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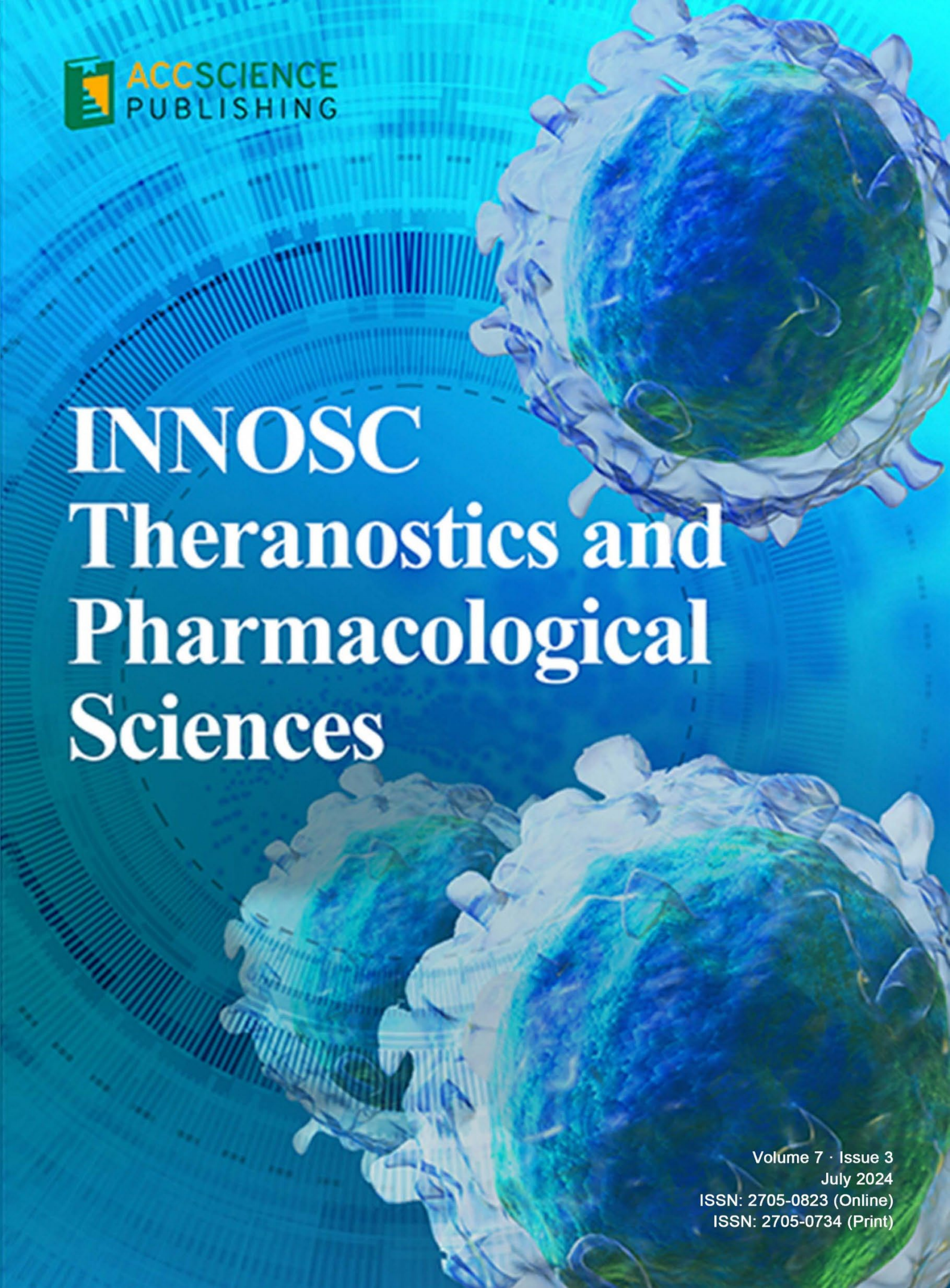

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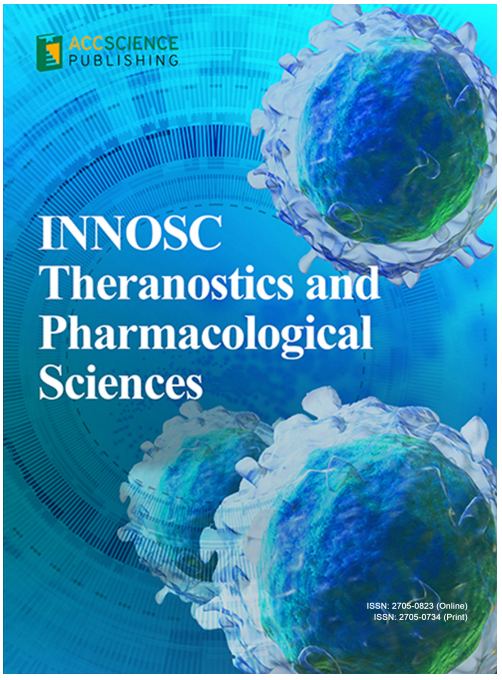

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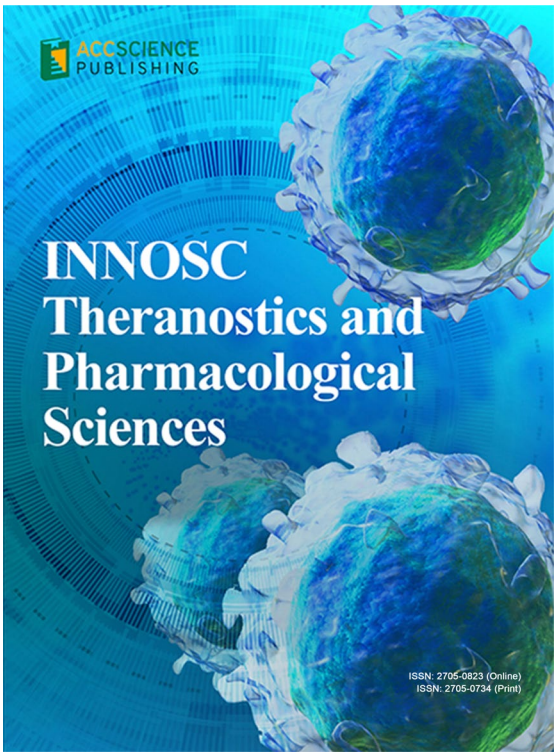

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INNOSC Theranostics and Pharmacological Sciences

EDITORIAL

Behavioral addictions beyond classic addictions and their future perspectives

Jo-Eun Jeong[1†] and Dai-Jin Kim[2†*]

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(This article belongs to the *Special Issue: Behavioral Addictions: From Bench to Bedside*)

1. Historical evolution of the concept of addictions

Over the past decades, the concept of addiction has undergone a profound evolution. This evolution has also been reflected in the Diagnostic and Statistical Manual of Mental Disorders (DSM), which is a widely used international diagnostic manual for mental disorders. The first edition of the DSM-I, published in 1952, considered addiction as primarily a personality issue and categorized it as sociopathic personality disturbance, which also encompassed antisocial personality disorder. Consequently, addiction was regarded as a secondary manifestation of personality disorders. In the DSM-II, published in 1968, addiction was separated from antisocial personality disorder; yet, it still remained within the realm of personality disorders.

It was not until the publication of the DSM-III in 1980 that addiction was finally recognized as a distinct category, designated as "substance use disorders (SUDs)." Furthermore, the DSM-III introduced criteria for substance abuse and substance dependence within the context of SUD. This edition placed particular emphasis on pharmacological criteria such as tolerance and withdrawal symptoms, rather than social problems related to substance use. Moreover, as pathological gambling came to be recognized as a disorder arising from impulse control issues, it was included under the category of impulse control disorders. In the revised third edition of the DSM (DSM-III-R), published in 1987, the diagnostic criteria for substance dependence underwent a significant refinement. This was evident in the shift from a focus on pharmacological criteria to a more comprehensive approach that encompassed behavioral syndromes, including impaired control, the giving up of activities, and the continued use of substances despite the presence of adverse consequences. The publication of the DSM-IV in 1994 saw the duration for substance abuse and dependence being revised from 1 month to 12 months.

The DSM-5,[1] published in 2013, presents a significant attempt in transforming the dimensional and structural aspects of addiction. First, the categories of abuse and dependence were merged into a single entity, and the criterion of "craving" was incorporated, while the item "recurrent substance-related legal problems" was eliminated. Another notable change was that DSM-5 expanded the scope of addiction to behavioral addictions. The term "pathological gambling" was replaced with "gambling disorder," and gambling disorder was reclassified from the "impulse control disorders" category to the "substance-related and addictive disorders" category. This change was based on the acknowledgment that gambling disorder shares numerous similarities with SUD in terms

†These authors are the guest editors for this Special Issue.

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of genetic predisposition, neurobiological underpinnings, personality and psychological characteristics, clinical features, comorbidities, course, and treatment methods. Furthermore, a new condition, "Internet gaming disorder", was included in Section III (Conditions for Further Study) of the DSM-5.

2. Behavioral addictions

The term "addiction" has traditionally been used to describe a chronic and relapsing brain disease caused by the action of certain substances or drugs on the brain. This results in neuroadaptation processes that lead to tolerance and withdrawal, as well as the repeated use of substances despite the negative consequences. However, the concept of addiction has evolved to include addiction caused by behaviors. Behaviors that were once performed for the purpose of survival now utilized as instruments to fulfill personal gratification beyond mere survival. These behaviors elicit pleasure, excitement, and mood enhancement, similar to addictive substances, and serve as a means to relieve or avoid stress and negative emotions. Examples of such behaviors include food consumption, sex, spending (shopping), working, and exercise. Behavioral addictions, such as gambling disorder, share common characteristics with SUD, including loss of control, preoccupation, tolerance, withdrawal, and a pattern of persistent behavior despite negative consequences. Moreover, therapeutic approaches for behavioral addictions are quite similar to those for SUD. These include motivational enhancement therapy to enhance intrinsic motivation, cognitive-behavioral therapy to identify and modify distorted thoughts or behaviors, the acknowledgment of one's powerlessness over addiction and recovery through spiritual awakening in the 12-step program, and the use of anti-craving medications like opioid antagonists, such as naltrexone, which are also applicable in behavioral addictions.

3. Beyond substance addictions

Given the distinct characteristics of behavioral addictions compared to SUD, it is imperative to adopt a different approach to comprehend behavioral addictions. First, in behavioral addictions, there are often no physical withdrawal symptoms. For instance, alcohol enters the brain and causes addiction, which can result in physical withdrawal symptoms such as hand tremors, nausea, vomiting, sweating, and seizures, in addition to psychological withdrawal symptoms like anxiety. Nevertheless, addictive behaviors such as gambling tend to manifest primarily psychological withdrawal symptoms, such as depression and anxiety, rather than physical ones.[2] In the case of Internet gaming disorder, withdrawal symptoms are most consistently referred to as irritability and restlessness following cessation of the activity.[3] It is important to differentiate between withdrawal symptoms associated with Internet gaming disorder and negative emotions resulting from abrupt cessation of gaming. Symptoms that persist for a few hours to a few days after the cessation of play can be regarded as withdrawal symptoms.[4] Second, behavioral addictions may have different motives from those of SUD. For example, in the case of gambling disorder, the presence of a financial motive within gambling behavior, as evidenced by the diagnostic criteria for persistence in gambling with the aim to recover lost money, can be observed. With respect to gaming motivations, there may be achievement, immersion, or social components.[5] Third, behaviors regarded as types of behavioral addictions, such as sex addiction and food addiction, frequently exhibit a high degree of interconnectedness with real-life experiences. This implies that the treatment goals for these behaviors differ from those of traditional addiction treatment, which is focused on abstinence. Treatment goals for behavioral addictions may include improving self-control, discontinuing specific behaviors, or minimizing the harm caused by addictive behaviors. Furthermore, while SUD are typically regarded as a progressive disease with worsening outcomes over time, some behavioral addictions may exhibit a spontaneous remission course.[6]

4. Future perspectives

The concept of behavioral addiction is evolving gradually, partly due to changes in societal environments, including the advancement of the Internet. Consequently, novel forms of behavioral addiction, such as Internet gambling, have emerged. To obtain a more comprehensive understanding of these behavioral addictions, it is essential to conduct in-depth research that examines not only the similarities with SUD but also the distinctive characteristics, motivational factors, risk factors, and other aspects of each behavior.

Conflict of interest

The authors declare that they have no competing interests.

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REVIEW ARTICLE

The potential therapeutic value of terpenes

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Abstract

Terpenes form part of a huge and diverse class of naturally occurring and volatile secondary metabolites produced by many plants, fruits, animals, insects, and other organisms. They are the largest group of naturally occurring metabolites, with over 55,000 types of terpenes produced by plants alone, primarily as essential oils. In humans, they contain significant biological properties such as antifungal, antiviral, antimicrobial, anti-inflammatory, antiparasitic, antihyperglycemic, anti-cancer, and analgesic agents. In plants, terpenes also play significant roles in defensive mechanisms against herbivores and invasive plants, disease resistance, chemical signaling and communication between plants, protection against photo-oxidation, plant-environment mediation, thermo-protection, and the attraction of pollinators. In addition, terpenes are responsible for a plant's scent, taste, flavor, and pigmentation, leading to their commercial use as fragrances and food dyes. Terpenes are also used in the production of synthetic polymers, natural rubbers (polyisoprene), organic solvents, varnishes, inks, adhesives, cleaning products, biofuels, pesticides, and food and drink products. For these reasons, terpenes have significant value in modern medicine, pharmacy, nutraceuticals, cosmetics, and other industries.

Keywords: Terpenes; Terpenoids; Metabolites; *Cannabis sativa*; Essential oils; Sterol; Squalene; Phytotherapeutics

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1. Introduction

1.1. History and general overview

The term terpene was first coined in 1866 by the German chemist August Kekule to classify camphene and all other hydrocarbons with the empirical formula $C_{10}H_{16}$.[1,2] The term has expanded to include other secondary metabolites such as limonene, myrcene, caryophyllene, linalool, ocimene, and many others, making terpenes the largest class of secondary metabolites. Notably, pinene is the most common terpene produced across plant species.[3,4]

Terpenes are found in many living organisms, most notably plants and animals, and have significant biological functions (Table 1). Owing to their chemical properties, terpenes have been used in the industrial production of fragrances (cosmetics, perfumes, etc.), inks, varnishes, adhesives, biofuels, rosin, solvents, flavorings, pesticides, insecticides, herbicides, aromatherapy, lubricants, biofuels, and agricultural chemicals.[5]

1.2. Classification of terpenes

While the terms terpenes and terpenoids (also known as isoprenoids) are often used interchangeably, terpenoids refer to an oxidized and denatured form of terpenes that contain an additional functional group with oxygen.[1,48] This oxidation occurs during drying and curing processes when the plant is exposed to open air.[49]

The building block of all terpene structures is an isoprene unit – a gaseous hydrocarbon (Figure 1). All terpenes and terpenoids are made up of varying combinations of these isoprene building blocks and can be further modified with a wide variety of functional groups, such as ketones, alcohols, and other substituents (e.g., fatty acids and sugars), to form a wide range of linear and cyclized hydrocarbon structures.[50]

Table 1. Functions of terpenes in plants

Function of terpenes in plants	Reference (s)
Host protection (i.e., the strong aroma may be a repellent to herbivores and may even detract invasive plant species)	5-8,9,10
Attraction of pollinators	5,11-16
Disease resistance	17-24
Combating oxidative stress	25
Combating abiotic and biotic stress, such as drought, temperature fluctuations, high light intensity, mechanical damage, and air and soil pollution	26
Inter-plant communication (chemical signaling)	16,27
Communication with the surrounding environment (e.g., beneficial species, such as pollinators, herbivores, predators, seed dispersers, and parasitoids)	16
Plant protection against ozone damage	28
Photoprotection	25,29-31
Light harvesting	31
Cloud-seeding	32-41
Modulation of cell growth	31
Plant elongation	31
Membrane permeability and fluidity control	31,42-47

The classification of terpenes is dependent on the number and organization of these isoprene units and the number of carbon atoms thereof. For example, monoterpenes have 10 carbon atoms (C_{10}), sesquiterpenes have 15 carbon atoms (C_{15}), and diterpenes have 20 carbon atoms (C_{20}). Figure 2 depicts the chemical structures of several popular terpenoids produced by *Cannabis sativa*. In a 2020 study, Jin *et al.* profiled secondary metabolites in cannabis inflorescences, leaves, stem bars, and roots for medicinal purposes, and the list of monoterpenoids and sesquiterpenoids is listed in Table 2.[51]

2. Production and extraction of terpenes

2.1. Terpene biosynthesis

Terpenes are synthesized from an isoprenoid precursor, isopentenyl pyrophosphate (IPP), and dimethylallyl pyrophosphate (DMAPP) through the plastidial deoxyxylulose phosphate/methyl-erythritol phosphate (DOXP/MEP) pathway (monoterpenoids) in plastids of plant cells[5,52] and the cytoplasmic mevalonate (MVA) pathway (sesquiterpenoids, triterpenoids, and sterols) in the cytosol of plant cells (Figure 3).[5,51,52]

2.2. *C. sativa* as a source of terpenes

C. sativa is well known for its agricultural and medicinal values in East Asian, African, and Indian cultures dating as far back as 6,000 years ago.[53-60] The medicinal properties

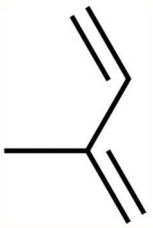

Figure 1. Chemical structure of an isoprene unit (C_5H_8), a five-carbon cyclic compound

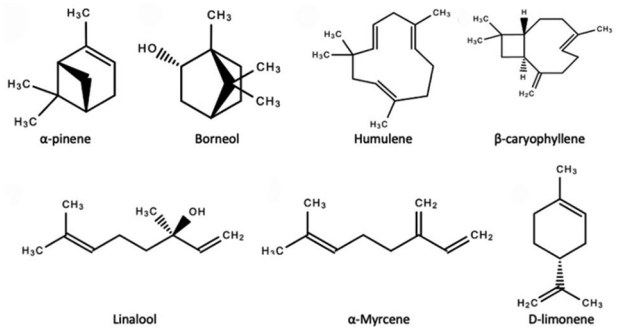

Figure 2. The chemical structures of some terpenoids produced by Cannabis sativa: α-pinene, borneol, humulene, β-caryophyllene, linalool, α-myrcene, and D-limonene

Table 2. Examples of monoterpenoids and sesquiterpenoids profiled in cannabis inflorescences, leaves, stem bars, and roots for medicinal purposes

Terpenoids	Examples
Mono-terpenoids	α-Pinene; eucalyptol; borneol; camphene; ocimene; terpinen-4-ol; sabinene; γ-terpinene; terpineol; (-)-β-pinene; sabinene hydrate; (+)-dihydrocarvone; β-myrcene; terpinolene; nerol; α-phellandrene; frenchone; pulegone; Δ^3-carene; linalool; carvone; α-terpinene; frenchol; geraniol; p-cymene; (-)-isopulegol; geranyl acetate; limonene; camphor.
Sesqui-terpenoids	(-)-β-Elemene; viridiflorol; β-caryophyllene; (-)-guaiol; aromadendrene; (+)-cedrol; trans-β-farnesene; β-eudesmol; α-humulene; α-bisabolol; valencene; ledene; trans-nerolidol; caryophyllene oxide; globulol.

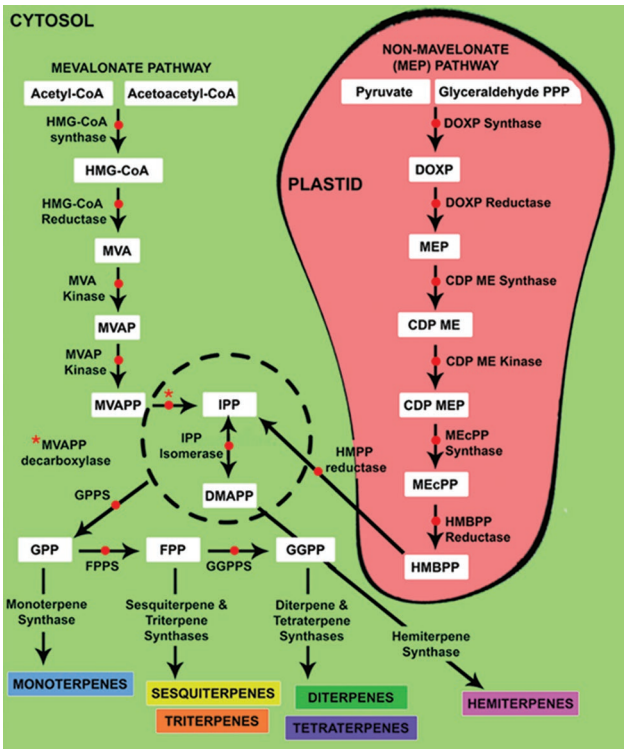

Figure 3. The mevalonate (MVA) pathways that occur in the cytosol of plant cells, the methyl-erythritol phosphate (MEP) pathway that takes place in plastids of plant cells and the Apicomplexa phylum of protozoa and bacteria, and the subsequent biosynthesis of terpenes from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) Abbreviations: CDP MEP: Diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate; DOXP: 1-Deoxy-d-xylulose 5-phosphate; HMG-CoA: 3-hydroxy-3-methyl-glutaryl-coenzyme A; FPP: Farnesyl pyrophosphate; FPPS: Farnesyl pyrophosphate synthase; GPP: Geranyl pyrophosphate; GGPP: Geranylgeranyl pyrophosphate; GPPS: Geranyl diphosphate synthase; HMBPP: (E)-4-hydroxy-3-methyl-but-2-enyl diphosphate; MEcPP: 2-C-methyl-d-erythritol cyclodiphosphate; MEP: 2-C-methyl-d-erythritol-4-phosphate; MVAPP: Mevalonate 5-diphosphate; PPP: PPP:3-phosphate.

of *C. sativa* primarily stem from its secondary metabolites, such as cannabinoids and terpenoids, the profiles of which vary across cannabis strains. Hence, it is suggested that these secondary metabolites work synergistically in a phenomenon known as the entourage effect to produce an enhanced pharmacological effect in humans.[61,62] This phytocannabinoid-terpenoid synergy could also be beneficial in the treatments of microbial infections, mental disorders, pain, inflammation, nociception, insulin resistance, diabetes, and comorbidities.[61,63-66] In this review, we will focus on the medicinal value of terpenoids instead of cannabinoids and their synergistic effects. Nonetheless, the terpenes of the cannabis plant have been well studied, and they shall be used as a reference in this review when discussing terpenes.

Over 150 different terpenes and 100 cannabinoids (e.g., tetrahydrocannabinol, cannabidiol, and cannabigerol) are produced in a resin that forms within glandular trichomes located on the surfaces of pistillate inflorescences as well as the foliage of a cannabis plant.[67,68] Specifically, the isoprenoid biosynthetic system of the cannabis plant produces the terpenes using the MVA and MEP pathways (Figure 3). Studies have indicated that the number of terpenes in cannabis plants from different origins can vary from 12 to 66.[67] The diversity of the hydrocarbon terpenes is mostly attributed to (i) the diversity of terpene synthase enzymes that are present and (ii) genetic and environmental differences. The terpenes that are most often found in cannabis plants include myrcene, β-caryophyllene, α-humulene, α-pinene, limonene, linalool, bisabolol, and (E)-β-farnesene.[67]

2.3. Extraction of terpenes

Terpenes are traditionally extracted through maceration, hydrodistillation, and soxhlet extraction. Other modern extraction methods have since been developed that are faster, use less solvent, and are environmentally safer, such as ultrasound,[69] microwave,[70] pressurized liquid, and supercritical fluid. Peres *et al.* reported that pressurized liquid extraction was easier and significantly more efficient to extract terpenes than both soxhlet and ultrasound-assisted extraction methods.[71] Similarly, Reddy *et al.* evaluated the comparison between supercritical fluid and conventional solvent extractions and reported that the use of butane or propane extracted more significant amounts of terpenes than supercritical CO_2 because of the mild nature of the solvents and the properties of the molecules (e.g., polarity).[72,73] In addition, terpenes can also be extracted through chemical-free methods, such as using heat, pressure, water, and mechanical approaches. For example, terpenes located in the glandular trichomes of the cannabis plant are extracted by mechanical sieving or centrifugation, where the separated glandular trichomes,

known as kief, are inserted in between hot metal plates to produce rosin containing both terpenes and cannabinoids.[74]

3. Biological and medicinal benefits of terpenes

3.1. Medicinal properties of terpenes

Terpenes were recognized for hundreds of years as essential oils from various plants and used in ancient ceremonies and for medicinal purposes in ancient civilizations. The primary constituents of essential oils are a combination of hydrophobic and volatile monoterpenes, sesquiterpenes, and phenylpropanoids. Plant families, such as *Asteraceae*, *Myrtaceae*, *Alliaceae*, *Apiaceae*, *Lamiaceae* (e.g., lavender and rosemary), *Poaceae*, and *Rutaceae*, produce a variety of essential oils of significant medicinal and industrial value.[75-77] In ancient times, the Arabs, Chinese, and Egyptians applied camphor as a topical medication for insect bites, itches, joint pain, swellings, sprains, and inflammation, as a decongestant, and as a therapeutic agent in the treatment of diarrhea, depression, fever, and dental cavities.[52,78-92] Terpenes are also traditionally used in exotic perfumes, incense, embalming fluid (e.g., in burial rituals, mummification, and religious ceremonies), as a fumigant and repellent against pests, and in cooking.[52,78-92] In modern days, terpenes and their derivates are widely used in industries, such as food (flavoring), toiletries, pharmaceutical, nutraceutical, and cosmeceutical industries (fragrances).

