standardly, medical secretaries were regarded as simply the back-office support responsible for filing, correspondences, booking the appointments. This perspective is too narrow for the current context. With the use of digital tools constantly coming into their everyday life at the workplace, secretaries have shifted their official roles to a much more holistic and collaborative position that is in direct partnership with clinical practice and administrative coordination (Bossen et al., 2019; Knudsen & Bertelsen, 2023). Their duties now involve activities such as patient documentation through the electronic health records (EHR), alignment of reporting systems, and communication aid between departments (Santavirta et al., 2021).

The change comes along with higher expectations of abilities. Just being organized, or mastering office software programs is not enough anymore. In this digital age, medical secretaries not only are required to have digital literacy, but also need to be knowledgeable about data privacy laws and learn to operate through multifarious information systems (Nazeha et al., 2020). They are supposed to be mannerly and meticulous, as well as to comprehend the flow of the system.

In the meantime, AI tools' incorporation such as voice-to-text transcription, automated scheduling, and predictive analytics has added a further transformative level. The tools and software that can transcribe and automate scheduling also result in changes to the system which make the task of the administrator easier; however, they equally come with the

introduction of fresh responsibilities. Secretaries begin to take on the role of overseeing the automated processes; they need to confirm the outputs and foster synergistic relations with the systems, which are not just mere instruments anymore, but semi-autonomous agents (Stanfill & Marc, 2019; Yahya et al., 2024). Of course, such things philosophically and legally arise, too, particularly with regard to matters of data accuracy, accountability, and the division of human and machine work (Gazquez-Garcia et al., 2024; Makrash et al., 2024).

In Türkiye, things have a tendency to be complicated. There is a positive facet though, due to the country's big infrastructures along with digital and HIS integration in place. However, less focus has been given to the aspect of human resources in this transformation (Işık & Akbolat, 2010; Engin & Gürses, 2018). The pace at which the tech sector is progressing is visible, yet the incapacity of the people to inarguably work in them becomes highly apparent. The medical secretary position is impacted by this dynamic to the point of having inadequate access to training and professional development facilities also needed to adapt to such swift changes (Düzcü et al., 2024).

This chapter is meant to investigate that very chasm. The qualification of medical secretaries as a specific professional group will be the focal point of the research that aims to explore the reshaping of their duties, such as the introduction of novel skills as well as possible education and policy adaptations. The approach is to regard the medical secretary from an alternative perspective of a passive prescriptive role, but of a proactive and a key agent in the digital transformation of healthcare, at least in some respects, the first beneficiary.

THE IMPACT OF HEALTH INFORMATION SYSTEMS ON THE PROFESSONAL ROLES AND COMPETENCIES OF MEDICAL SECRETARIES

Health Information Systems (HIS) constitute the core digital infrastructures designed to integrate and manage clinical and administrative processes within healthcare organizations (Bertelsen & Nøhr, 2005; Santavirta et al., 2021). These systems interface with a wide range of components, including hospital information management systems, electronic health records (EHR), patient registration and appointment modules, billing applications, and reporting tools, all of which share the common goal of accelerating healthcare delivery while enhancing accuracy and traceability. The discourse surrounding health information systems should therefore frame HIS not merely as technological advancements, but also as mechanisms for the reconfiguration of work within healthcare organizations (Knudsen & Bertelsen, 2021).

Core Characteristics and Functions of Health Information Systems

The core tasks performed by HIS electronic documentation of patient data, real-time sharing of clinical and administrative information, workflow standardization, and internal communication facilitation (Bertelsen & Nøhr, 2005). The systems convert critical processes-from admission and referrals to diagnosis, treatment, and discharge-into electronic versions, therefore, removing the need for paper-based documentation and allowing for the monitoring of healthcare delivery on a unified platform (Santavirta et al., 2021).

Paperless operations have become the central nerve center for hospitals. Accurate, complete, and timely data have, therefore, become direct contributors to positive clinical and also the administrative efficiency and organizational performance (Knudsen & Bertelsen, 2023). As the data-driven setup, medical secretaries have emerged as a necessary component in healthcare.