By various mechanisms of action, terpenes and their derivates demonstrate anti-cancer, antioxidant, neuroprotective, anti-inflammatory, cardioprotective, digestive, antitumor, antibacterial, anti-plasmodial, antiviral, antiseptic, antimalarial, immunoregulatory, antiaging, diuretic, antidiabetic, hypoglycemic properties, and more.[93-98] Limonene (from *Citrus limon/Citrus reticulata/Juniperus communis*) is among the most common terpenes used in folk medicine, particularly in Indian Ayurvedic medicine and traditional Chinese medicine (TCM) to treat oral cavities, digestive problems, abdominal colic pain, cough, cold, swelling, and urinary problems.[99-101]

Humulene (*Panax* sp./Ginseng) is also widely used in Ayurvedic medicine and TCM to boost energy and memory, reduce fatigue, and treat symptoms of menopause and muscular skeletal conditions (such as arthritis and rheumatism).[102-104] Garlic (*Allium sativum*) contains nerolidol, alpha-pinene, and terpinolene and is also widely used in folk medicine to treat wounds, headaches, heart disease, ulcers, and gastric/digestive problems.[105,106] Zingiberene (ginger/*Zingiber officinale*) is also a mainstay in folk medicine, and it is used to treat digestive problems, colds, coughs, asthma, wheezing, and joint pain.[107,108] Likewise, cinnamon (*Cinnamomum verum*) contains many medicinal terpenes, such as α-pinene, caryophyllene, cymene, linalool, humulene, α-phellandrene, and cymene. It is used to treat respiratory issues (e.g., cold, sinus, congestion, and bronchitis), digestive problems, diabetes, and chest pain.[109]

3.2. Biological properties of terpenes

The secondary metabolites of cannabis have a wide range of biological characteristics, such as anticancer, anti-inflammatory, analgesic, antimicrobial, neuroprotective, and antioxidant properties.[51,110-112] These characteristics of terpenes have enabled the treatment of various conditions, such as stress, anxiety, depression, decongestion, inflammation, pain, cancer, digestive disorders, bacterial infections, viral infections, fungal infections, metabolic diseases, and cardiovascular diseases (CVDs) (Table 3).[75,110,113-115]

The antioxidant properties of terpenes are of significance to counteract the effects of reactive oxygen species (ROS) involved in the aging process and the pathology of many conditions (e.g., diabetes, CVDs, liver disease, renal disease, cancer, and neurodegenerative diseases).[116-119] Other proposed mechanisms of antioxidant action of terpenes are through free radical scavenging mechanisms and by enhancing the activity of antioxidant molecules.[116] Notably, a patented combination of cannabis terpenes has demonstrated clinical significance in the treatment and prevention of COVID-19.[120,121]

Cannabis terpenes, such as ocimene, terpinolene, and β-myrcene, have exhibited anti-cancer effects in a taxol-resistant model of breast cancer.[122] Similarly, other cannabis terpenes, such as myrcene, linalool humulene, β-caryophyllene, limonene, pinene, eucalyptol, and borneol, have all demonstrated anti-cancer effects by various mechanisms of action such as induction of apoptosis, induction of cell cycle arrest.[61,122-138]

The pharmacological effects of *C. sativa* are primarily induced by the interaction of secondary metabolites with the endocannabinoid system (ECS). The ECS is primarily responsible for maintaining homeostasis.[162] In addition to regulating physiological processes, the ECS directly influences other biological aspects of a human being (e.g., physical, mental, and psychological),[163,164] and the multifaceted role of ECS has been implicated in several pathophysiological diseases, such as cancer, CVDs, and neurodegenerative diseases. In this regard, the genetic and pharmacological modulations of the ECS

Table 3. Biological properties of common terpenes in cannabis and other plants

Terpene (or its derivative)	Biological property	Other sources	Reference (s)
Myrcene	Potent analgesic	Ginger	139-141
	Antioxidant; neuroprotective; anti-inflammatory	Mango, guava, and hops	95
	Anticonvulsant	N/A	96
1,8-Cineole	Increases cerebral blood flow and enhances cortical activity	*Eucalyptus obliqua* L'Hér.	142
Limonene	Inhibits many species of bacteria and fungi; repellent	N/A	98
	Anti-inflammatory; antioxidant; antiviral; antidiabetic; anticancer; anti-hyperglycemic	Grapefruit, lemon, orange peel, and vitex	95,119,140,141, 143,144
	Antidepressant; anticonvulsant	N/A	95,98
α-Pinene	Antimicrobial; repellent	N/A	96
	Bronchodilator; anti-inflammatory	Basel	140,141
	Memory improvement/enhancement; acetylcholinesterase inhibitor, antiseptic, antibacterial, anti-inflammatory, bronchodilator, gastroprotective	Pine needle and rosemary	145
β-Pinene	Antimicrobial; antiseptic; anti-tumor	Mint, pepper, nutmeg, and pine needle	140,141,145
Linalool	Anxiolytic; anti-inflammatory; antimicrobial; anticancer; neuroprotective; antidepressant	Lavender	4,95,140
	Anti-influenza	N/A	146
	Sedative; induces apoptosis in cancer cells	N/A	95
α-Terpineol	Antimicrobial; repellent; anti-ischemic	N/A	98
	Anti-inflammatory	Cardamom and mugwort	141,147-149
	Analgesic	Pine and bitter orange tree (*Citrus aurantium* L.) essential oils	150
	Nociception inhibition	N/A	150,151
	Anticonvulsant	N/A	152
	Antimicrobial	N/A	96,97
	Gastroprotective	N/A	97
Borneol	Antimicrobial; repellent	Rosemary (*Rosmarinus officinalis*) and mugwort (*Artemisia absinthium* L.)	98
β-caryophyllene	Anti-inflammatory; analgesic	N/A	95
	Antispasmodic in gut muscles	Parsley	96
	Anti-inflammatory; gastroprotective; analgesic; anticancerogenic; antifungal; antibacterial; antihyperglycemic; antidepressant; anti-inflammatory; antiproliferative; antioxidant; anxiolytic; analgesic; neuroprotective	Clove, angelica, pennywort, and black pepper	61,63,140,141,143, 153-156
Caryophyllene oxide	Antifungal	Clove, rosemary, and hops	63
Humulene	Antiallergy; anticancer	Basil	95,141
Ocimene	Antifungal; antibacterial; antioxidant; antiviral; anti-inflammatory	Alfalfa and thyme	141,157-161
Eucalyptus oil	Expectorant (stimulates mucus); diuretic	N/A	52
Clover leaf oil	Analgesic	N/A	52
Menthol	Reduces flatulence and combats indigestion	N/A	52

Abbreviation: N/A: Not available.

have gained significant interest in medicine, research, and drug discovery and development (e.g., cannabinergic, cannabimimetic, and cannabinoid-based therapeutic drugs) to inhibit the metabolic pathways and/or agonism or antagonism of the ECS receptors.[165,166] Consequently, ECS regulation enables the treatment of a wide range of diseases, including (but not limited to) general pain, headache, migraine, glaucoma, mood and anxiety disorders, obesity/metabolic syndrome, osteoporosis, neuromotor, neuropsychological and neurodegenerative diseases, respiratory diseases (i.e., asthma), and vascular diseases such as stroke, atherosclerosis, myocardial infarction, arrhythmias, and hypertension.[49,165-170]

3.3. Antimicrobial properties of terpenes

The antimicrobial properties of essential oils are well established and are primarily attributed to the presence of a complex mixture of terpenes and other secondary metabolites.[18,19,24,77,171-180] In a 2019 study, Guimarães *et al.* investigated that the antibacterial activities of terpenes and terpenoids present in essential oils against bacteria such as *Salmonella enterica* and *Staphylococcus aureus*.[77] Terpenes (i.e., eugenol and terpineol) demonstrated rapid bactericidal action against *S. enterica* serovar Typhimurium and *S. aureus*, respectively, while other terpenes (i.e., carveol, citronella, and geraniol) exhibited significant bactericidal activity against *Escherichia coli*.[77] Therefore, terpenes may serve as a suitable alternative to current antibiotics when antibiotic resistance becomes a critical issue.

3.4. Analgesic and anti-nociceptive properties of terpenes

The demand for analgesic drugs continues to increase due to the increasing prevalence of pain (of all sorts) affecting people worldwide, leading to a growing interest in novel therapeutic options.[181] The analgesic potential of terpenes is also well studied and documented,[75,182] particularly in animals subjected to pain and nociceptive tests, such as hot-plate, formalin, tail-flick, and acetic acid-induced writhing tests.

Plant species, such as *Hyptis fruticosa* Salzm. ex Benth. (Lamiaceae), *Hyptis pectinata* Poit. (Lamiaceae), *Illicium lanceolatum* A.C. Smith (Illiciaceae), *Lippia gracilis* Schauer (Verbenaceae), *Matricaria recutita* L. (Asteraceae), *Mentha x villosa* Huds (Labiatae), *Pimenta pseudocaryophyllus* (Gomes) L.R. Landrum (Myrtaceae), and *Erythrina velutina* Willd. (Fabaceae), are known for their therapeutic essential oils and are widely used in folk medicine as analgesics and antinociceptives.[183-187] These properties are primarily due to the presence of terpenoids (monoterpenes and sesquiterpenes) and phenylpropanoids. Monoterpenes, such as linalool, myrcene, limonene, and 1,8-cineole in particular, are strong candidates for the treatment of chronic pain (e.g., chronic muscle pain, cancer-related pain, chronic inflammatory pain, and neuropathic pain).[139,182,188-191] Likewise, the combination of cannabinoids and flavonoids may be used to treat headaches and migraines.[64]

The analgesic activity of terpenes involves stimulating the release of anti-inflammatory cytokines,[182] regulating ion channels (e.g., transient receptor potential cation channels),[192] and directly acting on nociceptive afferent fibers[75] through multiple receptors (e.g., opioid, adenosine A1 and A2, and capsaicin).[151,191,193-196] The possible mechanisms of analgesic and antinociceptive actions of terpenes and their derivatives are displayed in Table 4.

Furthermore, terpenes can also enhance skin permeability and drug permeation by promoting interactions with the lipid bilayer of the stratum corneum of the human skin. Therefore, terpenes are good candidates for transdermal and transmucosal drug delivery systems, particularly for pain relief and management.[197]

3.5. Anti-inflammatory properties of terpenes

The pathological development of inflammatory diseases is regulated by NF-κB and is characterized by an overproduction of inflammatory cytokines (e.g., interleukin-6 [IL-6, IL-1β, IL-4, IL-8, IL-17, and tumor necrosis factor-alpha [TNF-α]). Nonetheless, terpenes have demonstrated the ability to inhibit the inflammation-associated signaling pathways by downregulating the expression of specific inflammatory cytokines.[208-213]

D-Limonene reportedly inhibited several pro-inflammatory cytokines, i.e., IL-1β, IL-4, IL-8, and IL-17,[197,214-218] thereby suggesting a protective role against various inflammatory disorders, such as asthma, rheumatoid arthritis, inflammatory bowel disease, skin inflammation, and psoriasis.[219-223] Artemisinin has reportedly treated inflammation-associated pain that is regulated by NF-κB signaling, promoted TNF-α-induced apoptosis, inhibited ROS production, and blocked the phosphorylation of p38 and ERK, both of which are pro-inflammatory processes.[208,221,224-226] In contrast, pinene, linalool, and 1-octanol-inhibited COX-2 overexpression, inducible nitric oxide synthase (iNOS), and nociceptive stimulus-induced inflammatory infiltrate.[182,197,208,221,227] Similarly, citral (geranial) and carvacrol activate the peroxisome proliferator-activated receptors (PPAR) α and γ.[197] α-Terpineol reportedly inhibited the intracellular formation of IL-6,[147] while borneol, linalool, catalpol, carvacrol, citronellol, geniposide, paeoniflorin, and 1,8-cineole (eucalyptol) inhibited the expression of

Table 4. Analgesic and anti-nociceptive mechanisms of action of terpenes and terpene derivatives

Terpene (and its derivative)	Plant species	Mechanism of action	Reference (s)
p-Cymene	*Hyptis pectinata* (L.) Poit. (Lamiaceae); *Protium heptaphyllum* (Aubl.) Marchand (Burseraceae)	Protective effects with a reduction of pro-inflammatory cytokines; inhibition of NF-κB and MAPK signaling	
(-)-Linalool	*Aniba rosaeodora* (Lauraceae)	Acts on several receptors, including opioids, adenosine A1 and A2, and cholinergic M2; regulation of changes in K^+channels	196,198-201
(-)-Menthol	*Mentha* species (including *M. piperita* and *M. arvensis*)	Acts on opioid receptors	202
Eugenol	*Abutilon indicum; Eugenia caryophyllata*	Acts on capsaicin, opioid, and α-adrenergic receptors; inhibition of high-voltage-activated calcium channels	151,193-195
Thymol	*Hofmeisteria schaffneri*	Inhibition of prostaglandin synthesis; inhibition of voltage-operated $Na^+$and K^+channels; activation of aminobutyric acid GABAA receptors	203-207
β-Pinene	*Hyptis fruticosa; Hyptis pectinata* (L.) Poit	Partial agonism of opioid μ receptors	191

Abbreviations: NF-κB: Nuclear factor kappa B; MAPK: Mitogen-activated protein kinase.

Table 5. Mechanisms of action of terpenes and terpenoids against inflammatory diseases

Terpene/ terpenoid	Origin	Inflammatory disease or experimental model	Mechanism of action	Reference (s)
Ginkgolide C	*Ginkgo biloba* leaves	I/R- related inflammation	Suppression of CD40-NF-κB signal pathway; inhibition of downstream inflammatory cytokines, IKK-β, and IκB-α phosphorylation	229,230
α-Pinene	Oils of coniferous trees and rosemary	LPS-induced macrophages	Inhibition of MAPK, NF-κB, and pro-inflammatory cytokines, such as IL-6 and TNF-α	231,232
Linalool	Essential oils of aromatic plants	Cigarette smoke-induced acute lung inflammation; LPS-induced inflammation in microglial cells	Inhibition of NF-κB activation; inhibition of pro-inflammatory cytokines (e.g., TNF-α, IL-6, IL-1β, IL-8, and MCP-1)	231,226,233,234
p-Cymene	N/A	Elastase-induced lung emphysema and inflammation	Inhibition of proinflammatory cytokines, such as IL-1β, IL-6, IL-8, and IL-17, in BALF	231,235
Malloconspur A and malloconspur B (diterpenoids)	*Mallotus conspurcatus* croizat (Euphorbiaceae) (Native to China)	Pelvic inflammatory disease and possibly other inflammatory diseases	N/A	236
α-Pinene	Essential oils of coniferous trees	Skin inflammation	Photoprotective effect against inflammatory signaling in human skin cells; inhibition of UVA-induced expression of inflammatory protein, such as TNF-α and IL-6; suppression of MAPKs pathway through inhibition of ERK and JNK phosphorylation	232,237
β-Pinene	*Boswellia serrata* Linn.	RA	Inhibition of inflammatory biomarkers (NF-κB, COX-2, and LOX-5) involved in the pathogenesis of RA	222
D-Limonene	Essential oils of citrus plants	Neuroinflammation	Inhibition of inflammatory cytokines, such as IL-1, IL-6, and TNF-α	219,234,238

Abbreviations: BLAF: Bronchoalveolar lavage fluid; I/R: Ischemia/reperfusion; LPS: Lipopolysaccharide; MAPK: mitogen-activated protein kinase; MCP-1: Monocyte chemoattractant protein-1; N/A: Not available; RA: Rheumatoid arthritis.

TNF-α.[197,228] Moreover, linalool, myrcene, carvacrol, α-phellandrene, paeoniflorin, and citronellal reportedly downregulated GluN2B receptors and, subsequently, glutamate, a pro-inflammatory amino acid.[182,197] Table 5 displays the mechanisms of action of several common terpenes (and terpenoids) as anti-inflammatory agents.

3.6. Anxiolytic and anti-depressant properties of terpenes

According to the World Health Organization (WHO), it is estimated that one billion people are affected by mental illness globally, and depression and anxiety-related disorders are reportedly the most prevalent mental conditions.[239-242]

Studies have suggested that terpenes may have anxiolytic and anti-depressant properties, but the molecular mechanism(s) of action remains unclear. It was hypothesized that anxiolytic and anti-depressant properties might be mediated through interaction with serotonergic 5-hydroxytryptamine receptors, noradrenergic, dopaminergic systems, or glutamate receptors.[243] In a study on *C. sativa*, it was suggested that the combination of terpenes, phytocannabinoids, and flavonoids synergistically induced the entourage effect to generate enhanced anxiolytic and anti-depressant activities,[244-246] warranting further studies to validate the anxiolytic and anti-depressant effects.

The aromatherapeutic and medicinal value of essential oils containing terpenes has been recognized in traditional medicine for hundreds of years based on their anxiolytic and anti-depressant properties.[52,78-92,247,248] Several common terpenes (i.e., linalool, geraniol, citronellol, citronellal, myrcene, β-caryophyllene, and limonene) have reported sedative, anxiolytic, anti-depressant, sedative, and anticonvulsant proprieties in both humans and murine models.[61,247,249-254,63,247,255-259] In particular, hydroxycitronellal (HC) exhibited anxiolytic and anti-depressant properties through regulating the GABAergic system ($GABA_A$ receptors).[260] Linalool and linalyl acetate reportedly inhibited voltage-gated calcium channels[252] and, subsequently, $5HT1_A$ receptor activity.[252,258] In murine models, D-limonene has demonstrated anti-depressant properties by downregulating IL-1, IL-6, and TNF-α.[234,238]

3.7. Cardioprotective properties of terpenes

CVDs collectively refer to conditions affecting the cardiac (i.e., heart failure, cardiac arrest, coronary heart disease, angina pectoris, myocardial infarction, hyperlipidemia, and cardiomyopathy) and vascular (i.e., atherosclerosis, peripheral artery disease, ischemic stroke, and pulmonary embolism) systems.[76,230] In 2021, CVDs were the leading cause of death globally with 17.9 million deaths.[261]

Ongoing research efforts have investigated terpenes as prospective novel therapeutics drug candidates for CVDs.[76] Limonene, carveol, linalool, and several diterpenes (and their derivatives) have demonstrated therapeutic potential against CVDs.[262-267]

Table 6 features several terpenoids that have reported cardioprotective effects against CVDs. Although the mechanisms of action of terpenes in the cardiovascular system have not yet been fully elucidated, it is suggested that they work by regulating heart rate and vasorelaxation.[263] Diterpenes such as dehydrocrotonin, 14-deoxyandrographolide, ent-18-hydroxytrachyloban-3β-ol, ent-18-hydroxyisopimara-7,15-diene-3β-ol, and neoorthosiphols A and B are used in folk medicine to treat hypertension.[266,268-270] Stevioside, a diterpenoid glycoside extracted from the plant *Stevia rebaudiana* Bertoni, also demonstrated vasodilatory effects and the ability to regulate mean arterial pressure.[76,266,267,271,272]

Another possibility is that terpenes, in synergy with phytocannabinoids and endocannabinoid-like molecules, regulate ECS and, subsequently, the cardiac and vascular systems.[264] In addition, terpenes may also ameliorate CVD through scavenging free radicals and enhancing the activity of antioxidant molecules.[116] The other more commonly proposed cardioprotective mechanisms include (i) inhibiting NF-κB and subsequently disrupting the NF-κB signaling pathway[116,208,236,269,209,236,269] and (ii) inhibiting NLRP3 inflammasome formation and subsequently disrupting the NLRP3 inflammatory pathway.[273]

Besides that, several terpenes (i.e., forskolin and marrubiin) have also displayed anticoagulant and anti-platelet properties.[274,275] Similarly, α-bulnesene, a sesquiterpene, exhibited anti-platelet-activating factor (PAF) properties,[276] while (+)-linalool and (-)-linalool could stimulate the cardiovascular system.[76,277,278] Citronellol has demonstrated antihypertensive and vasorelaxant properties that may be beneficial in the treatment of hypertension.[76] Thymol, a monoterpenoid, has vasorelaxant properties,[279] while limonene demonstrated cardioprotective and anti-hypertensive properties.[76]

Camphene, a plant-derived monoterpene, demonstrated hypolipidemic, hypocholesterolemic, and hypotriglyceridemic effects in rats, thereby reducing and preventing the accumulation of lipids, cholesterol, and triglycerides in arterial blood vessels.[280] In another study, Jiang *et al.* demonstrated that terpenoids from different polar extracts of *Cyclocarya paliurus* leaves had cholesterol-lowering effects in hyperlipidemic mice by converting cholesterol into bile acids.[281] These findings suggested that terpenes can regulate hyperlipidemia as well.

3.8. Anticancer properties of terpenes

Cancer is a major health burden that affected an estimated 19.3 million people globally in 2020 with an estimated 10 million deaths in the same year.[294-296] According to GLOBOCAN2020, global cancer cases are expected to

Table 6. Terpene derivatives (terpenoids, triterpenoids, and triterpenic acids) used to treat cardiovascular diseases

Terpene derivatives	Plant species	Mechanism(s) of action	Reference(s)
Rubiarbonone C	Roots of *Rubia yunnanensis* Diels	N/A	230,282
Platycodin D	Roots of *Platycodon grandiflorum*	Promotes atherosclerotic activity in HUVECs by increasing NO concentration, reducing the oxLDL-induced cell adhesion molecule expression, and regulating endothelial cell adhesion to monocytes	230,283
3,4-Seco-olean-18-ene-3,2,8-dioic acid	*Phoradendron reichenbachianum* (Viscaceae)	Not fully elucidated – possibly through inhibition of the NF-κB signaling pathway, DNA binding, or p65 translocation	230,284
Rubiarbonol C	Roots of *Rubia yunnanensis* Diels	Not fully elucidated – possibly through inhibition of the NF-κB signaling pathway, DNA binding, or p65 translocation	230,284
Triterpenic acid	Several plant taxa but primarily in plants of the mint family (*Mentha spicata* L.)	Not fully elucidated – possibly through inhibition of the NF-κB signaling pathway, DNA binding, or p65 translocation	230
Ursolic acid	Plants, such as elderflower, peppermint, oregano, and lavender	Not fully elucidated – possibly through inhibition of the NF-κB signaling pathway, DNA binding, or p65 translocation	230
β-Sitosterol glycoside	Saw palmetto (*Serenoa repens* [Bartram] J.K. Small); pygeum extract from the bark of the African cherry tree (*Prunus Africana* [Hook. f.] Kalkman)	Not fully elucidated – possibly through inhibition of the NF-κB signaling pathway, DNA binding, or p65 translocation	230
Astragaloside IV	*Astragalus membranaceus*	Not fully elucidated – possibly through inhibition of the NF-κB signaling pathway, DNA binding, or p65 translocation	230,285
Betulinic acid	White birch (*Betula pubescens*); Ber tree (*Ziziphus mauritiana*); self-heal (*Prunella vulgaris*)	Not fully elucidated – possibly through inhibition of the NF-κB signaling pathway, DNA binding, or p65 translocation	230,284
6β-OH-Betulinic acid	*Licania cruegeriana*	Not fully elucidated – possibly through inhibition of the NF-κB signaling pathway, DNA binding, or p65 translocation	230,286
Boswellic acid	*Boswellia* plant genus (such as *Boswellia serrata* Roxb.)	Not fully elucidated – possibly through inhibition of the NF-κB signaling pathway, DNA binding, or p65 translocation	230,287
Elatoside C	Longya *Aralia chinensis* L.	Increases cell viability, mitochondrial membrane potential, Bcl-2/Bax ratio, and pSTAT3/STAT3 ratio; decreases mitochondrial ROS, and the expression levels of CHOP, cleaved caspase-12, GRP78, and p-JNK	230,288
Ginkgolide C	*Ginkgo biloba* leaves	Enhances the survivability of H/R-induced ventricular myocytes; reduces inflammatory damage by reducing NF-κB p65 subunit translocation, phosphorylation of IκBα, and the activity of IκB kinase	230,231, 289-291
Forskolin	N/A	Promotes vasorelaxation	292
Kaurane	Roots of *Viguiera robusta*	Blocks extracellular Ca^{2+} influx to promote vasorelaxation and inhibit vasoconstriction	114,293
Pimarane-type diterpenes	Roots of *Viguiera arenaria*	Blocks extracellular Ca^{2+} influx to promote vasorelaxation and inhibit vasoconstriction	114,293
Marrubenol	*Marrubium vulgare* (Horehound, Lamiaceae)	Blocks L-type Ca^{2+} channels to inhibit smooth muscle contraction	114

Abbreviations: H/R: Hypoxia/reoxygenation; HUVECs: Human umbilical vein endothelial cells; N/A: Not available; NO: Nitric oxide; oxLDL: Oxidized low-density lipoprotein; ROS: Reactive oxygen species.

rise to 28.4 – 30.2 million.[294,297] Breast, lung, prostate, and nonmelanoma skin cancers are among the most prevalent cancers.[294,296] In addition, cancer occurs more often in males than in females, though this figure varied significantly in other parts of the world.[294]

The anti-cancer properties of terpenes are well established. Terpenoids modulate various intracellular pathways to (i) activate apoptosis, (ii) initiate cell-cycle arrest and subsequently suppress tumorigenesis, (iii) counteract oxidative stress (i.e., ROS scavenging and enhancement of antioxidant activity), (iv) inhibit

angiogenesis and metastasis, and (v) induce autophagy (e.g., through MAPK/ERK/JNK, PI3K/AKT/mTOR, AMPK, and NF-κB signaling pathways).[298-302]

Taxol (paclitaxel), a diterpenoid-based chemotherapy drug, is widely used to treat solid-tumor cancers (e.g., lung, prostate, breast, ovarian, esophageal, and melanoma)[303] and is considered one of the most effective terpenoid-based chemotherapeutic drugs. Taxol exhibits its anticancer effect by destabilizing microtubules, resulting in mitotic arrest, inhibition of cancer cell division, and ultimately cancer cell death.[304] Taxol also indirectly promotes apoptosis by binding to and inhibiting B-cell lymphoma 2 (Bcl-2) which prevents cell death.[304]

Terpenoids have demonstrated anticancer effects in several liver cancer cell lines through different mechanisms.[305] For example, geraniol reduced the expression of HMG-CoA reductase to inhibit HepG2 cell growth,[306] while asiatic acid-induced apoptosis through increased intracellular Ca^{2+} release and expression of p53.[307,308]

Oleanolic acid,[309] ursolic acid,[309] echinocystic acid,[310] lupeol,[311] and betulinic acid[312] reportedly promoted apoptosis of cancer cells, while the anticancer property of β-carotene is attributed to an antioxidant mechanism.[313] Various terpenes and their derivatives (e.g., geraniol and neral) are natural inhibitors of the NF-κB signaling pathway that plays a significant role in the pathological development

Table 7. Mechanisms of action of terpenes and terpenoids for treating various cancers

Terpene/ terpenoid	Origin	Type of cancer (cell line)	Mechanism(s) of action	Reference(s)
Taxol	N/A	Female breast cancer	Destabilizes microtubules to arrest cancer cell division and induce cancer cell death; promotes apoptosis through binding to Bcl-2	304,316
Geraniol (monoterpene)	N/A	HCC	Inhibits HepG2 cell growth through reducing HMG-CoA reductase production	305,306
Geraniol	N/A	Colon Cancer	Inhibits polyamine biosynthesis and metabolism (i.e., increased in cancer growth); apoptosis; DNA damage; cell cycle arrest; disruption of cell membrane	317-321
Andrographolide (diterpene)	*Andrographis paniculata*	HCC	Inhibits Hep3B cell growth (increase apoptosis, MAP kinases, ERK1/2, and pJNK); inhibits MMP-7 expression	305,306,322
Limonene	Lemon, orange, grapefruit, caraway, bergamot, peppermint, spearmint, dill, and tomato	Human bladder cancer cell (T24 cell line)	Inhibits human bladder cancer cell growth through apoptosis, caspase activation, and G2/M-phase cell cycle arrest	124
		Colon, mammary, pancreatic, and prostate cancers	Induces apoptosis; inhibits the post-translational isoprenylation of cell growth-regulating proteins	299,323,324
		Bladder cancer	Induces cellular apoptosis; caspase activation; G2/M-phase cell cycle arrest; inhibits cancer metastasis	124
Retinol	Carrot, spinach, pumpkin, broccoli, mango, papaya, cherry, tomato, corn, orange, cabbage, watermelon, and lettuce	Prostate cancer	Induces apoptosis; inhibits premalignant cell growth; suppresses carcinogens through retinoid nuclear receptors, retinoic acid receptors (RARs), and retinoid X receptors (RXRs)	325-331
Lycopene	Tomato, orange, carrot, pea, sprout, green, bean, and corn	Colon cancer	Suppresses Akt signaling and non-phosphorylated β-catenin protein levels; G-phase cell cycle arrest	299,332,333
		Prostate cancer	N/A	334,335
Latilagascenes C and D; 3β-acetoxy-helioscopinolide B and E	Extract from the *Euphorbia* species	Gastric (EPG85-257), pancreatic (EPP85-181), and colon (HT-29) carcinomas	N/A	336
Geraniol and neral (in combination)	*Zingiber officinale* (ginger)	Endometrial cancer cells	Promoted apoptosis by p53 activation	337
β-Caryophyllene	*Cannabis Sativa and Pamburus missionis* (SV1)	Taxol-resistant model of breast cancer	Activates apoptotic cascade; reduces cellular invasion	338

Abbreviations: HCC: Hepatocellular carcinoma; N/A: Not available.

of many cancers.[209,225,314,315] Other terpenes (e.g., menthol, β-caryophyllene, d-limonene, eugenol, and menthol) may be used independently or with chemotherapeutic drugs to promote anticancer activity by inducing apoptosis or necrosis and inhibiting cell proliferation.[5] Table 7 features several terpenes and terpenoids, as well as their mechanisms of action against various cancers.

3.9. Neuroprotective properties of terpenes

Neurological disorders are those characterized by the dysregulation of the neurons (nerve cells) in the brain or of the central and peripheral nervous systems. Neurodegenerative diseases (Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease) are a subgroup of neurological disorders characterized by progressive and inexorable damage to the nervous system and accompanied by loss of neurological function. In 2019, it was estimated that 10 million global deaths were attributed to neurological disorders.[339] Nonetheless, the increasing prevalence of neurological disorders with the aging of the global population has prompted the search for novel therapeutics.

Alzheimer's disease is the most prevalent neurological disorder, with an estimated 10 million global cases diagnosed annually.[340,341] The WHO has estimated that 55 million people are currently suffering from dementia. It has also been predicted that the number of Alzheimer's cases will reach 75 million by 2030 and up to 135 million by 2050.[341,342] Dementia (of which 80% are due to Alzheimer's disease) alone accounted for US$1.3 trillion worldwide in 2019 and is projected to exceed US$2.8 trillion by 2030.[342] In the United States alone, this amount is estimated to surpass $800 billion annually.[343] In addition, the increasing prevalence of Parkinson's disease and other neurological disorders (e.g., stroke and epilepsy) suggests that neurological diseases will be a substantial health burden, and their management may not be affordable or accessible to the less developed regions of the world.

Neuroprotective agents may increase the recovery and regeneration or slow the rate of deterioration of nerve tissues due to neurodegenerative diseases or other insults (e.g., ischemia or toxic injury). In one study, both monoterpenes and sesquiterpenes extracted from *Citrus sinensis* (orange) by-products demonstrated the ability to cross the blood–brain barrier, suggesting their potential as neuroprotective agents.[117] Sesquiterpenes (e.g., δ-cadinene and nootkatone) and other more common terpenes (e.g., limonene, pulegone, carvone, and γ-terpinene) have demonstrated the capacity to inhibit acetylcholinesterase (AChE) and butylcholinesterase (BChE), both of which are involved in the formation of amyloid β plaques in Alzheimer's disease.[117,344-347] The terpenoids of the orange extract also demonstrated the ability to inhibit IL-6, a pro-inflammatory mediator.[117] In addition, limonene reportedly inhibited the activation/phosphorylation of extracellular receptor kinase (ERK) that is linked to oxidative stress and pro-inflammatory immune responses in the pathogenesis of Alzheimer's disease.[348,349]

The aforementioned sesquiterpenes have also displayed the inhibition of lipoxygenase (LOX), the overactivity of which leads to synaptic dysfunction and neuroinflammation.[117,344] In another study, 1,8-cineole, bornyl acetate, terpinen-4-ol, and camphor (extracted from essential oils of *Rosmarinus officinalis* L.) reportedly inhibited LOX, while 1,8-cineole and 3-carene (also from the same extract) reportedly inhibited AChE.[350] The inhibition of AChE and BChE increases acetylcholine levels to promote neural activity and regulate Alzheimer's disease, respectively. Similarly, the inhibition of LOX also ameliorated the inflammatory and immune responses in the brain tissue of patients with Alzheimer's disease.[344] In a drosophila model of Aβ42-induced neurotoxicity in Alzheimer's disease, ρ-cymene, (+)-limonene, (-)-limonene, linalool, (+)-α-pinene, and (-)-β-pinene demonstrated partial suppression of amyloid β42 (Aβ42) expression and accumulation in the flies.[144]

Oxidative stress and the accumulation of ROS and reactive nitrogen species (RNS) are also characteristics of Alzheimer's disease due to the decline in the antioxidant activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx).[351] In this context, monoterpenes, such as limonene and nerol, with strong antioxidant properties can also be considered neuroprotective agents.[117-119] Besides that, the regulation of inflammation may play an important role in ameliorating neurological disorders.

Likewise, non-degenerative neurological disorders can be managed by terpenes in isolation or combination with other agents. For example, caryophyllene and β-caryophyllene have been studied for treating somatic pain and migraine, respectively. In contrast, linalool may directly target GABAergic neurotransmission in epilepsy and enhance the effects of other agents, similar to the entourage effect with cannabinoids.

3.10. Antidiabetic properties of terpenes

Diabetes is another major health burden that affects over 500 million people worldwide[252-354] with an estimated 6.7 million deaths annually. It is estimated that approximately 578 million people will be affected by diabetes by 2030 and up to 700 million people by 2045.[354] Notably, the global health expenditure for diabetes is estimated at US$966 billion.[352]

Diabetes mellitus is a chronic disease characterized by impairment in the regulation of glucose in the blood (blood

Table 8. List of terpene-related patents

Title of invention	Patent number	Treatment	References
Injectable formulation of paclitaxel for coronary artery disease treatment	EP1618879B1	Coronary artery disease	375
Anti-tumor terpene compounds	(i) WO2005049001A1 (ii) CA2545717A1	Cancer	376
Use of monoterpenes, sesquiterpenes and diterpenes for the treatment of cancer	WO1994020080A9	Prostate cancer, colon cancer, astrocytoma, and sarcoma	377
Monoterpenoid derivatives for treatment of cancer	WO1999045912A1	Cancer	378
Terpene and cannabinoid formulations	WO2015068052A2	Pain, allergies, inflammation, infection, epilepsy, depression, migraine, bipolar disorders, anxiety disorder, and drug dependency and withdrawal syndromes	379
Penetrating topical pain relief compositions and methods of use	WO2020243352A1	Topical pain	380
Device with compositions for delivery to the lungs, the oral mucosa, and the brain	AU2021201949A1	Pain, anxiety, depression, and plaque-related diseases.	381
Terpene-enriched cannabinoid composition	US20220323371A1	Treating acute and/or chronic pain, including chronic pain from multiple sclerosis, fibromyalgia, cancer, and peripheral neuropathy	382
Composition for preventing or treating neurodegenerative diseases, containing diterpene-based compound	EP3701946A1	Neurodegenerative diseases	383
Compositions comprising terpene compounds for treating negative sensory phenomena	US9415023B2	Negative sensory phenomena	384
Novel medicines based on sesquiterpene mixtures	WO2001080868A1	Cancer and treatment of epithelial and connective tissues	385
Compositions for improving skin conditions comprising alpha-bisabolol as an active ingredient	WO2000062744A2	Acne, aging, and skin inflammation	386

sugar). There are two types of diabetes: type 1 diabetes is characterized by the pancreas's inability to produce insulin, whereas type 2 diabetes is characterized by the body's inability to use insulin, often caused by a lack of physical exercise or obesity. Both types of diabetes are characterized by an increase in blood sugar (hyperglycemia) and ROS in the body.[143] In addition, diabetes is a significant risk factor for CVD, such as stroke, coronary artery disease, and blood vessel disease.[355-357]

Insulin is a hormone produced by the pancreas, and it regulates blood sugar by facilitating its uptake into cells and subsequent conversion into energy.[358] Insulin also regulates glucose storage in the liver, muscles, and fat cells of the body and plays a role in the metabolism of fats, proteins, and carbohydrates.[358] A dysregulation of these processes may lead to widespread damage to the body's tissues and organs, such as the heart, eyes, and kidneys.

Triterpenes have demonstrated multiple antidiabetic mechanisms, as well as hypoglycemic, hypolipidemic, and anti-obesity properties.[345] In addition, triterpenes can ameliorate insulin resistance, normalize plasma glucose and insulin levels, and inhibit the formation of advanced glycation end products (AGEs), commonly implicated in diabetic nephropathy, diabetic neuropathy, diabetic embryopathy, and impaired diabetic wound healing.[345,346]

Terpenes, such as D-limonene, β-caryophyllene, trans-anethole (TA), geranic acid, citral, farnesol, farnesal, and terpinen-4-ol, have demonstrated potent antihyperglycemic, anti-α-glucosidase, antioxidant, and anti-inflammatory properties,[143,347,359-363] suggesting their potential application as anti-diabetic agents.[345] In a study on streptozotocin (STZ)-induced diabetes rats, D-limonene was evaluated against oxidative stress parameters (i.e., lipid peroxidation by-products) and enzymatic antioxidants (i.e., glutathione-S-transferase, GPx, SOD, and catalase [CAT]),[347,361,364,365] and D-limonene demonstrated the ability to alleviate oxidative stress by enhancing the bioactivity of the enzymatic antioxidants and reducing the levels of lipid peroxidation by-products.[347]

Safranal, a monoterpenoid produced by the saffron plant (*Crocus sativus* L.), also demonstrated protection against oxidative damage in STZ-induced diabetes rats through enhancement of antioxidant enzymes such as GPx, SOD, and CAT.[366,367] There was also a reduction in the levels of lipid peroxidation markers, including nitric oxide

(NO) and malondialdehyde (MDA), lipids, triglycerides, and cholesterol.[366,367] These results suggest that safranal could be used as an anti-diabetic agent.

The sodium-glucose co-transporter-1/sodium glucose-linked transporter-1 (SGLT-1) regulates the reabsorption of glucose into the small intestines (and less frequently, the kidneys), thereby reducing blood glucose levels and improving glycemic control.[368] Farnesol and farnesal, two acyclic terpenoids, reportedly inhibit SGLT-1 to produce antihyperglycemic effects.[369]

ECS overactivity, particularly cannabinoid receptor type 1 (CB_1R), has been implicated in the progression of insulin resistance and diabetes (especially type 2) and its age-related comorbidities, such as atherosclerosis, retinopathy, neuropathy, and nephropathy.[65] Terpenes, in combination with phytocannabinoids, may also interact with CB_1R to ameliorate diabetes and its complications.[65] Cannabinoid receptor type 2 (CB_2R), also of the ECS, has been implicated in glucose and lipid metabolism and may also have therapeutic benefits against diabetes and chronic inflammatory diseases.[143] β-caryophyllene is reported to interact with CB_2R[143] and exhibit antiglycemic properties through interaction with PPAR-α and PPAR-γ.[143]

AMP-activated protein kinase (AMPK) is a stress kinase that increases fatty acid oxidation, inhibits lipid synthesis, improves insulin action, and facilitates GLUT4 translocation of glucose entry into adipocytes and muscle cells.[370] Two cucurbitane triterpenoids from *M. charantia*, karaviloside XI, and momordicoside, have demonstrated the ability to mediate the phosphorylation and activation of AMPK.[370-372] In the same study, momordicoside reportedly enhanced fatty acid oxidation and glucose disposal in both insulin-resistant and insulin-sensitive mice.[370]

4. Economic value of terpenes

Besides their medicinal value, terpenes and their derivatives also have high economic value as they are used widely in many industries, such as the food, toiletries, pharmaceutical, nutraceutical, and cosmeceutical industries. Notably, the COVID-19 pandemic has also greatly influenced the growth of the terpene market.

Terpenes are an important industrial raw material and play a significant role in the production of synthetic polymers, natural rubbers (polyisoprene), organic solvents, varnishes, inks, adhesives, cleaning products, and biofuels. For example, (-)-menthol (1-menthol) is widely used in the manufacturing of many beverages, cigarettes, and other products with a wild mint flavor or fragrance. Likewise, linalool is responsible for the distinct lavender fragrance and is also widely used in many industries.

As aforementioned, taxol (paclitaxel) is widely used to treat solid-tumor cancers, and its economic value has been increasing annually, correlating to the growing incidence of many cancers globally. In 2021, the global paclitaxel injection market was valued at US$4.51 billion and is expected to exceed US$11.16 billion by 2030 at a compound annual growth rate (CAGR) of 12.5% between 2022 and 2030.[373]

According to a 2022 market growth report, the global terpenes market was estimated to value around US$693 million and is expected to reach a valuation of over US$1 billion by 2028, with a CAGR of 6.4% between 2021 and 2027.[374] It should also be noted that several terpene-based patents contribute to this growth, and Table 8 presents several existing terpene-related patents for the treatment of various diseases.

5. Conclusion

Terpenes have significant therapeutic potential and a wide range of industrial applications. Owing to their high natural abundance and chemical diversity, terpenes are suitable candidates for novel and commercially valuable products and alternatives to synthetic products.

With the increasing incidence of cancer, there is also a growing interest in the therapeutic benefits of terpenes. Based on the treatment efficacy of taxol (paclitaxel), other terpenoids may have similar potential. However, there is currently a lack of definitive terpene and terpenoid profiles, and the biosynthetic systems and metabolic processes influencing the anticancer properties of terpenes remain unclear.

Given these limitations, further research is warranted to elucidate the bioactivity and potential synergism of terpenes and terpenoids when used in combination with cannabinoids and flavonoids. Consequently, this approach would result in more advanced commercial terpene- and terpenoid-based therapeutics in the market.

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

Medicinal plants as a source of natural remedies in the management of diabetes

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Abstract

Diabetes is a severe chronic illness that has impacted thousands of individuals worldwide. It is caused by the body's failure to produce insulin or insufficient production of insulin. While diabetes is not curable, it can be managed with injectable insulin, which decreases blood glucose levels. However, this treatment has several disadvantages that can affect a patient's health, and it is often unaffordable for some individuals. Previous studies have suggested that phytochemicals can improve insulin sensitivity. Due to the presence of therapeutic phytochemicals in natural plants, medicinal plants emerge as potential candidates for treating diabetes. In addition, compared to conventional diabetes treatments, phytochemical treatment may be affordable for all diabetics and has fewer side effects. This review primarily focuses on the symptoms and treatment options for the four known types of diabetes: type 1 diabetes mellitus, type 2 diabetes mellitus, type 3c diabetes mellitus, and neonatal diabetes. The article reviews medicinal plants that have been used to treat diabetes effectively with minimum side effects, such as *Momordica charantia* L., *Syzygium cumini* (L.) Skeels, and *Ocimum tenuiflorum* L., among others. In addition, some newly approved drugs, such as tirzepatide, sergliflozin, saxagliptin, and liraglutide, recommended for treating patients suffering from various forms of diabetes, are discussed.

Keywords: Diabetes; Symptoms; Treatment; Medicinal plants; Phytochemicals

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1. Introduction

There has been a startling rise in diabetes cases on a global scale in developing countries. Statistically, 537 million people had diabetes worldwide as of 2021, with about 10.5% of adults affected by diabetes mellitus (DM).[1] DM was recognized and explained by ancient Egyptians; the word "diabetes" is derived from the Greek word meaning "passer-by siphon," and "mellitus" means "sweet." DM is a chronic illness that occurs when blood glucose levels are high in the body, causing damage to internal organs such as nerves, kidneys, and blood vessels over a prolonged period.

The primary energy source in the human body, glucose, is obtained from food, specifically from proteins, lipids, and carbohydrates.[2] Blood glucose concentration is

regulated by several hormones, most of which increase glucose levels in circulation. Hormones such as glucagon, adrenaline, somatotropin, and cortisol are known to increase blood glucose levels. The endocrine gland secretes insulin, an endocrine hormone that helps food-derived glucose enters cells to be used as an energy source.[3] Typically, individuals develop diabetes when their body produces insufficient hypoglycemic agents or fails to use them effectively. As a result, glucose does not enter the cells and remains in the blood, increasing its concentration.[4]

The pancreas produces insulin, which helps regulate blood sugar. Insulin is produced by a group of small cells in the pancreas called the "islets of Langerhans". When blood sugar levels are too high, the islets release insulin, allowing glucose to enter cells.[5] The liver releases glucose into the bloodstream after being stimulated by glucagon to transform stored glucose (glycogen) into a usable form through a process called glycogenolysis. Several pathogenesis pathways are involved in the development of diabetes, including the destruction of beta-cells (β-cells) in the pancreas, where insulin is produced, and anomalies that lead to insulin resistance.[6] Diabetes has long-term complications, such as loss of vision, peripheral neuropathy with a risk of foot ulcers, and renal failure.[7]

2. Classification of DM

Diabetes is classified into several types, mainly type 1 DM (T1DM), type 2 DM (T2DM), and gestational DM (GDM). The terms "insulin-dependent" (describing T1DM, which requires external insulin to manage) and "non-insulin-dependent" (describing T2DM, which may be managed without insulin but can develop into insulin dependence) are used to classify the pathophysiological conditions of diabetes.[8]

2.1. T1DM

T1DM is common in children and young adults. It occurs due to auto-immune destruction of insulin-producing β-cells in the pancreas, leading to insulin deficiency resulting from the destruction of β-cells in the pancreas.[9] T1DM is sometimes referred to as insulin-dependent diabetes because the body is unable to produce sufficient insulin. T1DM is less common than T2DM, accounting for approximately 10% of all diabetes cases. Patients with T1DM require daily insulin injections to survive,[10] follow a specialized diet, and regularly monitor their blood glucose levels.

2.2. T2DM

T2DM, also known as adult-onset diabetes, is the most common form of diabetes, usually affecting middle-aged and elderly individuals, as well as those who lack exercise. In addition, overweight, obese individuals, and those with visceral fat are at greater risk of developing T2DM. T2DM accounts for about 90% of all diabetic cases worldwide. In T2DM, the body either produces insufficient insulin or uses it improperly,[11] or the body's muscle, fat, and liver cells do not respond to insulin due to insulin resistance. As a result, blood sugar cannot enter the cells to be stored and builds up in the blood. T2DM symptoms can be managed through exercise, weight loss, and a specialized healthy diet.[12] However, T2DM is a progressive disease that gradually gets worse, potentially requiring insulin therapy.

2.3. GDM

The third form of diabetes, GDM, usually occurs in the second or third trimester of pregnancy. This type of diabetes occurs only during pregnancy and can often be resolved after the baby is born, although it might develop into T2DM later in life.[13] Changes in hormones and weight gain during pregnancy can impair insulin functioning, resulting in high blood glucose levels. Although GDM usually disappears after pregnancy, most women who have had it have a 40 – 60% chance of developing T2DM within 5 – 10 years.[14]

2.4. Maturity onset diabetes of the young (MODY)

MODY is a non-autoimmune, monogenic form of diabetes characterized by the destruction of pancreatic β-cells and perturbed insulin biosynthesis.[15,16] It differs from T1DM and T2DM but is frequently misdiagnosed as one of these types. MODY is caused by a single gene mutation. Its diagnostic criteria include onset between adolescence and early adulthood (below 25 years), a familial background of autosomal dominant diabetes across a minimum of two generations, and β-cell dysfunction with no autoantibodies.[15-17] Assessment methods introduced in the 1990s identified mutations associated with MODY, including *HNF1B*, *HNF1A*, *GCK*, and *HNF4A*.[15] In MODY, disruption in insulin production results in hyperglycemia, which may damage blood vessels, eyes, nerves, and kidneys.[16]

2.5. Neonatal diabetes (NDM)

NDM, also termed congenital diabetes, is a rare cause of hyperglycemia caused by mutations in proteins essential for the normal functioning of pancreatic β-cells. NDM typically occurs before the age of 6 months, although it can rarely be present between 6 months and 1 year.[18,19] NDM arises through two primary mechanisms: the abnormal functionality of existing pancreatic β-cells or anomalies in the pancreas affecting the survival of insulin-secreting cells. The major genetic causes of NDM

involving abnormal β-cell function are mutations in the genes *KCNJ11* or *ABCC8*,[18,20] which code for the potassium channel in pancreatic β-cells, and anomalies in the 6q24 locus.[18] Patients with mutations in *KCNJ11* or *ABCC8*, as well as 6q24 abnormalities, are compliant with a successful switch from insulin injection (i.e., initially used as a mode of treatment) to oral sulfonylureas.[18,20]

2.6. Type 3c DM

Type 3c DM, also known as pancreatogenic diabetes or pancreatic DM, results from conditions affecting the exocrine pancreas, such as pancreatic cancer, chronic pancreatitis, and cystic fibrosis.[21] Due to its association with pancreatic diseases, patients with T3cDM often experience pancreatic exocrine insufficiency, leading to fat malabsorption and undernutrition.[21] The pathogenesis of T3cDM is due to decreased insulin production, facilitated by both the reduction in the functional capacity of the islets and a decrease in their number due to extensive sclerosis and fibrosis.[22] Similar to T1DM and T2DM, controlling hyperglycemia remains the primary target for minimizing the risk of macrovascular and microvascular complications.[22] However, there are few studies or randomized trials on the pharmacological treatment of T3cDM due to the unique and variable clinical and metabolic characteristics of these patients.[21,22]

2.7. Latent autoimmune diabetes in adults (LADA)

LADA is a subtype of T1DM that appears to bridge the gap between T1DM and T2DM. LADA is characterized by a slow progression of autoimmune diabetes and exhibits immunological markers typical of T1DM but does not necessarily require insulin treatment upon diagnosis.[23] Sometimes referred to as type 1.5 diabetes, LADA displays some characteristics closer to T1DM, while others are closer to T2DM.[24] The diagnosis of LADA is more commonly made in secondary care settings compared to primary care. The critical diagnostic criteria for LADA include adult onset (>30 years old), the presence of autoantibodies associated with diabetes, and no immediate requirement for insulin therapy post-diagnosis.[23,24] LADA patients retain functioning β-cells; therefore, it is imperative to initiate therapeutic strategies aimed at improving metabolic control while preserving the insulin-secreting capacity.[24]

3. Symptoms and treatment of DM

3.1. Symptoms

The symptoms of DM may appear harmless when considered individually, which is the primary reason why people may have diabetes without being aware of the predicament. The symptoms of the different types of diabetes may vary and develop at different rates. For example, the symptoms of T1DM usually develop over a short period, while those of T2DM develop more slowly. In some individuals, no symptoms are present at all. In GDM, symptoms may or may not develop during pregnancy, necessitating testing for the condition.[25] In general, DM often presents asymptotically, but some significant symptoms that develop or indicate a potential diabetic patient include those listed in Table 1.