Electronic Record Systems and Data Management

HIS, one of the electronic health records that introduced is the first and the most famous electronic health record. With the electronic health records, we get to store and access a combination of patient demographics, clinical notes, diagnostic tests, medications, and health care reports (Santavirta et al., 2021). The medical secretaries participate considerably in the development, storage, and structuring of the information (Bertelsen & Nøhr, 2005).

They are now given a lot more responsibilities than before, which have significantly changed: they do not only enter data now; they also make sure that the health information is continued, accurate, and traceable (Knudsen & Bertelsen, 2021). Data entries that are not accurate or complete may lead to grave consequences in clinical decision-making, administrative reporting, and patient safety. Thus, the medical secretary profession being data stewardship has become a core competency area (Bossen et al., 2019; Nazeha et al., 2020).

Expanded Responsibilities and Professional Visibility

The arrival of HIS has broadened the horizon of medical secretaries incredibly (Santavirta et al., 2021). While a few years back, they were just confined to them, filing, scheduling, and correspondence, now today's medical secretaries should work with digital platforms, safeguard data integrity, and create flows of information among the departments (Knudsen & Bertelsen, 2023).

The research of Bossen et al. (2019) presents the medical secretaries are indeed the most important digital link between the clinical staff and the

administrative department. Their role is key to ensuring information that is generated during clinical meetings is entered into the system accurately, the administrative reports are prepared reliably, and the data that is essential for managerial decision-making is readily available (Bertelsen & Nøhr, 2005). This intermediary function, aside from increasing the professional visibility of the medical secretaries, also puts them under a greater degree of scrutiny, as their actions affect institutional accuracy and efficiency directly. As a result, the chances for mistakes in this process have lessened, and the extent of professional liability has dramatically gone up (Knudsen & Bertelsen, 2021).

New Competency Areas: Digital Literacy, Data Privacy, and System Coordination

The implementation of digital technology in medical offices has led to the emergence of new competence areas for medical secretaries, the most fundamental one being digital literacy (Nazeha et al., 2020). One of the critical factors that the health care secretaries must be proficient in are software operation, module switch, and basic issue troubleshooting in health information systems (Santavirta et al., 2021).

Furthermore, besides data privacy, the medical secretary has also achieved the status of information security within her or his professional practice (Stanfill & Marc, 2019). Electronic health data is very personal, and the legal and ethical issues that arise when this information is safeguarded are more pronounced (Makrash et al., 2024). A medical secretary should know the rules and be able to handle computerization in their own way; this is the direct and substantial effect on the abiding of the healthcare institution (Gazquez-Garcia et al., 2024).

Although traditionally taught as technology-related obligations, system management is gradually evolving into a practical area provisioned for medical-secretaries-by (Knudsen & Bertelsen, 2023). Now, tasks like diagnosing user-side errors, explaining technical issues to the right departments, and securing uninterrupted daily workflows are very much dependent on how well medical secretaries' interface with and navigate the health information systems (Santavirta et al., 2021).

Benefits to Workflow Efficiency and Interdepartmental Communication

One of the major effects of the benefit of HIS is their tendency to cut workload and thus improve the general operational efficiency (Bertelsen & Nøhr, 2005). HIS prospect more agile and responsive healthcare settings by enabling quicker access to digital records, shrinking the repetitive manual tasks, and nurturing a more structured internal communication (Santavirta et al., 2021). According to medical secretaries, these upgrades result in time management improvements, a more even workload distribution, and better task coordination (Knudsen & Bertelsen, 2021).

Furthermore, HIS promote clearer and more uniform communication between clinical and administrative units, which helps to prevent the loss of information or misinterpretation (Bossen et al., 2019). Medical secretaries are right in the middle of this digital communication center and they are the ones who are mainly responsible for the transparency and for the healthcare and patients to have the more organized and reliable information flows (Knudsen & Bertelsen, 2023).

Challenges Encountered: System Interfaces, Training Deficiencies, and Adaptation Issues

Though HIS boast considerable advantages, their adoption is also fraught with obstacles. The most prevalent difficulty concerns the layout of the system interfaces. If the interfaces are not friendly or intuitive for the users, they will unintentionally make medical secretaries work harder and thus lead to higher possibilities of errors in data entry (Lærum et al., 2004; Santavirta et al., 2021). These types of usability problems can make workflows slower and, consequently, nullify the very efficiency that these systems are supposed to bring.