3.2. Treatment

There is currently no approved cure for DM; therefore, management and treatment of the disease are the primary courses of action. The treatment of DM is complex and involves a combination of exercise, pharmacotherapy, and nutritional therapy as interventions for successful disease management.[25] The primary goal of DM treatment is to maintain healthy blood glucose levels to prevent DM-related complications. Patients living with DM are encouraged to consume proteins, carbohydrates, and fats in appropriate nutritional proportions. This recommendation entails carbohydrate intake comprising 55 – 60% of total caloric intake, limiting fat intake to a maximum of 30%, and ensuring protein intake within 10–20% of total daily intake. The expected caloric intake is an average of 30 kcal/kg body weight.[27] As part of their treatment regimen, individuals with diabetes are advised to engage in at least 20 min of aerobic exercise per day (at least 150 min/week). This regimen of exercise helps in lowering blood glucose levels. Regular exercise also assists in regulating blood cholesterol levels, lowering blood pressure, and maintaining a healthy body weight.[25]

Pharmacotherapy treatment depends on the type of DM diagnosed. T1DM is primarily caused by the absence of insulin, so daily insulin injections or the use of an insulin pump is the recommended forms of treatment.[25] GDM also requires insulin administration as a mode of treatment. The initial insulin dosage is 0.5–1 unit/kg in individuals with T1DM. Several oral medications are available for managing T2DM (Table 2), while insulin injections may also be required. The choice of oral drug therapy is complex, and physicians rely on clinical judgment to determine the most effective combination of drugs for the patient. It is important to note that discretion is critically essential over a long period as the treatment of persistent, chronic diseases such as DM are ongoing and unrelenting, and the response to therapy may potentially change over time.[25-27]

While the oral medications listed in Table 2 are commonly used for managing diabetes, they often fail to completely control the condition and may exhibit

Table 1. Symptoms of diabetes mellitus (DM)

Symptoms	Description	References
Polyuria (frequent urination)	An increased glucose concentration from the blood accumulates in the kidneys and attracts large volumes of water, resulting in frequent urination (from 1.8 L in healthy people to over 3 L in those with DM).	25,26
Polydipsia (excessive thirst)	Loss of substantial amounts of water through excessive urination results in dehydration that, in turn, triggers a thirst that is difficult to satisfy.	25,26
Lethargy/Fatigue	If there is no hormone insulin or it is malfunctioning, there would be no glucose uptake into cells, resulting in listlessness and fatigue.	25,26
Polyphagia (intense hunger)	Large amounts of sugars in the blood are not used in the cells as an energy source due to the absence of insulin or its malfunction. The body's reaction is to try and seek energy, triggering the release of ghrelin and cortisol hormone (hunger hormones), and the patient experiences increased hunger.	25,26
Ketoacidosis	Due to the absence of sugar to fuel metabolism, the body turns to fats and proteins, and the breakdown of fats results in the generation of acetone (ketones) used in metabolism. Ketones lower blood pH (ketoacidosis) and are presented as a fruity smell on the breath, urine, and sweat, as well as Kussmaul breathing (increased breathing rate).	25,26

Table 2. Types of oral medications, their action, and the available drug(s) for diabetes mellitus

Type of oral medication	Action of drug	Available drug	References
Biguanides	Exhibits insulin-sensitizing properties in which it lowers basal and postprandial plasma glucose and increases glucose uptake by the periphery.	Metformin	25,26,28
Sulfonylureas	Stimulates insulin secretion by β-cells of the pancreas, leading to a drop in blood glucose level and a reduction of basal hepatic glucose secretion.	Gliclazide, glimepiride, glipizide, and glibenclamide	25,26,28
α-glucosidase inhibitors	Acts within the small intestines, inhibiting α-glucosidase enzyme, thereby delaying digestion/absorption of ingested carbohydrates and, in turn, lowering postprandial blood glucose.	Miglitol and acarbose	25,26,28
Thiazolidinediones	Targets insulin resistance, enhancing insulin sensitivity to tissues in muscle through activation of intracellular receptors.	Rosiglitazone and pioglitazone	25,26,28
Meglitinides	Increases insulin secretion by triggering pancreatic β-cells.	Netaglinid and repaglinide	25,26,28
Sodium-glucose co-transporter 2 inhibitors	Block glucose reabsorption in the kidneys and improves glucose excretion, in turn lowering blood glucose levels.	Canagliflozin, ipragliflozin, dapagliflozin, and empagliflozin	25,26,28
Dopamine D2 and its receptor agonist	Lowers resistance to insulin and improves insulin sensitivity.	Bromocriptine	25,26,28
Bile acid sequestrant	Develops glucose-lowering effect and improves glucose tolerance.	Colestimide and colesevelam	25,26,28

negative side effects. Therefore, ongoing research is aimed at developing improved treatments for DM.[29] Although the drugs that have been and are still being used have proven to be effective, novel classes of innovative targets for diabetic control and antihyperglycemic therapies have emerged to manage diabetes.[28] The United States Food and Drug Administration (FDA) approved tirzepatide, a new, unique, first-in-class medication for the treatment of T2DM, on May 13, 2022. The drug is also known as Mounjaro.[30] In the following subsections, tirzepatide and several other medications for T2DM are discussed in detail.

3.2.1. Tirzepatide

Tirzepatide is a dual incretin agonist that decreases blood sugar and promotes weight loss reduction more competently than the usual treatments used for T2DM.[30] After meal consumption, the gut secretes hormones known as incretins, which include glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic

polypeptide. Incretins trigger the liver to release stored glucose, which raises blood glucose levels and stimulates the pancreas to release insulin, a hormone that lowers blood glucose and blocks the hormone glucagon.[30]

3.2.2. Saxagliptin

In July 2009, the FDA authorized saxagliptin, which is generally recommended as an addition to diet and exercise to improve glycemic control in individuals with T2DM. One oral dose of 5 mg of saxagliptin is recommended once daily; this dosage produces broad absorption. Saxagliptin is mostly metabolized by the hepatic cytochrome P450 isoenzyme CYP3A4/5. It is eliminated by both metabolism and renal excretion.[31]

3.2.3. Sergliflozin

Sergliflozin, a sodium-glucose co-transporter 2 inhibitor, has shown dose-dependent urine glucose excretion in animal experiments. In clinical trials, a single oral dose of sergliflozin resulted in dose-dependent glycosuria during fasting and after meals but had no effect on the excretion of electrolytes in the urine. In addition, it significantly reduced weight in healthy obese adults compared to a placebo.[32] However, there have been a few mild side effects, such as headache, sore throat, and dyspepsia in diabetic patients. In terms of metabolism, it exhibits a pharmacokinetic profile comparable to that of remigliflozin. Consequently, phase II clinical trials for sergliflozin have halted despite the drug's encouraging profile when treating obese diabetic patients.

3.2.4. Liraglutide

As a GLP-1 counterpart, liraglutide is 97% similar to natural human GLP-1 except for a single amino acid substitution and a side chain of glutamate-linked fatty acids. When used to treat T2DM, it has demonstrated safety, effectiveness, and acceptability as a glucose-lowering medication.[33] Liraglutide reduces insulin resistance while lowering systolic blood pressure, body weight, visceral fat, and other cardiovascular risk factors. It also improves lipid profile. Liraglutide is a desirable alternative for the therapy of insulin resistance because of its positive effects on all aspects of the metabolic syndrome, also known as insulin resistance syndrome, with a low risk of hypoglycemia.[34] It has been demonstrated that liraglutide lowers triglyceride levels without changing the concentration of other lipids. The recommended initial dose is 0.6 mg, which can be increased to 1.2 mg or even 1.8 mg if necessary.[35]

4. Medicinal plants and their health benefits

Medicinal plants have garnered increasing attention from researchers and are reported as one of the natural sources with the potential to play an important role in the treatment or management of diabetes.[36] Approximately 410 medicinal plants have experimentally proven anti-diabetic activities. These medicinal plants contain important anti-diabetic properties without harmful side effects, and nowadays, many individuals from urban and rural areas depend on natural plants to treat various chronic diseases.[37] Tannins, alkaloids, flavonoids, and phenolics are rich sources found in medicinal plants, essential for improving the effectiveness of the pancreatic tissues by regulating the intestinal absorption of glucose and the secretion of insulin.[38] Therefore, medicinal plants can be used in the treatment of diabetes due to their therapeutically important phytochemicals present in natural plants. This treatment can also be more affordable for everyone suffering from diabetes, making medicinal plants the focus for the development of alternative therapies for DM.

The use of medicinal plants for healing and treatment of diseases dates back to ancient times. In antiquity, people turned to nature for remedies as a means of recovery from diseases. There exists a profound connection between the search for natural remedies and historical mandates, supported by ample evidence from various sources. Humanity's enduring struggle against disease has prompted the utilization of roots, seeds, fruits, stems, barks, and other plant parts as sources of medical drugs. This practice has heightened awareness of the use of medicinal plants.[39] To this day, traditional medicinal plant remedies continue to be the easily accessible and most affordable source of treatment within the health-care system, with ethnobotany serving as a rich resource for natural drug research and development in African and Asian countries.[40,41]

Plants, being living entities, possess the element of life, which is absent from artificial substances. They are capable of working in harmony with the physiological processes of the human body, supporting the normal operation of body systems and facilitating self-healing.[40] Most modern medicines are designed to suppress symptoms, and research has shown that these treatments are less effective and have more contraindications, making the use of traditional medicinal plants topical once again.[39]

A medicinal plant is any plant with one or more of its parts or organs containing substances that can be exploited for therapeutic purposes. They are also defined as any plants that are precursors in the synthesis of medicinal drugs for the treatment of various diseases.[42] Naturally, plants produce metabolites that are not food but are essential to their defense mechanism to protect themselves from harmful insects, pathogenic microbes, and unfavorable environmental changes.[42] These metabolites are responsible for the medicinal value of these plants and

exhibit physiological effects on people.[43] These metabolites are collectively known as phytochemicals (Figure 1).

Phytochemicals are naturally occurring chemical compounds that are biologically active and highly beneficial to human health. Based on their roles in plant metabolism, they are classified as primary metabolites (proteins, sugars, pyrimidines, and purines of nucleic acids, amino acids, and chlorophylls) and secondary metabolites (flavonoids, phytosterols, saponins, alkaloids, phenolics, terpenes, curcumins, glucosides, lignans, and anthraquinones) (Figure 2).[40,43,44] Phytochemicals can be found in fruits, vegetables, plant-based beverages, and cereal grains. These compounds accumulate in plant parts such as stems, roots, flowers, leaves, and seeds, and they are often concentrated in the outer layers of various plant tissues.[44] The levels of phytochemicals vary from plant to plant depending on growing conditions, variety, processing, and cooking. Due to the specific properties of phytochemicals, medicinal plants play indispensable roles in people's daily lives and serve as complements or potential substitutes for modern medical treatments.[40]

Phytochemicals can provide direct or indirect defensive mechanisms against harmful ailments or pathogens due to their anticarcinogenic, antimicrobial, antimutagenic, anti-inflammatory, antigenotoxic, anthelmintic, antioxidative, and antiproliferative properties (Figure 2).[43,44] The consumption of phytochemicals is associated with a decrease in risks of many different types of chronic diseases owing to their free radical scavenging effects and antioxidant properties. A recent review highlighted the potential roles of phytochemicals in increased blood flow and improved endothelial function, supporting the idea that plants containing phytochemicals may beneficially supplement the needs required by the human body.[44]

Phytochemicals offer diverse medical benefits across different health domains. For example, flavonoids and polyphenols exhibit anti-inflammatory and antioxidant properties that can accelerate the healing of wounds. Flavonoids and carotenoids also possess anti-cancer properties, inhibiting the growth of cancer cells by suppressing the formation of tumors and inducing apoptosis.[45] In oral health, phytochemicals found in plants, such as tea tree oil, contribute to oral health by exhibiting antimicrobial properties that inhibit the growth of bacteria that may potentially cause oral infections.[46] Certain phytochemicals, such as those in spices and herbs, possess neuroprotective properties. These phytochemicals can mitigate oxidative stress, nerve damage, or other conditions involving neuropathy. Phytochemicals, including quercetin and resveratrol, possess potential positive effects on metabolism, influencing insulin sensitivity, contributing to overall metabolic balance, and regulating blood sugar levels.[45]

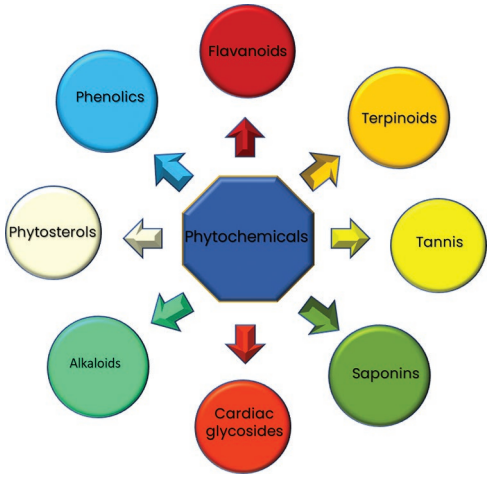

Figure 1. The main phytochemical components present in medicinal plants[43]

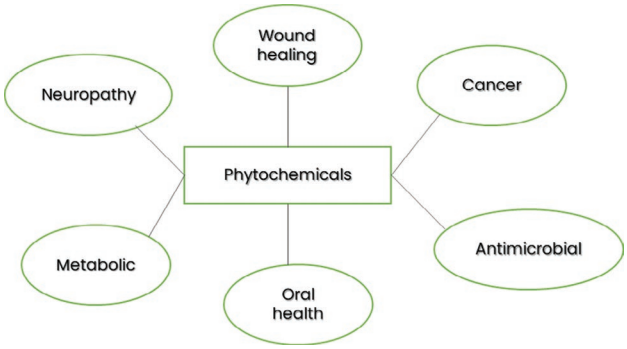

Figure 2. Medical benefits of phytochemicals from medicinal plants.[43] Phytochemicals derived from plants contribute to medical benefits due to their diverse bioactive properties, ranging from anti-inflammatory and antioxidant effects to neuroprotective and antimicrobial actions. These properties make phytochemicals valuable in promoting health and addressing various medical issues.

5. Medicinal plants with anti-diabetic activity

The use of medicinal plants for healing, treatment, and management of diseases has been extensively explored as an alternative with minimum side effects and lower cost. Here, we focus on medicinal plants readily accessible and known for their anti-diabetic properties with minimal side effects (Table 2).

5.1. *Cinnamomum verum*

Cinnamon, one of the most common spices used in our daily lives, contains active components, such as cinnamate, associated with numerous health benefits. Studies have demonstrated that cinnamon has a 20-fold higher insulin-potentiating effect

compared to many other spices.[47] In addition, cinnamon contains polyphenols that exhibit anti-diabetic properties, such as regulating glucose levels and repairing pancreatic β-cells responsible for insulin production.[48]

5.2. *Momordica charantia* L.

M. charantia L. has been used as an herbal medicine for years due to its biological properties, such as anti-diabetic, antioxidant, and anti-tumor effects. A study by Oguntibeju[49] on charantin-rich extract for T1DM and T2DM proved that charantin-rich extract increases plasma glucose tolerance. It was suggested that charantin-rich extract could improve insulin sensitivity in individuals with T2DM, rather than protect patients with T1DM from β-cell abnormalities.[50]

5.3. *Syzygium cumini* (L.) Skeels

The bark and the leaf extract of *S. cumini* have demonstrated the potential for significantly lowering blood glucose levels, as evidenced by the increased volume and size of the pancreatic islets observed in the treated diabetic rats.[47] In addition, the flavonoid-rich extract obtained from the seed of *S. cumini* has been associated with increased insulin sensitivity and a decrease in triglycerides and low-density lipoprotein (LDL) levels.[51]

5.4. *Ocimum tenuiflorum* L.

O. tenuiflorum, considered one of the holy and important medicinal herbs for diabetes treatment, has demonstrated that the oil extracted from its leaves significantly lowers blood glucose levels while concurrently increasing insulin levels.[52] Over an 8-week period, oral treatment of *Ocimum sanctum* aqueous extract in conjunction with diet significantly lowered serum lipid profiles, lipid peroxidation, and fasting blood glucose levels, while also improving glucose tolerance. Studies in rats found that ethanol, butanol, aqueous, and ethyl acetate fractions of *Ocimum sanctum* induced insulin secretion from the impaired rat pancreas and isolated rat islets, as well as from clonal pancreatic β-cells.[53]

5.5. *Zingiber officinale* Roscoe

Z. officinale, commonly known as ginger, is considered one of the most common species with several health benefits. Ginger rhizomes have been traditionally used to treat diabetes, fever, toothache, and muscular aches.[54] According to Gayar *et al.*,[55] taking a supplement of powdered ginger 3 times a day for 3 months can improve glycemic index and antioxidant activity in T2DM. In addition, a similar study by Ebrahimzadeh *et al.*[56] found that taking a ginger supplement twice a day can decrease insulin levels without any significant changes in fasting plasma glucose.

5.6. *Allium sativum*

The precursor of allium and garlic oil, S-allyl cysteine sulfoxide, has a higher level of molecular peroxidation regulation than glibenclamide and hormone due to the presence of amino alkanoic acid. A study conducted in 2021[57] revealed that juice and oil extracts from *A. sativum* reduced blood glucose levels by restoring insulin secretion from the pancreas cells.

5.7. *Coriandrum sativum*

In a review by Paari and Pari,[58] the stem and leaf extract of coriander demonstrated the ability to improve the antioxidant effects. Sobhani e*t al.*[59] found that the aqueous extract of coriander seeds can normalize decreasing blood glucose levels and increase insulin production from β-cells of pancreatic islets. Diabetic rats induced by streptozotocin demonstrated reduced serum glucose levels and increased insulin-releasing capacity of pancreatic islet β-cells when administered coriander seed ethanol extract (200 mg/kg body weight).[60]

5.8. *Aegle marmelos* (L.) Correa

The fruit of *A. marmelos* is commonly used in various traditional medicinal practices, such as laxatives and the treatment of dysentery, peptic ulcers, and chronic diarrhea.[61] The Tripuri tribe of North East India consumes the juice of this fruit daily on an empty stomach to treat gastrointestinal issues.[62] The leaf paste is also used to treat high malaria fever. In a study where *A. marmelos* fruit extract was administered orally, twice daily for 4 weeks, at a dose of 250 mg/kg body weight, a significant reduction in blood sugar levels was observed in streptozotocin-induced diabetic Wistar rats. In a complementary study,[63] the water-soluble fruit extract was found to protect the pancreatic system by partially reversing the damage caused to pancreatic islets by streptozotocin. It was discovered that rats with mild diabetes and sub-diabetes demonstrated enhanced glucose tolerance.[64] *A. marmelos* water-soluble fruit extract has been shown to restore the function of pancreatic β-cells and enhance insulin sensitivity by upregulating peroxisome proliferator-activated receptor-γ (PPAR-γ) expression.[65]

5.9. *Murraya koenigii* (L.) Spreng

In streptozotocin-induced diabetic rats, *Murraya koenigii* has been shown to significantly affect postprandial blood glucose levels and insulin sensitivity through koenidine, a metabolically stable alkaloid.[66] Insulin-resistant mice resulting from dexamethasone treatment showed improvements in insulin sensitivity and glucose

tolerance. Male albinos administered 150 mg/kg body weight of *M. koenigii* ethanolic extract orally for 30 days showed increases in insulin, C-peptide, hemoglobin, and protein levels, along with a decrease in blood glucose and glycosylated hemoglobin.[67,68] The inclusion of inorganic trace elements in *M. koenigii* leaf extracts may be beneficial in managing glucose tolerance disorders and may play an indirect role in the management of DM. These trace elements include zinc, copper, chromium-vanadium, nickel, iron, sodium, and potassium.

5.10. *Phyllanthus emblica*

P. emblica's high polyphenol content, mostly composed of tannin and flavonoid components, provides the majority of its health advantages. This plant is known to help control blood sugar levels and enhance glucose metabolism, in addition to possessing a variety of anti-inflammatory and anti-cancer effects.[69] In addition, it stimulates insulin production in the pancreas and the growth of β-cells. Diabetic participants who consumed a daily dose of 3 g of *P. emblica* powder demonstrated a significant reduction in total lipids and LDL cholesterol levels, accompanied by an improvement in high-density lipoprotein cholesterol.[70] In Wistar rats with T1DM, an improvement in hyperglycemia, oxidative stress, and hyperlipidemia was observed when they were given 1 ml of fruit juice per kg body weight orally every day for 8 weeks.[70] Furthermore, the cardiovascular function of streptozotocin-induced diabetic Wistar rats was improved by strengthening the antioxidant defense systems in the cardiac tissues.[71] Studies have shown that when the aqueous fruit extract of this plant is administered intraperitoneally to diabetic rats induced with alloxan, blood glucose levels significantly decrease. In addition, the extract induces hypotriglyceridemia in diabetic rats by lowering triglyceride levels. The aqueous extract was found to improve liver functioning by normalization of the activity of alanine transaminase, a liver-specific enzyme.[72] Some of the medicinal plants are listed in Table 3 with their main phytochemical components.

6. Anti-diabetic mechanism of natural phenolic compounds

Non-flavonoid phenolics consist of two basic carbon frameworks: hydroxycinnamic and hydroxybenzoic.[73] These structures can be divided into four groups: phenolic acids, quinines, stilbenes, and coumarins. Phenolic compounds have been investigated for their antimicrobial properties as well as their nutritional value and potential anti-diabetic effects. Some studies have suggested that phenolics offer nutritional benefits and could aid in managing diabetes.[74] However, further research is required to validate these findings, especially involving human subjects, as there is limited information about the benefits of pure compounds or diets enriched with phenolics. Cinnamic acid has been shown to enhance the activity of several enzymes, including lipase and angiotensin-converting enzyme, and to reduce the risk of heart and arterial issues in rats fed high-fat diets.[74]

Furthermore, cinnamic acid has been demonstrated to reduce triglyceride and cholesterol levels in rats on a high-cholesterol diet by blocking the actions of acyl-coenzyme A: cholesterol acyltransferases and β-hydroxy β-methylglutaryl-CoA reductase. In addition, it has been discovered to increase AMP-activated protein kinase phosphorylation and adiponectin production,

Table 3. Medicinal plants with anti-diabetic activity

Plant name	Useful plant part(s)	Main phytochemical components	Activity	References
Annona squamosa L.	Leaves and fruit	Flavonoids; alkaloids	Anti-diabetic	78,79
Cocos nucifera L.	Coconut water and drupe	Polyphenols; flavonoids; fatty acids	Antiglycation	80
Curcuma longa L.	Rhizome	Turmerones; curcuminoids	Antioxidant and anti-diabetic	81
Embelia ribes Burm. F.	Berries	Quinone derivatives; embelin	Anti-diabetic	82
Mangifera indica L.	Leaves and fruit	Polyphenols; flavonoids; mangiferin	Anti-diabetic	83
Olea europaea L.	Leaves	Flavonoids; hydroxytyrosol	Anti-hyperglycemic	84
Swertia chirayita (Roxb.)	Whole plant	Xanthones; amarogentin	Anti-diabetic	85
Punica granatum L.	Leaves and fruit	Ellagic acid; anthocyanins	Anti-diabetic	86
Phyllanthus niruri	Aerial parts	Flavonoids; phyllanthin	Anti-diabetic	87
Memecylon umbellatum Burm. F.	Leaves	Ursolic acid; flavonoids, betulinic acid	Anti-diabetic	88

enhancing insulin sensitivity to a degree similar to that of the conventional drug glibenclamide.[75] Interestingly, hydroxycinnamic acid, a derivative of cinnamic acid, has been demonstrated to inhibit protein tyrosine phosphatase 1B, a major negative regulator of the insulin signaling pathway. Therefore, cinnamic acid appears to possess anti-diabetic, anti-hypertensive, and anti-obesity properties.[76]

Gallic acid may have anti-diabetic effects, as suggested by research on its effects on adipocyte differentiation and adiponectin secretion.[77] It has been shown to increase the expression of PPAR-γ target proteins and fatty acid binding protein-4, both associated with adipocyte differentiation. In addition, it exhibits antioxidant effects that may be beneficial in reducing oxidative stress and depressive symptoms.

7. Conclusion and future perspectives

The increasing prevalence of diabetes highlights the urgent need for efficient treatment. Medicinal plants have been proposed as potential therapeutic agents for various types of diabetes, supported by both conceptual and empirical data. Clinical investigations have validated the use of phytochemicals derived from naturally occurring plants in the treatment of diabetes. This review summarizes certain medicinal plants with known anti-diabetic properties, proposing that further research is desperately needed to explore additional natural plants with potential anti-diabetic properties and to identify new avenues for future research.

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Conflict of interest

The authors declare that they have no competing interests.

Ethics approval and consent to participate

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Consent for publication

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Availability of data

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

Criteria for developing active cellular targeting miRNA oligonucleotide therapeutics with a peptide nucleic acid backbone: Combating cardiometabolic pandemics

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Abstract

Oligonucleotide therapeutics (ONTs) represent a growing new class of therapeutic agents aimed at addressing chronic diseases that remain untreatable by small molecules and antibodies. Our goal was to establish a selection of several criteria to design and develop miRNA-based ONTs, focusing on improved chemistry, pharmacokinetics/pharmacodynamics (PK/PD) profiles, and safety characteristics to combat cardiometabolic pandemics. By leveraging our own experimental data obtained from experiments involving miR-22-3p antagomirs and a careful review of the literature, we established a set of seven criteria to optimize the design of miRNA ONTs. These criteria prioritize simplified drug synthesis, optimized PK/PD properties, and reduced potential toxicities. This proposed set of seven criteria represents a novel strategy for developing active cellular targeting miRNA ONTs for various therapeutic indications.

Keywords: MiRNAs; Antagomirs; Oligonucleotide therapeutics; Active cellular targeted delivery; Cardio-metabolic pandemics

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1. Introduction

Twenty-five years after the initial discovery of RNA interference by Fire *et al.*,[1] innovations in RNA chemistry and delivery strategies have facilitated the design and advancement of RNA-based therapies.[2-4] Oligonucleotide therapeutics (ONTs) exhibit the ability to target almost any gene, including those associated with so-called "un-druggable diseases"—conditions where genes are implicated beyond the reach of small molecules and antibodies.[5] RNA-based ONTs are distinct from small molecules and protein drugs in several aspects,[6] particularly their mechanisms of action and clinical pharmacology features. This distinction is especially notable in ONT conjugates designed for specific tissue delivery.[7,8]

The different classes and characteristics of ONTs currently being developed are shown in Table 1.

Nineteen ONTs, mainly antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs), have been approved by regulatory authorities for the treatment of patients (Table 2).[9-12]

Table 1. Characteristics of the classes of oligonucleotide therapeutics

Class	Structure	Target	Site of action	Mechanism of action
Anti-sense oligonucleotides • Gapmers • Splice-switching	Single-stranded molecule	mRNA and pre-mRNA	Nucleus and cytoplasm	Gapmer: RNA degradation by RNase H and splice site switch
Small interfering RNAs	Double-stranded molecule	mRNA	Cytoplasm	mRNA degradation
miRNA antagomirs	Single-stranded molecule	miRNA	Cytoplasm	Steric inhibition
miRNA agomirs	Double-stranded molecule	mRNAs	Cytoplasm	Modulation of mRNA targets
Aptamers	Single-stranded molecule	Extracellular protein and cell surface	Extracellular site	Inhibition of protein function

Chemical modifications of ONTs have been introduced to improve the "druggability" of ONT candidates. Most of the ONT drugs currently approved for human use include (i) "first generation," which involves backbone modifications (mainly phosphorothioate [PS]) and (ii) "second generation," which involves nucleobase and sugar modifications (2'-fluoro [2'F], 2'O-methyl [2'-OMe], 2'-methoxyethyl [2'-MOE]), cytosine methylated at position 5 [m^5C], gapmers, 5'-phosphate stabilization, as well as backbone modifications (locked nucleic acid [LNA], phosphorodiamidate morpholino oligomers [PMO], and glycol nucleic acid).[9,13-15] These chemical modifications were introduced to:

(i) Improve compound stability, bioavailability, and efficacy
(ii) Avoid off-target toxicity
(iii) Elude innate immune response.

To enhance the efficacy, safety profile, and therapeutic index of ONTs, generation 2.5 ONTs (also referred to as third-generation ONTs) were developed. They include various delivery formulations (cholesterol, liposomes, lipids, nanoparticles, polymers, cell-penetrating peptides, and antibody conjugates)[16,17] and/or a cellular targeting moiety such as N-acetyl-galactosamine (GalNac) for specific delivery to the liver at a much-reduced effective dose.[18]

Our ultimate objective is to develop safe and effective therapies with reduced cost of synthesis and improved patient compliance, two key factors in prevalent and chronic diseases.

2. MiRNAs

Mature miRNAs are conserved 18 – 24 nucleotides long non-coding RNAs that post-transcriptionally regulate gene functions through direct degradation of their target messenger RNAs (mRNAs) and/or translational repression.[19] MiRNAs are stable, usually cell-type specific, easily measured in body fluids and tissues, and involved in exosomal cell-to-cell communications.[20-24]

MiRNAs serve as useful diagnostic, prognostic, and therapeutic markers for many human diseases,[25-30] including cancers.[31-34] Furthermore, miRNAs are relevant modulators of disease pathogenesis and valuable molecular therapeutic targets.[35,36] Due to their specific expression patterns and their ability to target multiple transcripts through a one-to-multiple pattern, miRNA modulators (known as agomirs and antagomirs) can simultaneously alter the expression of several genes and proteins involved in complex diseases that cannot be adequately treated by small-molecule drugs.[37]

3. MiRNA antagomirs

MiRNA antagomirs (or anti-miRs) are synthetic, single-stranded ONTs that bind by Watson–Crick base-pairing to their complementary miRNAs, preventing these miRNAs from modulating their target mRNAs (Figure 1). Antagomirs do not induce RNAse H-mediated target RNA degradation. The risk of off-target effects of antagomirs is low due to their high binding specificity and affinity.[13] Compared to other ONTs, the mechanism of action of antagomirs is straightforward (steric blockade) and localized into the cytoplasm (Figure 1).

Various agomirs and antagomirs are in pre-clinical development, especially for oncology indications (miR-10b, miR-21, miR-29, miR-34a, miR-221, and miR-710).[2,36,38-42] MiRNA-based therapies that have reached clinical trials target miR10b, miR-16, miR-17, miR-21, miR-34a, miR-92a, miR-103/107, miR-122, miR-132, miR-155, and miR-200a/c.[36,40] Several miRNA-based ONTs have entered clinical development (Table 3).[37,43-46]

4. Selection of structural and chemical criteria for new generation 2.5 antagomirs

We propose a set of seven chemical modifications to optimize the properties of miRNA antagomirs as therapeutic agents for prevalent and chronic diseases (Table 4).

Table 2. Oligonucleotide therapeutics approved by regulatory authorities

Year of approval	Compound (Trade name)	Category	Chemical changes	Coupling agent	Route of administration	Indication
1998	Fomivirsen (VITRAVENE®)	21-mer ASO	PS, DNA	-	Intravitreal	CMV retinitis
2004	Pegaptanib (MACUGEN®)	27-mer Aptamer	2'-OH, 2'-F, 2'-OMe, DNA	PEG	Intravitreal	Neovascular AMD
2013	Mipomersen (KYNAMRO®)	20-gapmer ASO	PS, 2'-MOE, m^5C	-	Subcutaneous	Hypercholesterolemia
2016	Eteplirsen (EXONDYS 51®)	30-mer SSO	2'MOE, PMO	-	Intra-venous	Duchenne muscular dystrophy
2016	Nusinersen (SPINRAZA®)	18-mer SSO	PS, 2'-MOE, m^5C	-	Intrathecal	Spinal muscular atrophy
2018	Patisiran (ONPATTRO®)	21-mer/21-mer siRNA	2'-OH, 2'-OMe	Liposomal	Intravenous	Hereditary transthyretin amyloidosis (ATTR amyloidosis)
2018	Inotersen (TEGSEDI®)	20-gapmer ASO	PS, 2'-MOE, m^5C	-	Subcutaneous	Hereditary ATTR amyloidosis
2019	Givosiran (GIVLAARI®)	21-mer/23-mer siRNA	PS, 2'-OMe, 2'-F	3 GalNAc residues	Intravenous	Acute hepatic porphyria
2019	Golodirsen (VYONDUS 53®)	25-mer SSO	2'-MOE, PMO	-	Intravenous	Duchenne muscular dystrophy
2019	Volanesorsen (WAYLIVRA®)	20-gapmer ASO	PS, 2'-MOE m^5C	-	Subcutaneous	Familial chylomicronemia syndrome
2020	Lumasiran (OXLUMO®)	21-mer/23-mer siRNA	PS, 2'-OMe, 2'-F	3 GalNAc residues	Subcutaneous	Primary hyperoxaluria
2020	Inclisiran (LEQVIO®)	21-mer/23-mer siRNA	PS, 2'-OMe, 2'-F	3 GalNAc residues	Subcutaneous	Hyper cholesterolemia
2020	Viltolarsen (VILTEPSO®)	21-mer SSO	2'-OMe, PMO	-	Intravenous	Duchenne muscular dystrophy
2021	Casimersen (AMONDYS 45®)	22-mer SSO	PMO	-	Intravenous	Duchenne muscular dystrophy
2022	Vultrisiran (AMVUTTRA®)	21-mer/23-mer siRNA	PS, 2'-F, 2'-OMe	3 GalNAc residues	Subcutaneous	Hereditary ATTR amyloidosis
2023	Eplontersen (WAINUA®)	20-gapmer ASO	PS, 2'-MOE, m^5C	1 trivalent GalNAc residue	Subcutaneous	Transthyretin-mediated amyloidosis
2023	Nedosiran (RIVFLOZA®)	22-mer/36-mer siRNA	PS, 2'-F, 2'-OMe	4 GalNAc residues	Subcutaneous	Primary hyperoxaluria
2023	Tofersen (QALSODY®)	20-gapmer ASO	PS, 2'-MOE, m^5C	-	Intrathecal	Amyotrophic lateral sclerosis with superoxide dismutase (*SOD1*) gene mutation
2023	Avacincaptad pegol (IZERVAY®)	39-mer RNA Aptamer	2'-OMe, 2'-F, inverted thymidine to cap 3' end	2 PEG chains	Intravitreal	Age-related macular degeneration

Abbreviations: ASO: Antisense oligonucleotide; AMD: Age-related macular degeneration; CMV: Cytomegalovirus; GalNAc: N-acetyl-D-galactosamine; m^5C: Cytosine methylated at position 5; PEG: Polyethylene glycol; PMO: Phosphorodiamidate morpholino oligomers; PS: Phosphorothioate; siRNA: Small interfering RNA; SSO: Single stranded oligonucleotide; 2'-F: 2'-Fluoro; 2'-MOE: 2'-methoxyethyl; 2'-OH: 2'-hydroxyl; 2'-OMe: 2'-O-methyl; 2'-MOE: 2'-Methoxyethyl.

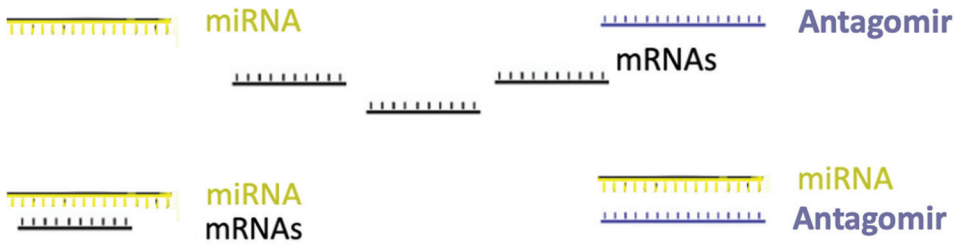

Figure 1. Mechanism of action of miRNA antagomirs. No binding of miRNA to complementary mRNAs in the presence of antagomir.

Table 3. MiRNA-based oligonucleotide therapeutics that have entered clinical development

Product	Sponsor	MiRNA	Stage of development	Indication
Miravirsen (SPC36490)	Roche/Santaris	MiR-122-5p antagomir	Phase II; NCT01200420; discontinued for strategic reasons	Hepatitis C
CDR132L	Cardior Pharmaceuticals	MiR-132-3p antagomir	Phase II; NCT05350969	MI and AHF
Lademirsen (SAR339375/ RG-012)	Sanofi/Genzyme	MiR-21 antagomir	Phase II; NCT03373786; NCT02855268	Alport nephropathy
RG-125 (AZD4076)	Astra-Zeneca	GalNAc-conjugated miR103/107 antagomir	Phase I/IIa; NCT02612662; NCT02826525; returned to Regulus	NAFLD/NASH
RGLS4326	Regulus Therapeutics	MiR-17-5p antagomir	Phase Ib; NCT04536688	PKD
RGLS8429	Regulus Therapeutics	GalNAc-conjugated miR17 Antagomir	Phase1b; NCT06621191	ADPKD
TargomiRs	Asbestos Disease Research Foundation	Nanoparticles delivered by miR-16 mimic	Phase I; NCT02369198	MPM and NSCLC
MRX34	Mirna Therapeutics	Liposomal miR-34a-5p mimic	Phase I; NCT018229971; terminated for immune-related SAE	Various cancers
Cobomarsen (MRG-106)	Miragen	MiR-155-5p antagomir (received Orphan Drug Product designation by the US FDA)	Phase I; NCT02580552; Phase II; NCT03713320	Cutaneous T-cell lymphoma
MRG-110 (S95010)	Miragen	MiR-92a-3p antagomir	Phase I; NCT03603431; NCT03494712	Wound healing
Remlarsen (MRG-201)	Miragen	Cholesterol-conjugated miR-29 mimic	Phase II; NCT026033224; NCT03601052	Keloid and scar formation

Abbreviations: ADPKD: Autosomal dominant polycystic kidney disease; AHF: Acute heart failure; US FDA: United States Food and Drug Administration; GalNAc: N-acetyl-D-galactosamine; MI: Myocardial infarction; MPM: Malignant pleural mesothelioma; NAFLD/NASH: Nonalcoholic fatty liver disease/nonalcoholic steatohepatitis; NSCLC: Non-small cell lung cancer; PKD: Polycystic kidney disease; SAE: Serious adverse event.

4.1. Criterion 1: Select a specific miRNA target that is unique, universal, phylogenetically conserved, and significantly expressed in the tissues and organs of interest

MiRNAs are present across the animal kingdom and are usually phylogenetically conserved.[47] About 2,000 human miRNAs have been identified and experimentally validated.[48,49] Furthermore, most miRNAs exhibit physiological effects at high expression levels in tissues and organs. Some of them belong to families (e.g., let-7 family of 15 miRNAs, miR-1-133 family, miR-34 family, miR-103/107 family, miR-17-92 cluster),[50,51] while others are unique. If one were to develop a specific miRNA antagomir, it would be intuitive to choose a miRNA target that is unique instead of a member of a miRNA family to avoid the risk of so-called on/off-target effects.[52]

To design generation 2.5 pharmacologic antagonists of mature miRNA guide strands, we used miR-22-3p as a therapeutic target for treating cardio-metabolic diseases.[53,54] The miR-22-3p nucleotide sequence is highly conserved across vertebrate species, including mice, rats, dogs, pigs, and primates (relevant information can be obtained from www.targetscan.org), suggesting that experimental data could be extrapolated from one species to another. We examined the expression of hsa-miR-22-3p across human tissues and organs using the TissueAtlas2

Table 4. Criteria selected to develop active cellular targeting miRNA ONTs

Criteria	Description
1	Select a specific miRNA target that is unique, universal, phylogenetically conserved, and significantly expressed in the tissues and organs of interest to optimize the benefit/risk ratio.
2	Replace the chemical modifications (phosphorothioate and locked nucleic acid) utilized in generations 1 and 2 compounds with a gamma peptide nucleic acid backbone to avoid potential toxicities linked to specific chemical groups.
3	Optimize the backbone and nucleoside modifications to increase resistance to nucleases and proteases/peptidases, improving the ONT PK/PD profile.
4	Avoid chirality to increase the ONT purity, stability, PK/PD profile, and safety.
5	Limit binding to serum proteins to favor uptake by the tissues and organs of interest before clearance/elimination by the liver and kidneys.
6	Optimize and simplify chemical synthesis to increase drug purity and reduce the cost of goods.
7	Conjugate the ONT to a fatty acid or a short peptide for enhanced and active targeted delivery to the tissues and organs of interest, delivering a much-reduced therapeutic dose with an extended mean residence time.

Abbreviations: PK/PD: Pharmacokinetic/pharmacodynamics; ONTs: Oligonucleotide therapeutics.

program.[55] The tissue specificity index (TSI) gives each non-coding RNA a value from 0 to 1, where 1 indicates that the RNA is detected in only one specific tissue and 0 indicates it is detected in all tissues. While miRNAs show a broad range of TSI values, the TSI for hsa-miR-22-3p is 0.855, signifying an elevated tissue-specific expression pattern, specifically in the adipocytes, myocardium, and skeletal muscle.[56]

We implemented a small throughput-high yield R and D strategy[56] as an alternative to the classic high throughput screening strategy implemented by pharmaceutical companies to find "hit compounds" from libraries of millions of samples. Several *in silico*, *in vitro*, and *in vivo* tools, we reported that miR-22-3p (a unique miRNA evolutionarily conserved from flies to mammals) modulates several metabolic genes involved in lipid oxidation, mitochondrial activity, energy expenditure, fat accumulation, inflammation, and necrosis.[53,54] In animal studies, we demonstrated that miR-22-3p antagomirs with first- and second-generation chemical modifications produced several metabolic improvements: Reduction of fat mass without change of lean mass while food intake remained unchanged, improvement of glucose, cholesterol, insulin sensitivity, and a marked reduction of liver steatosis.

In addition to our work, several other investigators have independently confirmed the metabolic roles of miR-22. Yang *et al.*[57,p.322] concluded that "miR-22 inhibitors may have potential as candidate drugs for nonalcoholic fatty liver disease (NAFLD) and obesity." Kaur *et al.*[58] reported abnormally high levels of miR-22-3p in the livers of *db/db* mouse models of insulin resistance and Type 2 diabetes. They showed that silencing miR-22-3p by antagomir administration lowered random and fasting glucose in diabetic mice and improved glucose tolerance and insulin sensitivity. Hu *et al.*[59,p2] concluded that "reducing miR-22 enhances hepatic fibroblast growth factor 21 and activates AMP-activated protein kinase, which could be a novel approach to treat steatosis and insulin resistance." In a more recent study, Castano *et al.*[60] reported that elevated circulating miR-22-3p is a relevant biomarker for the stratification of patients with metabolic dysfunction-associated fatty liver disease (MAFLD). These authors suggested that elevated circulating miR-22-3p levels may be used to identify patients with a high fatty liver index. Panella *et al.*[61] used knockout and transgenic mouse models to report that miR-22 loss-of-function protects against obesity and hepatic steatosis, while its overexpression promotes both phenotypes even when mice are fed a regular chow diet. These studies included the use of first- and second-generation antagomirs. A biotechnology company (www.resalistherapeutics.com) is developing LNA/DNA "mixmer" miR-22 antagomirs for the treatment of obesity. One of their candidates is conjugated to GalNAc to treat metabolic dysfunction associated with steatohepatitis.

4.2. Criterion 2: Avoid potential toxicities by replacing PS and LNA chemical modifications utilized in generations 1 and 2 compounds with a gamma peptide nucleic acid (PNA) backbone

The first generation of ONTs, already advanced into clinical trials, incorporated several medicinal chemistry modifications, including:[62-64]

(i) PS backbone modifications, which were used to diminish nuclease degradation and augment plasma protein binding to promote tissue uptake.
(ii) Gapmer oligodeoxynucleotides, which cause RNase H cleavage of the target RNA.
(iii) Ribose modifications (mainly 2' position modifications, e.g., 2'-F, 2'-OMe, 2′-MOE, and 2' – 4' LNA), which were used to increase stability and specificity.

A panel of experts (DARTER group [https://antisenserna.eu/]) recently published considerations in the preclinical assessment of the safety of ASOs.[65] Based on animal and human data, they suggested that blood platelets and complement activation should be examined for PS-ASOs that renal and hepatic toxicities should be assessed for LNA gapmers. Therefore, alternative chemical

modifications that are not hampered by these potential toxicities are needed.

PNAs were discovered by Nielsen[66] and Nielsen *et al.*[67] in 1991. PNAs are synthetic DNA mimics with the deoxyribose phosphate backbone replaced by N-(2-aminoethyl)-glycine units (Figure 2). PNA molecules combine the properties of both peptides and nucleic acids. The neutral pseudopeptide backbone of PNAs enhances binding affinity, improves biological stability, and prolongs half-life.[68] Nucleobases (adenine, guanine, cytosine, and thymine/uridine) are attached to the backbone through a carboxy methylene linkage. The chemical properties of PNA ensure a similar distance between nucleobases, allowing tight hybridization with their complementary nucleic acid targets through Watson–Crick base-pairing. A given PNA recognizes its complementary nucleic acid strand with high affinity and excellent specificity, surpassing those of natural nucleic acids and their analogs.[69] Unlike gapmer ASOs, PNA–RNA hybrids are not targets for RNase H1 enzyme degradation.

The initial PNA structure included a neutral and achiral backbone, which is hampered by poor cellular penetration. Consequently, several chemical modifications (α-PNAs, β-PNAs, and γ-PNAs) were implemented to improve their hybridization properties and cellular penetration (Figure 3).

Gamma backbone modifications convert a randomly folded PNA into a helical structure.[70,71] The introduction of an L-serine side chain at the γ-position produces a right-handed helix PNA and enhances hybridization with its target. Regarding the risks of on/off-target effects, a PNA–RNA heteroduplex is more stable and more susceptible to mismatches than a PNA–DNA heteroduplex. A single base-pair mismatch in a PNA–RNA heteroduplex significantly diminishes the thermal melting temperature by approximately 12°C. PNAs require only 9 – 12 monomers to effectively discriminate and efficiently bind to their specific targets.[72] For reference, a 17-mer oligonucleotide sequence is expected to occur only once in the human genome.[9] Therefore, a 17 – 22-mer PNA ONT would not trigger on/off-target effects.

Mechanistically, PNA antagomirs bind to their target miRNAs by Watson–Crick base pairing, consequently sterically blocking downstream miRNA-mRNA interactions.[73] Thus, our single-stranded miR-22-3p ONTs act by Watson–Crick base-pairing complementary to the human miR-22-3p sequence in a specific manner.

The preclinical efficacy and safety of chemically modified PNAs have been established for multiple targets in several animal models. Furthermore, the capacity of PNAs to evade immune surveillance is a substantial benefit over ASOs.[68]

PNA antagomirs have been successfully tested to target miR-155,[74-76] miR-122,[77,78] miR-122-3p,[79] miR-21,[80,81] miR-101-3p, miR-145-5p and miR-335-5p,[82] miR-210,[83] miR-584-5p and miR-425-3p,[84] miR-141-3p,[85] miR-155-5p and miR-221-3p,[86] miR-10b and 21,[87] and miR-33.[88,89] As reported recently by Pradeep *et al.*, PNAs targeting mRNA, pre-mRNA, and miRNA can be used to treat various human pathologies.[68]

The therapeutic relevance of PNAs was initially hampered by their poor cellular permeability and the absence of efficient delivery methods. Recent advances in delivery methods of PNAs, using lipid or peptide conjugates or nanoparticles, have improved their *in vivo* efficacy and safety.[74,90] This topic is specifically addressed in Criterion 7 below.