The other significant barrier is related to the training which is not enough during the adoption process of new systems. In many situations, medical secretaries lack the proper kind of training or sufficient hands-on practice to navigate through new digital platforms effectively (Makrash et al., 2024). The lack of programmed onboarding tier complicates the changeover and may reduce inter-team system adoption in healthcare.

For medical secretaries specifically, who often have a background from the traditional administrative field, no continuous targeted training in digital skills is a big challenge (Işık & Akbolat, 2010). Without consistent in-service training which is designed based on HIS dynamic technical requirements, it becomes hard for them to completely interact with these systems. This knowledge shortfall not only diminishes the potential utilization of digital resources but might also be an additional factor of stress, role strain, and dissatisfaction in the job aspects (Davies et al., 2022; Santavirta et al., 2021).

THE IMPACT OF ARTIFICIAL INTELLIGENCE ON THE PROFESSIONAL ROLES AND COMPETENCIES OF MEDICAL SECRETARIES

The Artificial Intelligence (AI) technology is a new revolutionary healthcare force in the world of medicine that has the most articles in both clinical and administrative areas (Stanfill & Marc, 2019). From helping to improve diagnostic performance and treatment planning to the improvement of documentation and operational workflows, the health sector especially

relies on AI technologies such as machine learning, natural language processing, speech recognition, and process automation due to their efficiency in operations and less human error (Gazquez-Garcia et al., 2024). While AI's implications for clinical roles have taken the center stage, its footprint in administrative functions, particularly the medical secretaries, is no less significant (Bossen et al., 2019).

With the further inclusion of AI in the organizations within the healthcare field, a significant discussion has been sparked about the future of medical secretaries. Mainly, the issues have arisen regarding the assurance of whether automation will actually be developed by this group of people which will replace most of the time routine administrative tasks carried out by them automatically (Stanfill & Marc, 2019). However, the available evidences do not point to this situation, but rather indicate a more elaborated path: instead of the complete extinction of medical secretaries, the AI technology will be the main driver of their roles that will be more analytical, supervisory, and integrative within the (Makrash et al., 2024).

General Applications of Artificial Intelligence in Healthcare

Healthcare, being a terrain where AI is configured across different healthcare sectors, the highest level of applications this technology has is in clinical decision support systems, medical imaging analysis, patient risk assessment, and resource optimization (Gazquez-Garcia et al., 2024). For the past few months, it seems that AI has also started its interference in the executive branches of healthcare institutions, namely with tools that perform the task of reducing symptomatic workload and thus improving operational efficiency (Yahya et al., 2024).

Among the several AI tools for administrative processes are those like automated patient appointment programs, intelligent document classification tools, coding assistance applications, and reporting networks (Stanfill & Marc, 2019). The abovementioned different diagnostic and therapeutic strategies are the key elements through which hospitals can speed up their operations considerably and above all that, cut down the time wasted and errors made with manual data processing (Bossen et al., 2019).

The integration of such systems is of particular importance for professionals who perform office medical secretarial duties. The changes brought about by these programs reflect not only the reorganization of tasks required to be done every day but also the essential consideration of professional boundaries and responsibilities. Consequently, the trend of customization of duties in the functional lines of work has pursued administrative medical employees in the direction of such tracking and reporting that the very typology of their function in the framework of automated health care has been remodeled (Makrash et al., 2024).

Automation, Speech Recognition, and Data Analytics Applications

The development of AI-based robotic technologies is moving toward the design of assistive or direct substitute robotic systems of a variety of medical secretaries' duties that were performed traditionally (Stanfill & Marc, 2019). Routine operations that are now under the automation of man include the scheduling of appointments, the arrangement of the patient record, the filling of standardized forms, and the production of routine reports-each of them decreasing manual workload and administrative redundancy (Yahya et al., 2024).

Among them, the speech recognition and speech-to-text channels are not only taking the center stage but also are creating a paradigm shift in the field of clinical documentation (Stanfill & Marc, 2019). These technologies that can automatically convert the doctor's oral notes to written documents, therefore, eliminate the requirement for manual transcription and get the work done faster. On the flip side of this transformation, medical secretaries now face additional responsibilities, such as reviewing, editing, and validating the clinical texts that have been generated automatically in order to ensure their accuracy and contextual coherence (Bossen et al., 2019).