4.3. Criterion 3: Maintain resistance to nucleases and proteases/peptidases

In contrast to small molecules and protein drugs, which are respectively metabolized or catabolized, ONTs can be degraded by endogenous RNases. ONTs share similar absorption, distribution, metabolism, and excretion (ADME) profiles within each chemistry category.[91,92] ONTs undergo prompt cellular uptake enabled by chemical conjugates and delivery methods, leading to a short circulating blood half-life. Such conjugated ONTs have a unique pharmacokinetic/pharmacodynamic (PK/PD) profile whereby their local PD effects in the targeted organs and tissues are distinct from their systemic PK.[6,93] After cellular/tissue uptake, the intracellular concentration of the ONT, not its systemic concentration, interacts with the targeted mRNAs, leading to an extended PD half-life and dosing frequency schedule. The PK/PD and ADME features of GalNAc-conjugated siRNAs are similar across species, predictable, and precisely scaled up to human subjects, allowing for safe and effective dosing regimens before assessing human liver PK profiles.[94]

The peculiar backbone of PNAs provides resistance against both peptidase and nuclease degradation.[68,95] No known endogenous nucleases and/or proteases recognize PNAs as substrates.[96]

4.4. Criterion 4: Avoid chirality

ONTs often include a PS backbone to decrease nuclease degradation.[97] Given that the substitution of oxygen in the ONT backbone with sulfur introduces chirality, a full PS 16-mer ONT includes 2^{15} (32,768) distinct stereoisomers, with inherent risks of inconsistent therapeutic effects and unintended off-target effects.[97-99] Other chemical

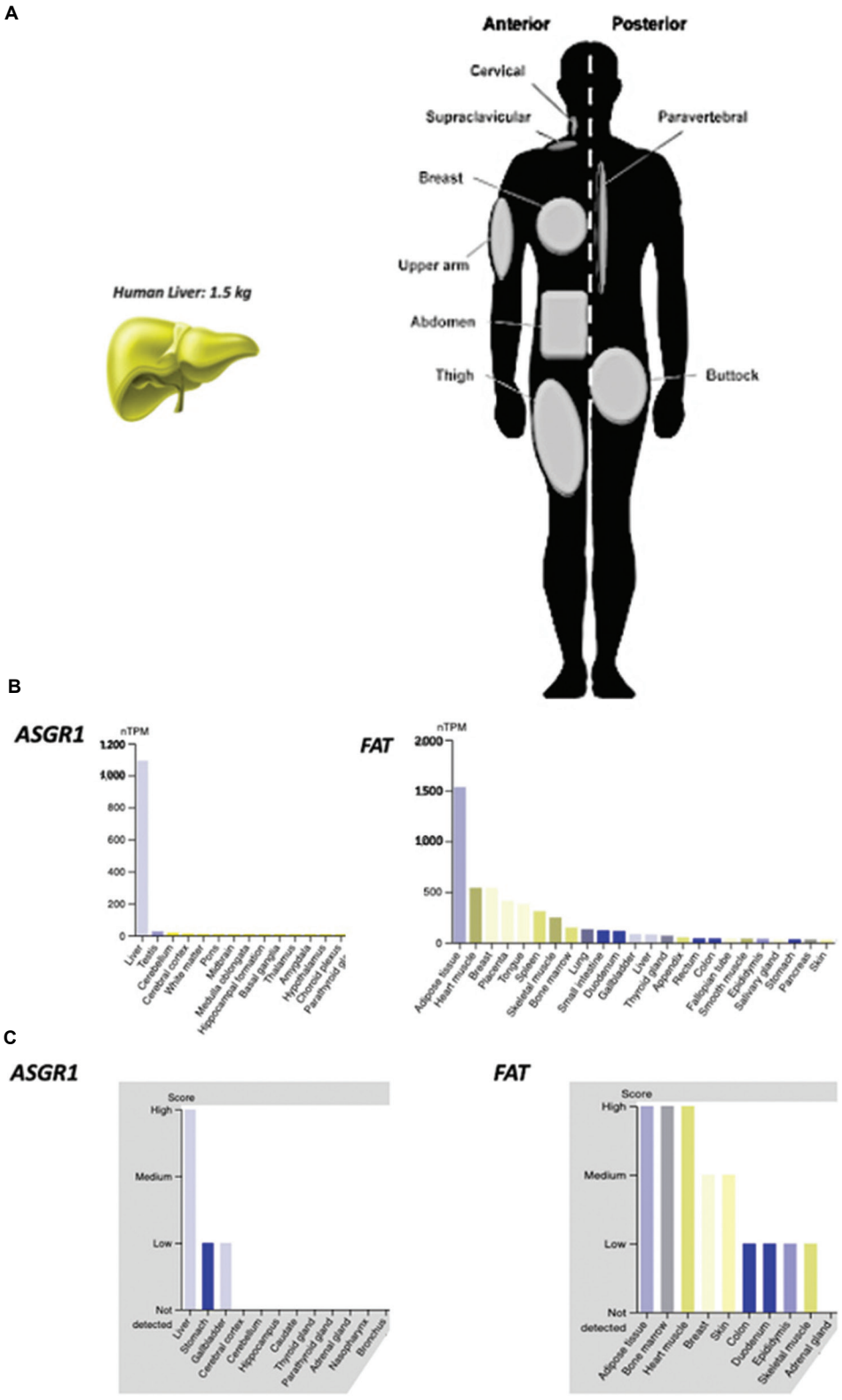

Figure 2. Comparison of targeted organs/tissues in various human tissues and organs of the asialoglycoprotein receptor 1 and the fatty acid translocase membrane transporter. (A) Size of organs/tissues: Human liver (1.5 kg), adipose tissue (40% of body weight in an obese individual), (B) mRNA expression levels, and (C) protein expression levels. Images adapted from.[56]
Abbreviation: nTPM: Normalized transcript per million.

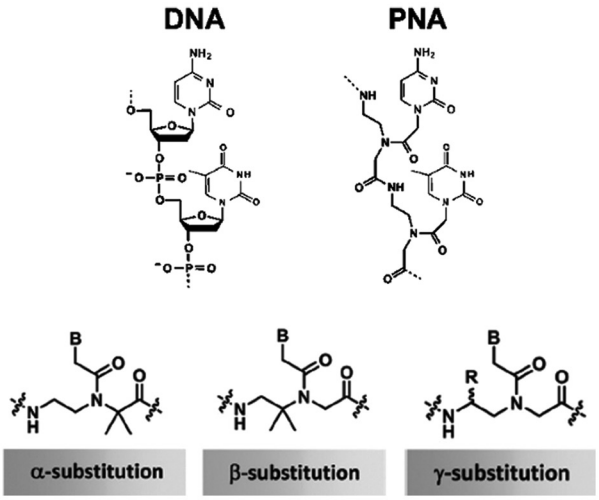

Figure 3. Various chemical modifications are applied to the structure of peptide nucleic acids (PNAs). Note: B=Nucleic acid base.

modifications, such as PMOs, also exhibit chirality. This issue of chirality is a matter of concern for regulatory authorities.[100] Since the 1980s, there has been a regulatory preference to approve single enantiomer drugs.[101] Consequently, strategies were implemented to reduce the number of PS modifications[9] or synthesize stereopure ONTs.[98,102] Scientists from Alnylam® Pharmaceuticals demonstrated that stereo-defined PS linkages at the termini of siRNAs improved their *in vivo* pharmacology.[103] Scientists from Wave Life Sciences Company reported that stereopure PS and phosphoryl guanidine backbone chemistries increase the potency and durability of mRNA silencing. They showed that a GalNAc-siRNA targeting human HSD17B13 leads to 80% silencing for at least 14 weeks after administration of a single subcutaneous dose in transgenic mice.[104]

Another option is to rely on chemical modifications that do not result in chirality, as is the case for PNA compounds, which have an achiral protein-like backbone.[105,106]

4.5. Criterion 5: Limit binding to serum proteins

The tissue bioavailability and potential toxicity of ONTs are modulated by plasma protein binding.[91,107] PS modifications increase plasma stability and plasma protein binding. In contrast, ONTs that lack charges or are less extensively bound to plasma proteins (e.g., unmodified and unformulated siRNA, PNAs, and PMOs) are rapidly cleared from the blood through either metabolism or excretion in urine. The systemic distribution of generations 1 and 2 ONTs is broad, with the highest concentrations achieved in the liver and kidneys. Gapmer PS-ASOs with constrained ethyl, LNA, and/or 2'-MOE modifications bind with high affinity to cellular proteins, modifying their functions, distribution, and half-lives.[108] Coupling an ONT to a cellular targeting agent dramatically alters the systemic PK/PD profile of the conjugate and reduces concerns about serum protein binding, as shown for the generation 2.5 N-acetyl-galactosamine conjugates.[95,109]

4.6. Criterion 6: Optimize and simplify the chemical synthesis of ONTs

Various chemical modification and conjugation strategies have been introduced to improve ONT drug characteristics, such as nuclease resistance, RNA-binding affinity, and PK profiles.[110,111] PNA compounds are synthesized using a straightforward method similar to peptide synthesis. This cost-effective solid-phase peptide synthesis method is detailed in the literature.[106,112] Furthermore, using a Fmoc/Boc protection scheme for both PNA monomers and amino acid building blocks, in association with microwave-assisted solid-phase synthesis, allows continuous assembly of PNA-peptide conjugates.[113,114]

To efficiently select PNA sequences of interest, a group of scientists recently developed machine learning algorithms and automated synthesis technology to predict PNA and peptide-PNA conjugates' synthesis efficiency and to guide rational PNA sequence design.[115,116] Another group of scientists incorporated an ultrasonication step in the solid-phase synthesis of PNAs.[106] This "United States (US)-PNAs" approach improves the purities and yields of synthesized PNAs, such as short or medium compounds (5-mer and 9-mer), complex purine-rich sequences, and longer oligomers.

4.7. Criterion 7: Conjugate the ONTs to a fatty acid or a short peptide for greater and active targeted delivery to the tissues and organs of interest at a much-reduced therapeutic dose

For therapeutic efficacy, after systemic or local administration, an ONT must be selectively delivered to the organs/tissues of interest, efficiently transported into cells, and translocated to the proper cellular compartment to interact with its molecular target. The rapid renal clearance and potential toxicity of unconjugated ONTs can be mitigated by using local targeted delivery methods. Therefore, the conjugation of ONTs is crucial to achieve safe and effective druggability.[13,41,110]

For instance, ONTs targeting the liver, the central nervous system, and muscles have been successfully developed and approved for clinical use.[9,109,117,118] The use of triantennary N-acetylgalactosamine (GalNAc3) ligand and the hepatocyte asialoglycoprotein receptor illustrates the advantages of receptor-mediated uptake of ligand-conjugated antisense drugs targeting RNAs in

hepatocytes.[119] Fifteen GalNAc3-conjugated 2'-MOE-modified ASOs are currently in clinical trials. Scientists from Ionis Pharmaceutical compared the tolerability and safety of four GalNAc3-conjugated and four identical unconjugated 2'-MOE ASOs, demonstrating an enhanced safety and tolerability of the GalNAc3-conjugated compounds versus their unconjugated controls.[119] They concluded that "the improved tolerability of the GalNAc3-conjugated 2'MOE ASO likely reflects the use of reduced doses and smaller sub-cutaneous injection volumes while increasing the ASO potency."[119,p24]

The therapeutic utility of PNAs was initially hindered by their own poor cellular permeability and the absence of effective delivery strategies. However, recent advances in PNA delivery using nanoparticles, ligands, and lipid or peptide conjugates have improved their efficacy and safety *in vivo*.[71,90,116,120-122] For instance, the South Korean drug company OliPass is developing PNA compounds for five different therapeutic indications using bases containing cationic lipid groups to increase stability and cell penetration.[123] Effective therapeutic doses in animal studies were as low as 10 ng/kg, several orders of magnitude lower than existing ASOs.

We opted to conjugate our miR-22-3p antagomir drug candidates to a fatty acid or a short peptide for enhanced targeted delivery to adipocytes and metabolic organs through the cellular membrane transporter fatty acid translocase (FAT). Our goal is to deliver a significantly reduced therapeutic dose to metabolic tissues and organs of interest with an extended intracellular duration of action (measured in mean residence time).

We chose the cellular membrane transporter FAT (FAT/CD36/SCARB3), which is the main route of uptake by adipose tissues of long-chain fatty acids as well as short peptides, such as hexarelin, prohibitin, and thrombospondin peptide-1.[124] It is worth noting that FAT is present not only on the cell surface membrane but also in intracellular compartments, notably the endosomes.[125] Thus, the reversible translocation of FAT from endosomes to the plasma membrane facilitates fatty acid cellular uptake.

The membrane transporter FAT is present in cells and tissues such as adipocytes, hepatocytes, skeletal and cardiac myocytes, pancreatic β-cells, kidney glomeruli and tubules cells, monocytes, and macrophages, which are sensitive to metabolic dysfunctions (Figure 4). In an obese male patient weighing around 200 lbs with 40% adipose tissue, there is a substantial quantity and density of FAT available to transport our new generation 2.5 of miR-22-3p antagomirs coupled to a fatty acid or a peptide into the adipocytes. To illustrate this point, we compared the

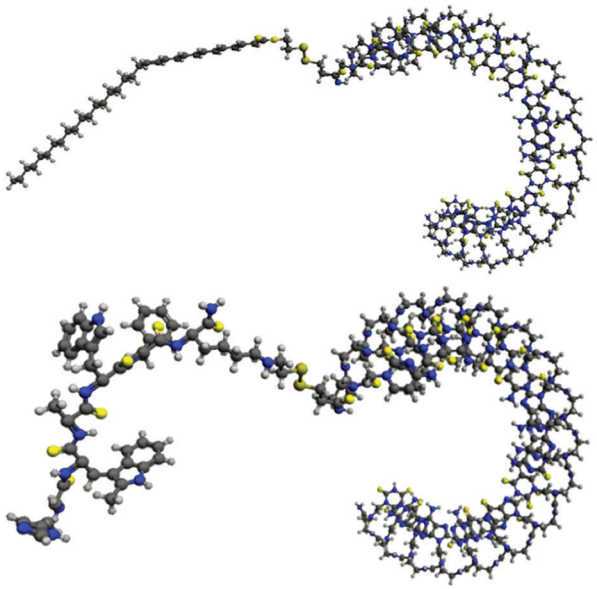

Figure 4. Graphic representations of an 18 mer miR-22-3p antagomir with a PNA backbone coupled with a long chain fatty acid (C32:6 dotriacontahexaenoic acid) (A) and a hexapeptide (hexarelin) (B). Images adapted from Thibonnier and Ghosh.[56]

mRNA and protein expression levels of FAT across human tissues to those of the asialoglycoprotein receptor 1 used for the preferred delivery of ONTs to the liver (Figure 2). This comparison underpins our rationale for targeting FAT for the preferred delivery of ONTs to metabolic organs.

Examples of the structure and composition of AptamiR's generation 2.5 active targeting miR-22-3p antagomirs are shown in Figure 4.

5. Discussion

MiRNA-based ONTs could provide an effective and convenient management of diseases, especially chronic and prevalent ones. By shifting from the classical "one drug-one target" strategy to a "one drug-many targets" paradigm, miRNA ONTs should produce significant therapeutic benefits while reducing polypharmacy, offering substantial advantages in drug cost, efficacy, safety, and patient compliance.

Our work, along with others,' has identified miR-22-3p as a promising candidate for the treatment of metabolic disorders associated with obesity and metabolic dysfunction-associated steatotic liver disease (MASLD).[53,54,56,61] We have established the efficacy of miR-22-3p antagomirs both *in vitro* in human cells in culture and *in vivo* preclinical animal models. The metabolic roles of miR-22 and the benefits of miR-22-3p antagomirs have been confirmed by several independent investigators, as summarized in a review paper by investigators at the

University of California, Davis, United States.[126] Early clinical trials for miR-22-3p antagomirs should be initiated in the near future.

Relying on an extensive and critical review of the literature and our own experience, we selected a set of seven criteria based on scientific parameters, PK/PD profile, ease, and cost of development to design generation 2.5 miRNA ONTs. Once the target miRNA of interest is selected according to criterion 1, five other criteria (criterions 2 – 6) can be simultaneously addressed by selecting a modified PNA backbone. The critical criterion 7, which involves enhanced selective delivery to the tissues and organs of interest, can be satisfied by coupling the PNA antagomir to a fatty acid or peptide actively transported by a cellular membrane transporter expressed in the tissue/organ of interest. Therefore, the modified PNA with its targeting moiety can achieve all these criteria (reduced potential toxicity, chemical stability, no chirality, superior PK/PD profile, ease, and cost of synthesis). This set of criteria could be applied to the development of miRNA ONTs for various therapeutic indications.

In the case of specific organs or tissues, such as the eyes, the brain, and the lungs, the mode and type of local administration represent another targeted delivery option. Several ONTs approved for ocular diseases (cytomegalovirus retinitis and age-related macular degeneration) are delivered through intravitreal administration (Table 1). Various ONT formulations directly delivered to the lungs using aerosolized inhalers for the treatment of fibrotic lung diseases, as well as asthma and lung cancer, are currently in clinical trials and display few systemic side effects.[127]

The clinical relevance of ONTs in the context of cardiometabolic pandemics can now be addressed by the example of inclisiran, the first-in-class siRNA targeting PCSK9 approved by both the US Food and Drug Administration (FDA) and European Medicines Evaluation Agency. Inclisiran is an effective and convenient way to tackle the huge burden of dyslipidemia and atherosclerotic cardiovascular disease.[128] The ORION/VICTORIAN clinical trials have shown that inclisiran is both safe and efficient for up to 5 years.[129] Ongoing animal and human studies are testing ASO, siRNA, and miRNA compounds to treat metabolic syndrome, which afflicts a large percentage of the world population.[130] Recently, Nappi *et al.*[131] reviewed the roles of miRNAs in the cardiovascular system, focusing on clinical translation.

Obesity affects one-third of the world's population, including millions of children.[132] This report suggests that "on current trends, overweight and obesity will cost the global economy over US$4 trillion of potential income in 2035, nearly 3% of current global gross domestic product." MASLD affects 25% of the global adult population, and the prevalence of liver steatosis is 76% in obese patients. At present, there is no FDA-approved medication for MASLD treatment.[133] Obesity and MASLD are growing and costly pandemics in need of safe, effective, convenient, and affordable therapies.

Ten years ago, there was little "appetite" to invest in new drugs for obesity and related cardiometabolic disorders due to limited efficacy and serious adverse events leading to drug non-approval or even removal from the market. Regulatory authorities required long and costly outcome studies, causing large pharmaceutical companies to shy away from this market. However, 10 years later, the landscape has changed significantly, thanks to the development of new classes of agents, especially single, double, and triple incretins agonists for obesity and diabetes mellitus, and thyroid hormone receptor β agonists for MAFLD.[134,135] In a phase II clinical trial, the triple-hormone receptor agonist retatrutide achieved up to 24% weight reduction at 48 weeks, comparable to metabolic/bariatric surgery outcomes.[136] In the MAESTRO-NAFLD-1 52-week randomized, double-blind, placebo-controlled phase III trial in adults with NAFLD, the thyroid hormone receptor beta-selective agonist resmetirom was safe and well tolerated.[137] Data from a phase III randomized controlled trial revealed resolution of NASH and/or fibrosis improvement after 52 weeks of treatment with resmetirom.[138,139]

Treatment selection for combating chronic cardiometabolic pandemics should rely on efficacy, safety, convenience, and cost/affordability parameters. The details are further elaborated as follows:

(i) Efficacy: Incretins and metabolic/bariatric surgery have now set the bar for body weight reduction in human subjects at the -24% level that new drug candidates should meet

(ii) Safety and tolerability: Several years of pre- and post-approval monitoring are required to accurately identify the type and incidence of potential adverse events of a given drug.[140]

(iii) Convenience and compliance: Twice-a-year subcutaneous administration of the siRNA inclisiran (Leqvio®) demonstrates improved adherence to therapy and lower low-density lipoprotein cholesterol over time, which in turn is postulated to improve clinical outcomes.[141-143] Such benefits should apply to targeting ONTs developed for combating cardiometabolic pandemics.

(iv) Cost/affordability: Chronic cardiometabolic pandemics affect a large fraction of the world's population. As far as ONTs are concerned, there is relevant experience with

Table 5. Checklist to develop an oligonucleotide therapeutic

Step	Task
1	Choose a relevant disease or unmet medical need
2	Select a specific RNA target and related network of genes and proteins
3	Elect an ONT type (ASO, dsRNA, miRNA agomir or antagomir, aptamer)
4	Incorporate chemical modifications for achieving optimized drug-like properties
5	Pick a targeting agent/delivery method to achieve the best PK/PD profile
6	Validate lead candidate with machine learning, *in vitro*, and *in vivo* studies
7	Optimize and simplify the chemical synthesis of the lead candidate to reduce costs

Abbreviation: PK/PD: Pharmacokinetic/pharmacodynamics.

inclisiran.[144] At the publicly available price of $3,250 per dose, Leqvio® was found to have "an incremental cost-effectiveness ratio (ICER) just above the $50,000 per quality-adjusted life year threshold of $51,686." By comparison, the ICER of the dual GLP-1/GIP analog Tirzepatide® is $70,147 for the 10 mg dose and $54,699 for the 15 mg dose versus the GLP-1 analog semiglutide 2 mg as recently reported in patients with Type 2 diabetes mellitus in the US.[145] In US adult patients with overweight and obesity, semaglutide 2.4 mg was also estimated to be cost-effective compared with no treatment and other anti-obesity medications, with ICERs ranging from $27,113 (vs. no treatment) to $144,296 (vs. phentermine-topiramate).[146]

Thus, an active cellular targeting miRNA antagomir with a PNA backbone produced by solid phase synthesis and a bi-annual administration regimen should be cost-effective, an assumption that will need to be confirmed by clinical studies and cost-effectiveness analyses.

6. Conclusion

ONTs represent a new class of potent therapeutic agents that are now achieving regulatory approvals for use in humans. Within a few years, they have evolved from treating a specific disease of the eyes, muscles, or brain to addressing cardiometabolic pandemics (e.g., inclisiran [Leqvio®] for elevated cholesterol and zilebesiran for arterial hypertension). These developments are the results of improvements in nucleic acid chemical modifications, conjugation to targeting ligands, and/or combination with delivery systems.

The road map to the successful development of an ONT is summarized in Table 5.

Compared to other therapeutic classes, ONTs are reasonably easy to synthesize with high purity at a competitive cost.[147,148] The new generation 2.5 ONTs allow the convenient administration of reduced doses on an extended schedule (e.g., bi-annual for inclisiran [Leqvio®]), which should reduce costs and improve patients' compliance with treatment.

Using the example of miR-22-3p as a therapeutic target, we propose a set of seven criteria to optimize the design and PK/PD profile of active cellular targeting miRNA ONTs. These criteria could be easily adapted to other miRNA targets and human diseases, especially chronic and endemic ones.

Obviously, the validity of such modifications must be confirmed by testing the lead candidate in animal models and, eventually, human subjects.

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Conflict of interest

Marc Thibonnier is the founder and a shareholder of AptamiR Therapeutics, Inc.

Author contributions

This is a single-authored article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Data presented in this article can be accessed in our previously published work[53,54,56] or by request to the corresponding author.

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REVIEW ARTICLE

Enhancers and super-enhancers as master regulators in cancer

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Abstract

Gene expression regulation is one of the most fundamental cellular processes, enabling the activation of a gene to produce either the translatable protein-coding transcript (mRNA) or a functional non-coding RNA with gene regulatory functions, ultimately determining cell identity and function. Although gene expression regulation can occur at transcriptional, translational, and post-translational levels, transcription initiation is the first and the most important step in gene expression, facilitating the transfer of biological information from DNA to protein. Enhancers and super-enhancers are among the master regulators of tissue- and cell-specific transcription regulation involved in cell differentiation and tumor formation. Despite four decades passing since the first discovery of enhancers in eukaryotes and extensive efforts undertaken to identify enhancers on a genomic scale during the last decade, the discovery of enhancers still faces certain limitations and needs further investigation. The perturbation of enhancer function due to genetic or epigenetic changes is closely linked to a range of human disorders, including the development and progression of cancers. Thus, the detection of early cancer-related enhancer activity and the subsequent normalization of expression abnormalities using enhancer-targeting CRISPR epigenetic editing, as well as enhancer-targeting pharmaceuticals, are regarded as groundbreaking therapeutic tactics in preclinical stages.