Simultaneously, data-analytic tools penetrate deeper and deeper into administrative routines. The machines gather different issues, make analyses of the whole inventory, and generate summary reports while processing the huge dataset. Still, such results need a human being to interpret and prove their point, whereas the data may become misleading and irrelevant if not correctly contextualized within the specific institution (Gazquez-Garcia et al., 2024).

Transition from Routine Tasks to Analytical Responsibilities

The modification of medical secretaries' workload is mainly due to the inclusion of ai in their daily tasks (Makrash et al., 2024). Cut-and-paste medical secretaries take on analytical tasks that are related to the time-consuming tasks they have been told to cut down on (Stanfill and Marc, 2019). Through this, the job can also be diversified to not only the operational side but also the evaluative side (Bossen et al., 2019).

The transformation from routine to analytical work comes with the active participation of medical secretaries in areas such as data quality, process humidity and system output consistency (Knudsen & Bertelsen, 2023). The shift is indicative of a transcendent transformation rather than a mere quantitative change in the area of professional abilities. The shift is not evaluated in terms of the reduced volume but rather in terms of the increasing complexity and intensity of responsibilities (Makrash et al., 2024).

New Functions Include Process Management, Oversight, and Data Validation

The rise of AI-based systems in the medical field has not only increased the number of new tasks but also introduced the new role of medical secretaries (Stanfill and Marc, 2019). Among various duties, the most popular ones include current process management, monitoring automated processes, and data validation (Bossen et al., 2019). Automation has nothing to do with the assumption of total-reliance on the system's faults; on the contrary, the part where human beings are involved becomes so considerable.

Instead of incorrect data AI systems, medical secretaries see the documents, records, and reports generated by these systems, note down the possible errors, and do the required correction (Yahya et al., 2024). So, there is an addition to the error-prevention, quality-control aspect of this profession (Makrash et al., 2024). Yet, medical secretaries perform the role of a coordinator by making sure that the software activities adapt to how the company operates.

Applications of Ethics: Privacy, Human-Machine Collaboration, and Shared Responsibility

There would always be questions about the ethical use of AI tools in medical related activities (Gazquez-Garcia et al., 2024). Matters like the confidentiality of medical data, the use of automated systems in medical decision-making processes, and liability for errors would all impact the methodologies of medical secretaries (Stanfill and Marc, 2019).

In the context of working with AI systems, the role played by medical secretaries is considered imperative from the perspective of data protection and ethics (Makrash et al., 2024). For a harmonious human-artificial system interface to be achieved, it is imperative for medical secretaries to be knowledgeable about the limitations and risks posed by AI systems (Nazeha et al., 2020). In this respect, being ethically informed should be considered a duty beyond the realm of law (Nazeha et al., 2020).

Professional Empowerment and Transformation with AI-Related Work Processes

Despite the fact that the role of the medical secretary profession is often analyzed concerning the role of AI in the potential automation of the profession and the resultant job displacement, the present state of evidence draws progressively more on the themes of occupational transformation and empowerment rather than job displacement (Bossen et al., 2019; Stanfill and Marc, 2019). Rather than making the services of the medical secretary unnecessary, the use of AI technology changes the nature of the work involved for the profession towards higher levels of analysis, visibility, and responsibility (Makrash et al., 2024).

This paradigm shift also leads to an improvement in the professional recognition of medical secretaries in the healthcare setting. With the integration of AI-based systems in the day-by-day routine of a healthcare organization, medical secretaries become involved in more than task-oriented activities but also in quality management/critical decision-making tasks (Makrash et al., 2024). Nevertheless, in order for this enabling potential to become achieved, medical secretary competency in terms of awareness of information technology, AI, ethics, and critical evaluation of system output needs to be developed (Nazeha et al., 2020; Gazquez-Garcia et al., 2024). At this point, a comparative overview of the impacts of Health Information Systems and Artificial Intelligence on the professional roles and competencies of medical secretaries is presented in Table 1.