Keywords: CRISPR; Epigenetic editing; Enhancer-promoter loop; Enhancer-targeting drugs; Non-coding transcript; Super-enhancers; Transcription

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1. Introduction

Enhancers are non-coding regions of DNA, ranging from 200 to 2,000 base pairs in length that can be bound by transcription factors (TFs) to modulate the transcription of cell-specific genes. Importantly, the action of enhancers on gene expression is not restricted by their position or distance from the target gene. Interestingly, enhancers can be located upstream, downstream, adjacent to promoters, or even up to one million base pairs away from the target gene.[1] Regardless of their distance, distal enhancers can form an enhancer-promoter loop complex to physically interact with the promoter of a target gene. Enhancers typically contain specific DNA elements recognized by tissue-specific TFs. Research has shown that enhancers recruit transcription complexes at the enhancer-promoter loop, including cell-specific TFs such as OCT4, SOX2, KLF4, and Nanog, RNA polymerase II (RNA pol II), co-activators, the mediator complex, enhancer

RNAs (eRNAs), and histone-modifying enzymes including methyltransferases, histone acetyltransferase EP300, and CBP. This cooperative binding initiates and promotes transcription (Figure 1A).[2-4] Although most enhancers are located in intergenic and intronic regions of the genome, some are located within exons.[5] Super-enhancers (SEs) are extensive genomic regions formed by clusters of enhancers. SEs exhibit a higher (several-fold) binding enrichment for transcriptional factors than typical enhancers, spanning more than 20 kb on average.[6] SEs have a greater impact on the transcription of specific genes in comparison to regular enhancers and have the ability to simultaneously activate a significant number of promoters. These SEs are typically found in close proximity to genes crucial for cell differentiation.[7,8] Scientific findings suggest that active enhancers are often marked by the co-occurrence of H3K4me1 and H3K27ac. However, certain enhancers can become active solely through H3K4me1 modification.[9,10] Genes linked to the H3K27ac enhancer mark exhibit higher expression levels compared to those associated with the H3K4me1 enhancer mark.[11] Further studies described CBP/EP300-mediated H3K27 acetylation as a marker of active enhancers, since repressing this modification reduces enhancer activity, indicating that H3K27ac is causative, not just correlative, to enhancer activity.[12] An illustration of this concept is the discovery that EP300 regulates enhancers in neuroblastoma (NB) by adding the H3K27ac mark to colorectal cancer-associated SEs. This process involves interaction with the recently identified TF TFAP2β in NB cells.[13] Moreover, EP300 has been shown to disrupt the activity of epigenetic modifiers known to regulate enhancers, such as histone deacetylases and non-coding RNA (ncRNAs), hence promoting pulmonary fibrosis.[14] Intriguingly, genome-wide RNA sequencing has

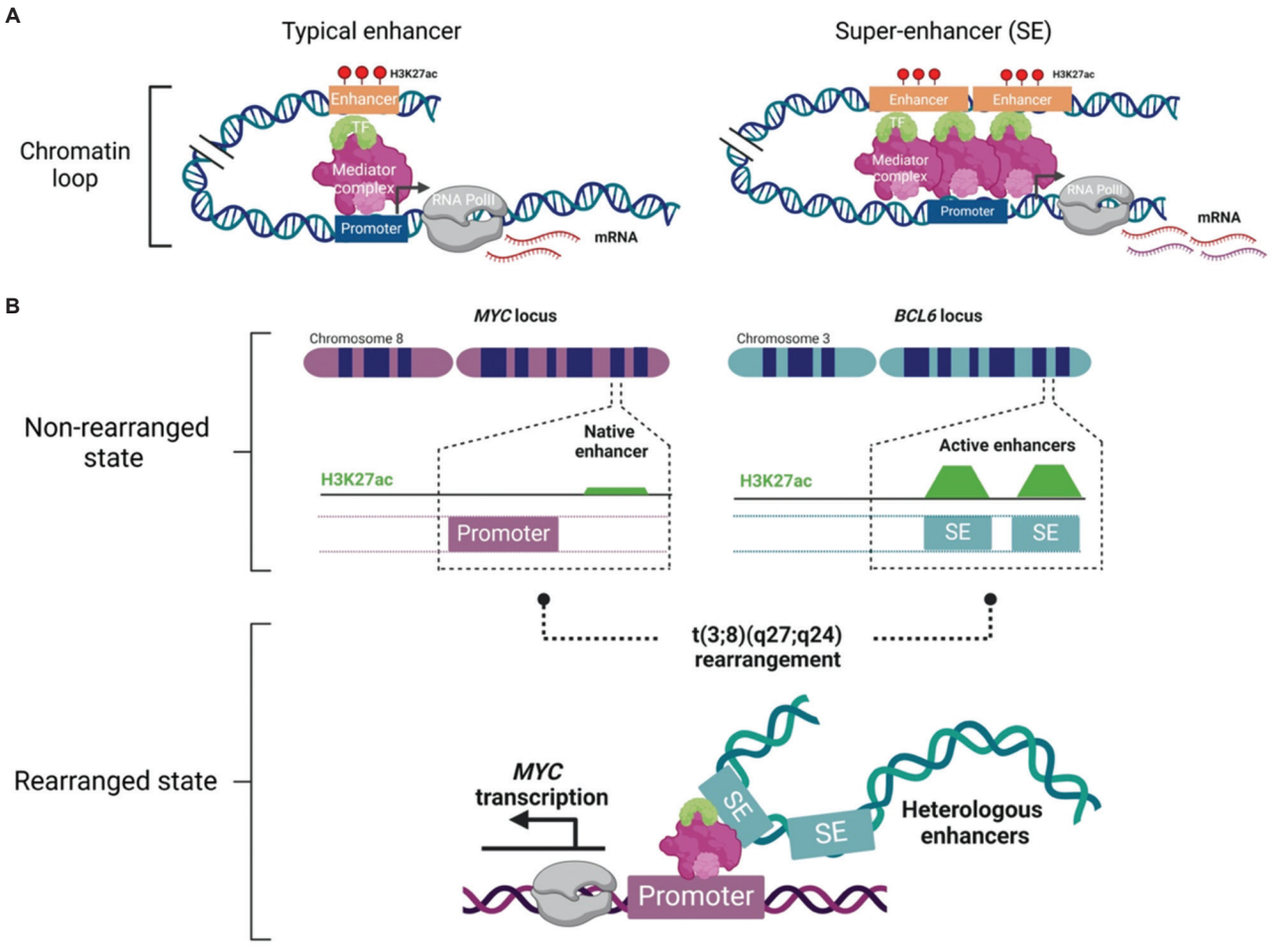

Figure 1. Enhancer hijacking and *MYC*-activating rearrangement. Heterologous genomic rearrangements linking the *BCL6* and *MYC* loci can activate *MYC* promoter through *BCL6* enhancers in B cell lymphomas. (A) Enhancers/super-enhancers elements. (B) Compared to a non-rearranged state, the genomic rearrangement t(3;8)(q27;q24) results in enhanced activation of *MYC* due to the interaction with BCL6 active distal enhancers, which are enriched with the active H3K27ac chromatin immunoprecipitation-seq mark. This rearrangement, which activates *MYC*, is linked to germline polymorphisms that modify the risk of developing lymphoma. Figure created using BioRENDER.com.

discovered that a significant portion of enhancers and SEs can be transcribed to give rise to eRNAs/SE RNAs that can further facilitate enhancer-promoter interactions, RNA pol II elongation, and can even act as decoys for repressive cofactors.[15-18]

2. Oncogenic enhancer and SEs activation by genomic rearrangements and variations

Chromosomal rearrangements, such as deletions, inversions, duplications, or translocations, can misplace active enhancers within the genome, causing abnormal gene expression. Such chromosomal rearrangements can lead to the activation of oncogenes or the silencing of tumor suppressor genes, commonly associated with cancer development.[19-22] Recent findings suggest that genomic rearrangements can lead to the repositioning of distal enhancers to the promoters of oncogenes specific to certain tumor types. These enhancers, which were not originally intended for these oncogenes, contribute to the initiation and progression of tumorigenesis by activating oncogenic signals. This phenomenon, known as enhancer hijacking, is a crucial cancer-driver mechanism.

For instance, enhancers specific to the lymphoma subtype within the *MYC* locus were shown to be silenced in lymphomas and associated with germline polymorphisms that alter the risk of developing lymphoma. Additionally, enhancers within the *BCL6* locus are subject to acetylation and possess the capability to undergo genomic duplication. Moreover, they can activate the *MYC* promoter, thereby functioning as an enhancer donor in a translocation phenomenon referred to as enhancer hijacking (Figure 1).[23]

In another study, Gröschel *et al.*[24] demonstrated that chromosomal 3q rearrangements result in the relocation of a distal *GATA2* enhancer (located 110 kb away from the *GATA2* gene at 3q21) to the *EVI1* locus. This event leads to the ectopic activation of *EVI1* expression, which is a crucial oncogenic driver in acute myeloid leukemia (AML). Furthermore, the elimination of the ectopic *EVI1* enhancer using the CRISPR/Cas9 genome-editing system resulted in decreased cell proliferation and increased apoptosis in the MUTZ-3 cell line derived from myeloid leukemia. These effects were remarkably similar to those observed when EVI1 knockdown was achieved using small hairpin RNA in the same cells.

These findings propose that chromosomal rearrangements can lead to the repositioning of a single enhancer, consequently impacting the regulation of two distinct distal genes, ultimately contributing to cancer development. In addition to chromosomal rearrangements, genetic variations such as deletions, mutations, or epigenetic modulations, such as methylation at the enhancer site, can affect the interaction among transcription regulatory elements (enhancer, promoter, and TFs binding site). Such genetic variations or epigenetic modifications often result in loop formation between proto-oncogenes and enhancers, leading to the upregulation of neighboring oncogenes and tumorigenesis. For example, a single nucleotide polymorphism (SNP) within the 15q15.1 chronic lymphocytic leukemia risk locus can create SEs that are correlated with decreased proapoptotic *BMF* expression. This SNP also hinders the interaction between the TF RELA (p65) and SE, resulting in an enhancement of BCL2's antiapoptotic function, thereby facilitating tumor growth.[25]

In addition, chromosomes are folded and arranged into 3D genomic segments that are megabases in length and have the ability to self-interact. However, interactions with regions beyond the designated topologically associated domains (TADs) are infrequent. Interactions of enhancers with their target genes are constrained due to the TAD boundaries enriched with insulator proteins such as CTCF in mammalian cells.[26,27] TAD boundary disruption due to chromosomal rearrangements or mutations can result in enhancers interacting with genes outside of the original TAD, resulting in inappropriate enhancer-promoter interactions. Such interactions have been implicated in rare diseases such as adult-onset demyelinating leukodystrophy,[28] and human limb malformation.[29,30]

A few studies have investigated TAD boundary disruption in carcinogenesis.[31,32] Interestingly, mutations in the CTCF motif at the TAD boundary result in NOTCH1 misregulation associated with ovarian cancer, due to the aberrant activity of enhancers caused by the disruption of the TAD.[33] However, more studies are required to unravel TAD boundary disruptions and their connection to cancer initiation and development.

3. Influence of SEs on tumor microenvironment

Cancer cells exhibit modified patterns of SE regulations, which contribute to the activation of oncogenes and other genes associated with key cancer features.[34,35] Chromosomal translocations in malignant lymphomas can relocate SEs to immunoglobulin loci near *MYC*, resulting in elevated levels of *MYC* expression.[36] Mechanistically, *MYC* overexpression may be reciprocally related to hijacking histone deposition, which alters cancer genome organization, as observed in the U2OS osteosarcoma cell line. Immunofluorescence labeling demonstrated that MYC molecules formed punctate foci at active transcriptional SEs, which were abrogated on removal of the architectural protein CTCF.[37]

Hijacking histone variants and chaperones, which transport histones across the cell and deposit them in

chromatin, is altered in solid tumors. H2A.Z, a highly conserved histone variant with 60% identity with H2A, is related to transcriptional activation. In mammals, there are two paralogues of H2A.Z: H2A.Z.1 and H2A.Z.2. Their expression is typically upregulated in numerous tumor types. MYC, ERα, and AR TFs can drive the addition of H2A.Z.1 to genomic sites in hormone-regulated malignancies such as breast and prostate cancer.[38] Furthermore, SEs activate the histone chaperone HJURP, resulting in abnormally high HJURP expression in t(4;14)-positive multiple myeloma. Overexpression of HJURP enhances tumor cell proliferation and is linked to poor outcomes in t(4;14)-positive multiple myeloma patients.[39] Enhancer hijacking may potentially increase resistance to treatment, rendering SEs more vulnerable to epigenetic therapies than canonical enhancers.[40] This is because SEs arise when master TFs attach to each component enhancer, attracting unusually high densities of cofactors (mediators and coactivators) that are proposed to interact with enhancers.

However, not all cofactors are essential for SEs activation. In HCT116 cells, enhancers have been classified based on their cofactor dependencies, highlighting different mechanisms for activating their correlated SEs and, thus, transcription.[41] This framework of categorization permits us to comprehend how enhancers contribute to gene expression programs and regulatory specificity.[41] Furthermore, the amount of mediators is elevated compared to other regions, making it a useful indicator for identifying SEs. Therefore, the transcription-activated complexes recruited by SEs display about 10-fold their molecular density of conventional enhancers. These complexes require a stable structure to preserve their conformation in optimal conditions.[42] High-mobility group proteins, such as HMGA1, are necessary for preserving the enhancer substructures of coactivators such as mediator subunit 1 (MED1) and bromodomain-containing protein 4 (BRD4).[43] BRD4 functions as an epigenetic reader that targets and interacts with acetylated lysine residues on histone H3 and H4. When BRD4 binds to these residues, it recruits the mediator complex, RNA pol II, and the positive transcription elongation factor b, facilitating the process of transcription initiation and elongation.[44,45]

The high levels of RNA pol II and cofactors in SEs create a condensate by establishing multivalent interactions, resulting in the formation of liquid droplets. This phenomenon may be explained by a model based on the process of liquid-liquid phase separation. The model, proposed by Hnisz *et al.*,[46,47] suggests that the dense concentration of TFs, RNA pol II, cofactors, and eRNAs enables the formation of localized phase separation through weak multivalent interactions among molecules associated with SEs. This process would be more difficult to achieve with typical enhancers. It allows for the rapid formation of a highly concentrated and dynamic environment that promotes effective transcription.[48,49] Growing data from *in vitro* and *in vivo* studies strongly support the notion that phase separation may be employed to elucidate the characteristics of SEs, encompassing their function, development, and susceptibility. Nevertheless, this model attempts to elucidate the precise order of events involved in the development of long-distance chromatin connections or the generation of transcriptional condensates. Research has demonstrated that the levels and alterations of RNA molecules have a regulatory impact on the creation and dissolution of condensates.[47] Condensate production is facilitated by synergistic interactions among polyvalent molecules, such as RNA, DNA, and intrinsically disordered regions (IDRs) in proteins.[50]

Plenty of evidence indicates that SEs undergo sudden modifications in formation and dissolution. They arise by a single nucleation event and disassemble when chromatin factors or nucleation regions are removed. These features were observed in murine embryonic stem cells. The disruption of MED1 and BRD4 by 1,6-hexanediol leads to the formation of distinct structures at specific enhancer elements within the cell nuclei. This disruption also led to the excision of MED1 and BRD4 from the chromatin at enhancers, as well as the loss of RNA pol II.[51] RNA pol II selectively accessed the mediator condensates through the IDR located at the phosphorylated C-terminal domain of the large subunit. RNA-binding proteins located near the promoter[42] of downstream stemness genes, such as *TP63*, *MET*, and *FOSL1*, recruit RNA pol II to activate cancer stemness features in squamous cell carcinoma (Figure 2).[52] The administration of bromodomain and extra-terminal domain (BET) inhibitors effectively disrupted SEs, resulting in a strong inhibition of cancer stem cells (CSCs) self-renewal and the complete eradication of CSCs in a mouse model of human head-and-neck squamous cell carcinoma (HNSCC). Furthermore, the disruption of SEs also hinders the spread and migration of CSCs derived from human HNSCC to the lymph nodes.[52] Nevertheless, the use of anti-BRD4 agonists as a therapeutic option remains restricted due to their high toxicity and delivery limitations.[53] As a result, new methods combining genomic and computational frameworks have been developed to identify BRD4-enriched SEs and confirm their involvement in promoting the growth and movement of cancer cells through CRISPR knockouts.[40,54] Within this perspective, drug design can be accomplished through a physicochemical mechanism of action, which offers a new method to target cellular components that were previously considered difficult to drug, such as intrinsically disordered proteins.[47,55]

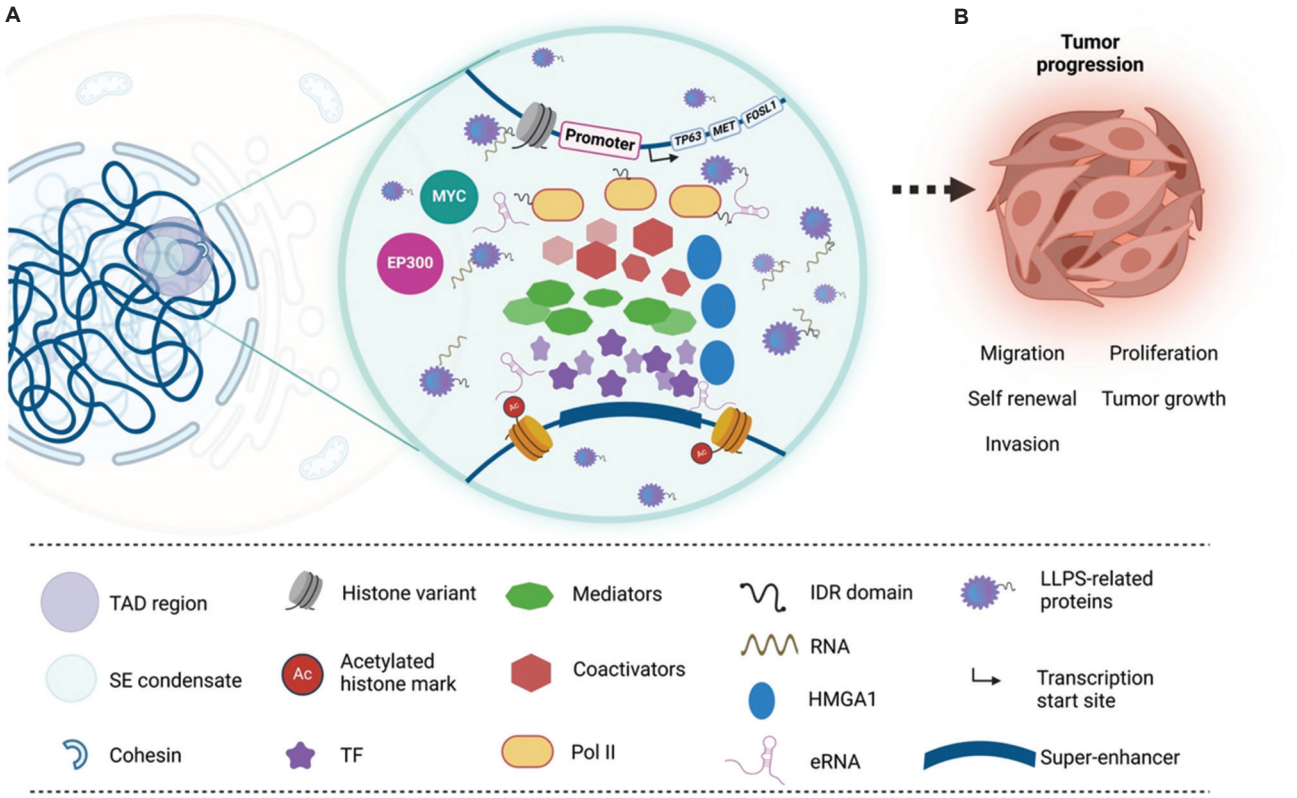

Figure 2. Oncogenic SEs assembled through LLPS processes. (A) At SE condensates, the transcription-activated complexes recruited by the SEs have about 10 times the molecular density of conventional enhancers. Such complexes require a stable structure to preserve their conformation under optimal conditions. The dense concentration of TFs, RNA polymerase II, cofactors (mediators and coactivators), and enhancer RNAs enables the formation of localized phase separation foci. SE condensate activation is facilitated by synergistic interactions among multivalent molecules, such as RNAs, DNA, and IDRs in proteins (LLPS-related proteins). RNA-binding proteins located near the promoter of downstream stemness genes, such as TP63, MET, and FOSL1, recruit Pol II to (B) activate cancer stemness features in squamous cell carcinoma, thereby creating a tumor microenvironment conducive to tumor progression. Figure created using BioRENDER.com.

Abbreviations: IDR: Intrinsically disordered regions; LLPS: Liquid-liquid phase separation; SE: Super-enhancer; TAD: Topologically associated domains; TF: Transcription factor.

4. eRNAs as modulators of the epigenome in cancer

eRNAs are a subclass of ncRNAs, known as long non-coding RNAs. These molecules are mostly unspliced and bidirectionally transcribed from enhancer elements by RNA pol II.[56] Although ncRNAs play integral roles in tumor formation and development in various ways,[57-60] the exact biological functions of eRNAs are still under investigation. Interestingly, cap-analysis gene expression technology has estimated the presence of approximately 40,000–65,000 eRNAs in humans, indicating a significant abundance.[61,62] eRNAs lack polyadenylation (polyA) modification at their 3'-end and are retained in the nucleus and chromatin-enriched fractions.[17,61] Moreover, eRNAs have 90–100-fold less stability than mRNAs,[17] making them prone to degradation by exosomal complexes in the nucleus.[63] Enhancer transcription is considered the most common rapid transcriptional change occurring when cells undergo a state change, peaking as early as 15 min after the transition trigger in some time courses across multiple biological systems.[62] However, enhancer activity is no longer required once the target promoter has been activated, leading eRNA levels to frequently return to baseline. In some instances, enhancers are rapidly activated and then continuously expressed, suggesting that these generated eRNAs may have additional functional roles in promoting elongation.[64]

Multiple studies have demonstrated that eRNAs play a key role in transcriptional regulation, mainly during cellular differentiation.[65,66] For instance, the tumor suppressor TP53 has been shown to bind to regions with enhancer activity located distantly from any known TP53 target genes, known as TP53-bound enhancer regions (p53BERs). In addition, p53BERs generate eRNAs in a TP53-dependent manner, which are involved in the transcriptional enhancement of target genes interacting with the enhancer they are

produced from.[67-69] Furthermore, research indicates that eRNA presence can be indicative of enhancer activity,[70,71] and the levels of eRNA transcription can reflect the degree of enhancer or promoter activity.[72] Thus, eRNAs may serve as biological markers for active enhancer regions.[73-75] Moreover, studies show that eRNAs stabilize enhancer-promoter loops by attracting cohesin complexes, which are essential for the formation and stabilization of chromatin loop structure.[76]

Increasing lines of evidence gradually revealed the regulatory role of eRNAs in various diseases, including cancer.[77-80] For instance, Jiao *et al.*[81] identified a SE and its derived eRNA that facilitated the expression of heparanase (HPSE), an endo-β-D-glucuronidase essential for cancer invasion and metastasis. They demonstrated that HPSE eRNA was highly expressed and positively correlated with HPSE levels in cancer tissues, promoting tumorigenesis and aggressiveness of cancer cells both *in vitro* and *in vivo*. In addition, HPSE eRNA was shown to promote cancer progression by driving chromatin looping and regulating hnRNPU/p300/EGR1/HPSE axis. Consequently, HPSE eRNA serves as an important prognostic marker for cancer patients with poor outcomes. Qin *et al.*[82] applied genome-wide profiling of eRNAs in Chinese lung adenocarcinoma patients, integrating RNA-seq data analysis to present a comprehensive description of eRNAs in lung adenocarcinoma. They discovered that highly upregulated eRNAs identified upstream of *TERT* may contribute to lung cancer development by upregulating *TERT* expression. *TERT* is a well-known predisposition gene for lung cancer, encoding human telomere reverse transcriptase, which maintains telomere ends.[83-85] Intriguingly, they discovered that *FOXO6* expression was elevated in lung adenocarcinoma, attributed to the copy number amplification of *FOXO6* eRNA in lung adenocarcinoma patients.

Another study showed that CCAT1, an enhancer-templated RNA, forms a complex with TFs TP63 and SOX2, regulating *EGFR* expression by binding to the SEs of *EGFR*. This interaction activates both the MEK/ERK1/2 and PI3K/AKT signaling pathways in squamous cancer cells, promoting tumorigenesis.[86] Similarly, *NET1e*, an eRNA located about 90 kb downstream of the oncogene *NET1*, was highly expressed in breast cancer.[80] In addition, in the study, CRISPR activation of *NET1e* was found to accelerate cell growth in MCF7 breast cancer cell lines. Conversely, its knockdown by locked nucleic acids antisense RNA significantly reduced cell proliferation in the MCF7 breast cancer cell line, suggesting its therapeutic potential in clinical eRNA-targeted therapy. Therefore, eRNAs offer considerable therapeutic potential and warrant further intense investigations for their roles in cancer and other diseases.

5. Limitations and future perspectives of enhancer-targeted cancer therapy

After almost 40 years since the first discovery of enhancers in the Simian virus 40 genome,[87] the precise mechanisms by which enhancers exert their effect on gene activation remain elusive. The limitations arise from the intrinsic complexity of enhancers and our limited knowledge, which needs further advances in molecular techniques for elucidation. As discussed earlier, the locations of enhancer elements can be identified by genome-wide profiling of histone marks, with H3K4me1 and H3K27ac being the two major histone marks flanking active enhancers.[88] Recently, the application of molecular biology techniques such as chromatin immunoprecipitation (ChIP) followed by high-throughput sequencing has proven beneficial for genome-wide enhancer identification.[89,90] Nevertheless, the discovery of enhancers throughout the genome remains limited, and determining their target gene is even more challenging.