Table 1: Comparative Impact of Health Information Systems (HIS) and Artificial Intelligence (AI) on Medical Secretaries' Professional Roles

Dimension	**Health Information Systems (HIS)**	**Artificial Intelligence (AI)**
Primary function	Digitalization and integration of administrative and clinical records	Automation, analytics, and decision-support
Impact on work processes	Standardization and acceleration of workflows	Reduction of routine tasks and process optimization
Role transformation	From clerical support to data and system coordinator	From system user to supervisor and process manager
Key responsibilities	Data entry accuracy, record maintenance, system coordination	Output validation, monitoring automated processes, quality assurance
Required competencies	Digital literacy, data management, system navigation	AI awareness, analytical skills, ethical judgment
Potential challenges	Interface complexity, training deficiencies	Ethical concerns, accountability, increased cognitive workload

In addition, there is a significant connection between the empowerment of professionals working with AI-enabled tasks and organizational as well as learning-based support systems. In the absence of proper training and learning outcomes or proper definition of roles, and

within a context that views shift in task responsibilities as part of one's duties, the added complexity of working with AI may cause not only role strain but also work-related stress (Santavirta et al., 2021). In fact, empowerment here would be a conditioned state of affairs produced as a consequence of technological advancement accompanied by skill development (Stanfill and Marc, 2019).

Accordingly, work processes in AI technology not only aim to automate the already-existing administration work of medical secretaries. Instead, the professional identity of medical secretaries is about to undergo a radical shift. If the required education and governance structure is in place, the new role of medical secretaries will enhance their professional status and make them essential players in the sustainability of digital health services (Bossen et al., 2019; Makrash et al., 2024).

EDUCATION AND PROFESSIONAL DEVELOPMENT REQUIREMENTS

With the advent of HIS and AI technology, the changing roles and responsibilities of medical secretaries are so demanding that they need an entire revamp of the recruiting and training systems (Davies et al, 2022). The online healthcare setting functionality is now no longer based on the term of technology; instead, it calls for a more sophisticated combination of skills that includes reading, writing, decision-making, and learning (Nazeha et al., 2020). Therefore, it is necessary for the medical secretary education program to adapt and embrace the dynamic changes in technology that are taking place in healthcare organizations.

The speed at which digital health technology advances means that skills needed to work with the technology have to be part of the training that goes beyond the vocational model. The medical secretary's education and training are, therefore, crucial components for them to be equipped with mastery levels and efficiency in tasks curbed by AI and HIS technologies (Santavirta et al., 2021).

Recommendations on the Curriculum Based on Digitization

Historically, the traditional educational curriculum for a medical secretary has included office management, medical terms, correspondence, and fundamental administrative tasks (Işık and Akbolat, 2010). Though these remain pertinent skills, the integration of HIS and AI technology into practice necessitates that a curriculum also include content related to healthcare technology (Nazeha et al., 2020).

"Education should enable medical secretaries not only to become savvy users of health care information systems, but also informed actors in health care-related computer processes." With this perspective in mind, education should cover Health Information Systems' structure and operation,

Health Information Systems skills, and legislation related to confidentiality and protection guidelines concerning personal health-related information (data) (Stanfill and Marc, 2019; Gazquez-Garcia et al., 2024).

Moreover, foundational-level literacy and conceptual learning concerning automation and Artificial Intelligence can facilitate a more informed and critical attitude towards technology for medical secretaries. This type of learning material empowers medical secretaries to understand the role of AI in various administrative procedures and allows them to critically analyze the result produced by the technology instead of accepting it blindly (Makrash et al., 2024).

In-Service Training and Lifelong Learning

Digital transformation is a continuous process and goes beyond education as learned in the school system and extends to other areas of one's career in life. HIS and AI-based systems are constantly being updated with various new modules and applications being added to them in a continuous manner. Hence, it becomes necessary to go beyond the education received prior to graduation to have continuous skills in one's profession (Davies et al., 2022).

Employee training initiatives play an important role in facilitating the medical secretaries' adoption of new technologies. Employee training initiatives should not be limited to the introduction of new systems but be developed on the basis of practice-focused, role-centered, and continuous learning (Santavirta et al., 2021). Adopting the philosophy of lifelong learning will also help decrease resistance towards change and new technologies, and build an innovation-cultivating professional environment (Nazeha et al., 2020).

Enhancing Technology-Related Working Skills

The development of their ability to work efficiently in a technological environment is a key challenging element for their performance and well-being in a digital health context (Knudsen and Bertelsen, 2023). This set of skills encompasses not only usage but also problem-solving and process improvement in a technological context.

Medical secretaries might be the pioneering point of where the technical downtimes or workflow-related problems are rooted in HIS- and AI-based systems. The identification of those issues, communication to the technical department, and the establishment of workflow continuity are their attachments to the technology use (Santavirta et al., 2021). Regular educational and personnel development opportunities which target these performance issues specifically may not only result in increased performance but also support the digital resilience of healthcare organizations (Davies et al., 2022).

CONCLUSION

Digitalization is a healthcare sector change made through the introduction of Health Information Systems and Artificial Intelligence tools. Since this is a paradigm shift, it is not only in the technical infrastructure aspect but it is also in the area of expertise and professional roles of the people involved in delivering the service (Bertelsen and Nøhr, 2005; Stanfill and Marc, 2019). In this transitional phase of digitalization, there clearly arise very essential effects on the medical secretaries who are working at the interface of clinical and administrative activities. Nowadays, medical secretaries have transformed from the traditional role of administrative assistants only to becoming the active part of the digital healthcare system (Bossen et al., 2019).

The findings listed in this chapter indicate that Health Information Systems have influenced the workflow of the medical secretaries and have helped them assume further responsibilities (Santavirta et al., 2021; Knudsen and Bertelsen, 2023). Medical secretaries make electronic medical records and integrated information systems possible and are now, in that process, not just data entry personnel but important agents in checking the data for accuracy, integrity, and consistency (Santavirta et al., 2021; Knudsen and Bertelsen, 2023). This results in a higher tolerance for errors and, at the same time, increases the visibility and significance of the work of medical secretaries within a medical organization. Nonetheless, it should also be acknowledged that the usage of the HIS system not only advances but also integrates better communications and cooperation in medical and administrative activities, thus providing a holistic service (Bossen et al., 2019).

The integration of Artificial Intelligence applications into the field of healthcare moves this process to a more advanced level. The use of Artificial Intelligence applications such as automation, speech recognition, and data analysis is transforming most of the regular administrative tasks hitherto done by a medical secretary into a more analysis, supervisory, and coordinating kind of role (Stanfill & Marc, 2019; Makrash et al., 2024). In this case, Artificial Intelligence can be seen not as a replacement for the profession of a medical secretary, because it indeed transforms the essence of the said profession (Makrash et al., 2024), but rather as a factor that enhances the cognitive, ethic, and professional requirements of a medical secretary, thereby requiring them to be equipped with the skills to carry out their new role effectively (Gazquez-Garcia et al., 2024).

The current chapter is also pointing out the educational and development aspects of digital transformation with a view to indicate that the current education structure for medical secretaries might not be sufficient enough to support competency alignment with HIS and AI-based work environments (Nazeha et al., 2020; Davies et al., 2022). Digital literacy

skills, privacy awareness for data, basic AI literacy skills, and technology-based work skills are part of new essential competency skills for medical secretaries these days. Therefore, an updated curriculum with new professional development activities for medical secretaries through lifelong learning activities are required to overcome current difficulties based on digital transformation (Santavirta et al., 2021).

Regarding the Turkish context, although the healthcare information systems have been widely adopted and the infrastructure has received a substantial amount of investment, there is a scarcity of scientific studies concentrating on the roles and skills of the medical secretaries during the current changing era (Işık and Akbolat, 2010). It is clear that there is a marked contrast between the progress achieved on the infrastructure front and the development of human resources that could efficiently exploit it. This situation continues to underline the importance of repositioning the role of the medical secretary profession from a reactive supporting profession towards a prominent profession that helps sustain the quality and dependability of the healthcare services provided via digital infrastructure (Engin and Gürses, 2018).

The growing use of Health Information Systems and Artificial Intelligence does not signal a decline in employment for medical secretaries; instead, it reflects a gradual redefinition of their professional roles. When training frameworks and educational strategies keep pace with the realities of digital change in healthcare, this transformation can support both professional development and improvements in service delivery (Bossen et al., 2019; Davies et al., 2022). In this sense, the adaptation of medical secretaries to digitally driven work environments represents a significant opportunity for the profession, contributing to more sustainable and efficient healthcare services. Moreover, empirical research that centers on the experiences and roles of medical secretaries is essential for shaping realistic and long-term workforce policies in digitally integrated healthcare systems.

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