Next-generation sequencing technologies, such as mapping RNA interactome *in vivo* (MARIO), *in situ* mapping of RNA-genome interactome (iMARGI), multinucleic acid interaction mapping in single cells (MUSIC), CAGE, global RNA interactions with DNA by deep sequencing (GRID-seq), and global run-on sequencing (GRO-seq), open new horizons for understanding the interactions of genomic regions with RNA. Despite the broad spectrum of applications for RNA-seq technology, its utilization in the detection of eRNAs on a large scale has been limited primarily due to the poor stability of eRNAs and insensitivity of the RNA-seq technique. The MARIO technique involves cross-linking RNA molecules with their associated proteins before ligating them to a biotinylated RNAlinker, resulting in a chimeric RNA in the form of RNA1-Linker-RNA2. These linker-containing chimeric RNAs are then separated using streptavidin-coated magnetic beads and subjected to paired-end sequencing.[91] This technology allows for an equitable selection of interacting RNAs, enabling comprehensive mapping of an RNA-RNA interactome on a global scale.[91] This approach bypasses the necessity of having a specific antibody for a protein and eliminates the constraint of studying only one RNA-binding protein at a given time. In addition, this technique exclusively captures RNA molecules that are co-bound with a solitary protein molecule, preventing the capture of RNA molecules bound independently to multiple copies of a protein. This precautionary measure ensures the avoidance of reporting false interaction.[91-93]

The IMARGI method is employed for the identification of chromatin-associated RNAs (caRNAs) and the elucidation

of their specific genomic interaction sites. The IMARGI procedure initiates with *in situ* crosslinking and genome fragmentation, then converts each nearby RNA-DNA pair into an RNA-linker-DNA chimeric sequence.[94] Subsequently, the chimeric sequences are transformed into a sequencing library optimized for paired-end sequencing. To analyze paired-end sequencing data and unveil caRNA-DNA interactions, researchers can utilize the standardized bioinformatic software package known as iMARGI-Docker, available at https://sysbio.ucsd.edu/imargi_pipeline.[94]

The MUSIC GRID technique enables simultaneous profiling of multiple chromatin interactions, gene expression, and RNA-chromatin associations at the single-nucleus level. It represents an effective tool for investigating chromatin structure and gene expression at the cellular level within intricate tissues.[95] Of significant note, GRID-seq is capable of identifying both coding and ncRNAs that interact with tissue-specific promoters and enhancers, particularly SEs. Consequently, it enables the generation of a comprehensive map illustrating the connectivity between promoters and enhancers on a global scale.[96]

The FANTOM consortium utilized the CAGE technique to analyze extensive transcriptomes from various cell types, leading to the identification of 43,011 enhancer elements that were transcribed into eRNAs.[61]

The utilization of GRO-seq, a cutting-edge methodology, enables the identification of the precise genomic locations and orientations of all RNA polymerases actively involved in transcription. This powerful approach proves invaluable in monitoring the transcription of nascent enhancers. In addition, the distinctive transcription pattern exhibited by enhancers can be utilized to identify these regulatory elements, even in the absence of any information regarding the underlying TFs.[97] Consequently, GRO-seq proves to be a proficient approach for the identification, characterization, and comprehension of enhancer transcription regulation. The detection of enhancer transcription through GRO-seq analysis serves as a highly dependable method for identifying active enhancers. This approach can be effectively utilized to study and characterize enhancers and is considered the most reliable indicator of enhancer activity, surpassing the histone modifications commonly enriched at enhancers.[16,97,98]

A more integrative approach combining ChIP with high-throughput molecular biology techniques such as Hi-C, Hi-ChIP, ATAC-seq, and their single-cell sequencing alternatives would be more efficient in uncovering the mechanisms by which enhancers and SEs regulate transcription and oncogenesis.[99] Furthermore, experimental validation *in vitro*, *in vivo*, and *ex vivo* using enhancer reporter vectors would not only help in identifying enhancers and SEs and their target genes but also in assessing their pathological functions and cancer-driving potential.[100-103]

The application of enhancer-targeting drugs, such as BET inhibitors, is known to block the family member of BET proteins, which are preferentially located at active enhancers (H3K27ac). BET proteins have the ability to identify and attach to acetylated lysine residues. Inhibiting BET proteins shows significant potential for advancing cancer treatment strategies in the future.[104] For instance, in an *in vitro* investigation, the pharmacologic inhibitor GNE987 reduced NB cell growth and survival, promoted apoptosis, and caused cell cycle arrest by degrading BRD4. These observations were consistent with a reduction in xenograft tumor size. Chen *et al.*[105] also identified a new oncogenic gene, *FAM163A*, enriched with the H3K27Ac mark in GNE987-treated cells using RNA-seq and ChIP-seq data.

The application of genome engineering tools to produce targeted mutations across different species has been described in various studies.[106-108] A pioneering study using CRISPR-Cas9 enhancer correction in treating sickle cell disease and β-thalassemia resulted in patients no longer needing transfusions and eliminated vaso-occlusive episodes.[109] These findings suggest that CRISPR genome/epigenome editing is not only a useful tool for generating and investigating chromosomal aberrations but also holds promise for correcting disease abnormalities, including cancer and age-related diseases.[110,111] For instance, it was shown that the application of CRISPR-Cas13a to knock down the SMAD7 enhancer, an estrogen-responsive eRNA, inhibited cell proliferation and migration while promoting cell apoptosis. This knockdown led to the suppression of cell invasion in bladder cancer in 5637 and T24 cells.[112] In another study, Mill *et al.*[113] discovered that using the CRISPR/Cas9 system to disrupt the SE region related to the *RUNX1* gene promoted apoptosis in acute leukemia cells (OCI-AML5), consequently modifying the survival rate of mice with AML. Additionally, Vincent *et al.*[114] demonstrated that epigenomic disruption of EGFR enhancers using CRISPRi (dCas9-KRAB) technology curtailed the invasive and proliferative competency of glioblastoma cells and enhanced their sensitivity to temozolomide treatment.

The landscape of drug delivery techniques in cancer treatment has undergone a remarkable revolution with the discovery of nanoparticles (NPs) and small-sized molecules. This breakthrough in nanotechnology has completely transformed conventional methods of delivering drugs to cancer tissues, presenting novel possibilities and renewed hope for effective cancer treatment. The distinctive characteristics of NPs, including decreased

toxicity, enhanced permeability, and precise targeting of cancer cells, offer a significant benefit in the treatment of cancer and aid in addressing the constraints and obstacles associated with traditional cancer treatment modalities[115] Huang *et al.*[116] made a significant discovery using ChIP-seq to identify top SE-associated genes, which they found to be promising oncogenes in pancreatic ductal adenocarcinoma (PDAC). These genes were shown to be highly susceptible to treatment with the cyclin-dependent kinase 7 inhibitor, THZ1, and BRD4 inhibitor, JQ1. In addition, it was shown that utilizing NPs containing a significant amount of JQ1, in combination with THZ1, could serve as a potentially effective therapeutic approach for treating PDAC by inhibiting SE-associated oncogenic transcription. While the study presents a novel approach for targeting SEs through the application of nanocarriers and opens new horizons for cancer treatment, the risks and hazards related to NPs still need to be addressed before approval for clinical applications.

6. Conclusion and perspectives

Enhancers play a crucial role in transcription regulation. The aberrant activation of enhancers and SEs due to chromosomal rearrangements and genetic/epigenetic variation drives oncogene activation, resulting in uncontrolled cell proliferation, resistance to apoptosis, and, consequently, tumor formation and progression. Enhancer-mediated regulation of genes is determined not only by their location but especially by their capability to physically bind to an appropriate promoter, which can even occur in a different chromosome topology. The epigenetic state of enhancers is crucial for their function, often used to identify their genomic locations. Active enhancers initiate the production of eRNAs and usually possess high levels of H3K4me1 and H3K27ac. However, more factors are required for robust enhancer identification.

The recent discovery of eRNAs adds another layer of complexity to the human transcriptome, encouraging intense research on the features and potential functions of this new class of ncRNAs. Evidence suggests that eRNAs may be powerful biological markers in cancer treatment and therapy. Abundant evidence shows that eRNAs are abnormally expressed in various cancers and that their expression is closely related to tumorigenesis. Investigations into these eRNAs reveal new oncogenic pathway activation in tumor cells and propose new potential targets for combination therapies. However, these findings on eRNAs and their association with cancer have been mainly established through genomic research, with limited supporting evidence from molecular assays, leaving the underlying molecular mechanisms involved unclear. Further molecular analyses are required to fully understand the complex molecular mechanisms of eRNAs in tumorigenesis. Moreover, a better understanding of enhancers and SEs structure, as well as reliable structural conformations of eRNAs and their interactions with target genes in three-dimensional space, is needed. Finally, CRISPR genome/epigenome-editing and enhancer-targeting drugs, such as BET inhibitors, present promising tools for correcting enhancers and SEs abnormalities in cancer therapy.

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The authors declare that they have no competing interests.

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

Medical imaging technology: Principles and systems

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Abstract

Medical imaging technology is an important course in biomedical engineering. It is a multidisciplinary field integrating advanced technologies from physics, electronic engineering, computer science, engineering mathematics, material science, and fine processing. This course lays the foundation for the implementation of imaging diagnostics essential for medical automation. It enables participants to systematically grasp the fundamental knowledge in medical imaging principles, equipment, and system analysis, as well as to understand the direction of the latest developments in this field. This paper discusses the basic principles and performance of various basic imaging devices, such as X-ray imaging, magnetic resonance imaging, nuclear medicine imaging, and ultrasound imaging. In addition, it explores the systems involved and the future prospects of medical imaging technology.

Keywords: Medical imaging; Human security; X-ray; Magnetic resonance imaging; Ultrasound

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1. Introduction

With the advancement of science and technology, medical imaging technology has made significant progress. Medical imaging refers to the process of creating medical image data and the technology or devices used to form these images (modern medical imaging). It is a science that relies on the interaction between specific energy and living organisms to extract information about the shape, structure, and certain physiological functions of tissues or organs, providing essential imaging information for biological tissue research and clinical diagnosis. The convergence of medical imaging technology with artificial intelligence (AI) has sparked transformative changes in the field, fostering mutual advancements and driving innovation. AI applications in medical imaging aim to streamline image recognition and analysis processes, ultimately bolstering diagnostic accuracy, efficiency, and reliability. Therefore, medical imaging occupies an important position in clinical diagnosis,[1] representing the intersection of high-tech and medicine.

The future development of medical imaging will primarily depend on advancements in computer-led high technology, which will subsequently enable modern medical imaging to progress rapidly. Modern medical imaging has evolved beyond traditional single ordinary X-ray and angiography examinations to include ultrasound, radionuclide imaging, X-ray computed tomography (CT), digital subtraction angiography, magnetic resonance imaging (MRI), digital imaging (computed radiography and digital radiography) of conventional X-ray examinations, and image storage and transmission of medical imaging system composed of multiple technologies such as picture archiving and communication systems. Overall, understanding the strengths and limitations of medical imaging technology is essential for health-care professionals to effectively utilize these modalities in clinical practice, ensuring optimal patient care and outcomes.

2. Common characteristics and types of medical imaging equipment

Medical imaging equipment is primarily composed of an energy emission source, effect tissue, detector, processor, display, and other components. As shown in Figure 1, the main types of medical imaging technology include: (i) X-ray imaging, (ii) MRI, (iii) nuclear imaging (nuclear medicine imaging technology), (iv) ultrasound imaging, (v) impedance imaging, (vi) thermal-microwave imaging, and (vii) optical imaging.[2] Among these technologies, the first four are the most widely used and easily popularized. Different types of medical imaging have complementary advantages, as detailed in Table 1.

3. Principles of medical imaging

In recent years, the advent of new imaging methodologies such as high-field MRI, spectral CT, and hybrid equipment has revolutionized medical imaging practices, offering enhanced diagnostic capabilities and clinical insights. High-field MRI, characterized by magnetic fields exceeding 3 Tesla, has enabled unprecedented spatial resolution and tissue contrast, which is particularly beneficial for neurological and musculoskeletal imaging. The improved signal-to-noise ratio provided by high-field MRI has facilitated enhanced anatomical detail and heightened sensitivity to pathological changes, advancing the detection and characterization of various diseases. Similarly, spectral CT, which utilizes energy-sensitive detectors to capture photon energy information, has emerged as a promising technology for spectral imaging and material differentiation. By discerning subtle differences in tissue composition and molecular properties, spectral CT enhances diagnostic accuracy and enables personalized treatment strategies, particularly in oncology and cardiovascular imaging. Moreover, the integration of hybrid imaging systems, such as positron emission tomography (PET)/CT and PET/MRI, synergistically combines the functional information from PET with the anatomical detail provided by CT or MRI.

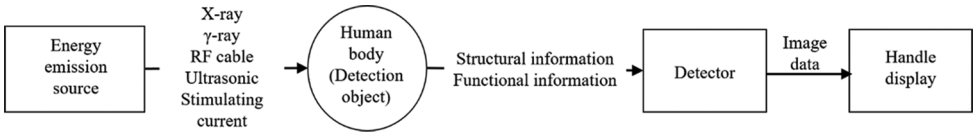

Figure 1. Medical imaging process
Abbreviation: RF: Radiofrequency.

Table 1. Types of medical imaging technologies

Medical image classification	Dominant form	Description content
Structure image: Describe the physiological anatomy of the human body	X-ray, CT	Tissue density distribution
	UI	Changes in tissue acoustic impedance
	MRI	Magnetic resonance density distribution and relaxation characteristics
Functional images: Describe the functional activities of human body tissues and organs in different states	RI (SPECT, PET)	Physiological and metabolic functions of tissues and organs
	fMRI	The functional activity of the human brain
	DFI	Blood flow imaging
Other types of imaging	EIT	Internal impedance or change in impedance
	Thermal and microwave imaging	Infrared signal on the body surface and microwave radiation signal in the body
	Optical imaging	Using optical and television technology to observe the shape of some human organs

Abbreviations: CT: Computed tomography; DFI: Detective flow imaging; EIT: Electrical impedance tomography; fMRI: Functional magnetic resonance imaging; MRI: Magnetic resonance imaging; PET: Positron emission tomography; RI: Radioisotope; SPECT: Single-photon emission computed tomography; UI: Ultrasonic imaging.

This integration offers comprehensive insights into disease processes and treatment responses. These advancements in imaging methodologies hold immense potential for optimizing patient care, guiding therapeutic interventions, and advancing our understanding of complex diseases. However, challenges concerning cost, accessibility, and standardization must be addressed to fully realize the clinical benefits of these innovative technologies. Future research efforts should focus on refining imaging protocols, validating diagnostic accuracy, and expanding the clinical utility of these cutting-edge imaging modalities across diverse medical specialties.

3.1. X-ray imaging

Roentgen discovered X-rays in 1895, which was the greatest discovery in medical diagnostics in the 19th century.[3] X-ray fluoroscopy and photography technology, as the earliest forms of medical imaging technology, remain among the most commonly used medical diagnostic methods today, holding considerable clinical value. The signal detected by the X-ray imaging system is the intensity of X-rays after they penetrate the tissue, reflecting differences in the X-ray absorption coefficients of various human tissues. This difference is due to variations in tissue thickness and density, which the image captures, showing the shape of tissues, organs, and diseased areas. For more than half a century, medical imaging science progressed steadily but slowly alongside advancements in X-ray technology.[4] In the early 1970s, the introduction of computerized X-ray tomography (XCT) marked a period of rapid development in medical imaging. CT involves taking thousands of X-rays from different angles. The substantial amount of data collected is processed by computers to form a three-dimensional image of any part of the body.[5] Doctors can instruct the computer to display a two-dimensional cross-section at any depth from any direction. CT scanning has brought revolutionary changes to medical diagnosis, allowing doctors to detect brain bleeding in head injuries or visualize the shape and extent of tumors in cancer patients.[4]

CT uses a digital signal input to the computer for processing. The processing of image formation involves dividing the selected layer into several cuboids of equal volume, called voxels.[6] The information obtained through scanning is used to calculate the X-ray attenuation coefficient or absorption coefficient of each voxel, which is then arranged into a digital matrix. Digital matrices can be stored on magnetic or optical disks. A digital-to-analog converter transforms each number in the digital matrix into small squares of grayscale, ranging from black to white, called pixels. These pixels are arranged in a matrix to form a CT image.

The emergence of XCT introduced a new concept in medical imaging. XCT uses extensive X-ray absorption data obtained from scanning around the body's organs to reconstruct tomographic images. When a beam of thin (fan-shaped) X-rays passes through a section of the body's organs, the total attenuation coefficient along the X-ray path is the line integral of the voxel attenuation coefficients, which can be measured with a detector.[4] Detectors convert the intensity of the rays into electrical signals, which are digitized and processed by computers. By conducting multiple measurements at different angles around the organs, the absorption coefficient related to each voxel at a specific level is calculated. The resulting two-dimensional absorption coefficient matrix is stored in the computer, using different gray scales.[7] This matrix information is then displayed as an image. The grayscale of each pixel on the displayed image corresponds to the absorption coefficient of the respective voxel, thereby providing information on the distribution of the attenuation coefficient on the fault plane. XCT technology is called tomography because it obtains a cross-sectional image of the body's organs.

This digital imaging technology has evolved from the first generation to the fifth generation. At present, the third and fourth generations are commonly used. The continued development of these two generations of CT technology has primarily focused on improving speed, enhancing image quality, developing new functions, expanding the scope of applications, and facilitating operations. For example, spiral scanning can reduce the time needed to scan the same coverage length by one-quarter to one-fifth or increase the scanning coverage length by 25 – 33% in the same amount of time. Another manifestation of increased speed is the shortened image reconstruction time. Some CT products adopt new image reconstruction technology, increasing the reconstruction time to one second, thus speeding up the scanning cycle. High-end CTs with continuous imaging or fluoroscopy functions can display six to eight images per second. CT fluoroscopy is very important for interventional radiology, as it reduces the number of scanning layers and the patient's X-ray exposure dose. Some products can achieve a 512 × 512 matrix image with a scanning dose of only 15 mA. Further studies are ongoing on CT fluoroscopy technology using an X-ray exposure dose of 10 mA. In terms of image quality, the spatial resolution of high-end, low-voltage spiral CT machines has reached 20 lp/cm, with high low-contrast resolution, significantly improving the ability to differentiate soft tissues.

The new generation of CT is called electron beam CT (EBCT). In EBCT, an electron beam is emitted by an electron gun, and a deflection coil changes the direction

of the electron beam to hit a set of semicircular target rings, producing X-rays. When the X-rays pass through the subject's body, they are received by the detector group arranged on the opposite side. Since there is no mechanical movement, the scanning speed can be as high as 50 ms/layer. While the temporal resolution is excellent, the spatial resolution is not as high as that of high-end third- and fourth-generation machines. Further, the development of EBCT is anticipated. Some hospitals in China have officially launched EBCT, which, due to its imaging speed being 20–40 times faster than ordinary CT, can provide exceptionally clear images of moving organs such as the heart. After many generations of development, XCT has been widely used in medicine. At present, it is primarily used in diagnosing spinal and head injuries, intracranial tumors, brain–blood clots, early heart disease prevention and treatment, soft-tissue injuries, gastrointestinal diseases, and malignant lesions of the waist and pelvis.

3.2. MRI

Since the 1980s, nuclear magnetic resonance technology has been applied to clinical medicine. The strong magnetic field interacts with the nuclei of the body tissue in the imaged part of the human body. The nuclei and their physiological conditions resonate under the magnetic field's influence and change their positions. The images generated by the magnetic field not only avoid the damage caused by CT machines to human tissue cells but also detect small physiological changes before pathological changes occur. Consequently, MRI has become a new branch of medical imaging diagnosis.

MRI is currently one of the most advanced imaging examination methods. It is an emerging imaging diagnostic technology that non-invasively displays the internal structure of the human body. Although this technology has been developed for <20 years, it has rapidly advanced, with equipment manufacturing technology and diagnostic theory continually improving. At present, MRI equipment is widely used in hospitals in large- and medium-sized cities. Its high-resolution images of human tissues and organs provide more intuitive image information about the internal structure of the human body, offering richer and more meaningful diagnostic and treatment information for clinical use.[8]

According to the principle of MRI, atomic nuclei are positively charged, and the nuclei of many elements, such as hydrogen-1, fluorine-19, and phosphorus-31, undergo spin motion. Normally, the arrangement of nuclear spin axes is irregular, but when placed in an external magnetic field, the spatial orientation of the nuclear spins transitions from disorder to order. The magnetization vector of the spin system gradually increases from zero, and when the system reaches equilibrium, the magnetization intensity stabilizes. If the nuclear spin system is affected by external influences, such as radiofrequency excitation of the nucleus at a certain frequency, a resonance effect occurs. After the radiofrequency pulse stops, the excited atomic nuclei cannot maintain this state and return to their original arrangement in the magnetic field. During this process, they release weak energy, which becomes radio signals. When these signals are detected and spatially resolved, an image of the distribution of atomic nuclei in motion can be obtained. The process of returning atomic nuclei from an excited state to an equilibrium arrangement is called a relaxation process, and the time it takes is called relaxation time. There are two types of relaxation times: T1 and T2. T1 is known as spin-lattice or longitudinal relaxation time, and T2 is known as spin-spin or transverse relaxation time.

Although MRI images are also displayed in different grayscales, they reflect differences in magnetic resonance signal intensity or the length of relaxation times T1 and T2, unlike CT images, where grayscales reflect tissue density. MRI images can generally be divided into three basic images: T1-weighted image, T2-weighted image, and proton density image. Fat appears as a high signal in these images, while muscles, liver, pancreas, and other tissues and organs show a medium signal on T1-weighted images and a low signal on T2-weighted images. Lung tissue, large blood vessels, and calcification generally appear as low signals in these images. In contrast, tissues and organs such as the kidneys and spleen have lower signals on T1-weighted images and higher signals on proton density and T2-weighted images. The contrast of CT images depends on the X-ray attenuation properties of the tissue. Fat has low density, calcifications have high density, and large blood vessels have a density similar to that of the liver and kidneys. Tumors often have a density similar to soft tissue, and contrast agent injection is generally required for better visualization and characterization.

The amount of information provided by MRI is not only greater than many other medical imaging techniques but also fundamentally different. Therefore, it has significant advantages in the diagnosis of diseases. MRI can directly produce tomographic images of cross-sections, sagittal planes, coronal planes, and various oblique planes without producing artifacts common in CT scans. MRI does not require the injection of contrast agents, exposes the patient to no ionizing radiation, and has no adverse effects on the body. Compared to CT, MRI has no radiation damage or bone artifacts, can perform multi-faceted and multi-parameter imaging, offers high soft-tissue resolution, and is suitable for diagnosing various diseases across different

body systems, such as tumors, inflammation, trauma, and degenerative conditions. MRI is particularly effective for examining sexual lesions and various congenital diseases. Its imaging of the brain, spine, and myelopathy is superior to that of CT. MRI can display the structure of blood vessels without using vascular contrast agents, making it unique in distinguishing between blood vessels, masses, lymph nodes, and vascular structures. Its soft-tissue resolution is several times higher than that of CT, allowing it to sensitively detect changes in water content in tissue components. Therefore, MRI often detects lesions earlier and more effectively than CT.

MRI can clearly and comprehensively display the cardiac chambers, myocardium, pericardium, and other small structures in the heart. It is a reliable method for diagnosing various heart diseases and assessing cardiac function. The images obtained using MRI are exceptionally clear and detailed, greatly improving diagnostic efficiency and reducing the need for exploratory surgeries such as thoracotomy or laparotomy. Since MRI does not use X-rays, which are harmful to the human body, or contrast agents that can often cause allergic reactions, it is considered safe. MRI can image various parts of the human body from multiple angles and planes with high resolution, providing detailed views of anatomical structures and their relationships, and better localizing and characterizing lesions. It is of great value in diagnosing diseases in various body systems, especially in detecting early-stage tumors.

3.3. Ultrasound imaging

Ultrasound is one of the best non-invasive and painless examination methods for human lesions today. In the 1960s, ultrasonic technology was applied to clinical diagnosis, leading to the development of type A, M, B, and C ultrasonic diagnostic machines. These machines can be used to observe the internal structure of the human body, diagnose tumors and cysts, and check the normality of organs, fetuses, and more. Long-term use, observation, and analysis have shown that the frequency and intensity of ultrasound imaging equipment are harmless to human health. Type A (amplitude modulation type) uses amplitude to indicate the strength of the reflected signal, displaying an "echogram." M type (photoelectric scanning type) uses the vertical direction to represent the spatial position from shallow to deep and the horizontal direction to represent time, displaying a graph of photoelectric movement over time. Both of these machine types are one-dimensional displays with limited application ranges. Type B (intensity modulation type) is an ultrasonic section imager, commonly referred to as "B-ultrasound." It uses photoelectric signals of varying brightness to represent the strength of the received signal. As the probe moves horizontally, the light point on the display screen moves synchronously, creating a two-dimensional cross-section image scanned by the ultrasonic sound beam. B-mode ultrasound is the most important diagnostic method in clinical applications. D-type ultrasound is based on the Doppler principle.

The working principle of ultrasonic diagnosis relies on the good directivity of ultrasonic waves and physical properties such as reflection, refraction, and attenuation, similar to light. Using an ultrasonic instrument and various scanning methods, ultrasonic waves are emitted into the body and spread through tissues. When there is a difference in acoustic impedance between normal and pathological tissue, the echo signal is received and processed to form a two-dimensional cross-sectional sonogram. Due to the different interface morphology, motion status, and degree of ultrasound absorption of various tissues, their echoes have specific commonalities and characteristics. By combining physiological, pathological, and clinical knowledge with a series of human body section sonograms, the location, nature, or function of the lesion can be determined, allowing for an accurate diagnosis.

During ultrasound imaging, ultrasound beams are used to scan the human body, and images of internal organs are obtained by receiving and processing reflected signals. Ultrasound diagnostic technology is non-damaging, painless, and can be performed repeatedly. It is particularly suitable for examining soft tissues, obtaining dynamic information, and conducting dynamic research. It has high sensitivity and resolution, is small in size, and is highly automated. Recent advancements in ultrasound imaging technology include grayscale and color display, real-time imaging, ultrasound holography, and intrabody cavity ultrasound imaging. Ultrasound imaging methods are often used to determine the location, size, and shape of organs; delineate the scope and physical properties of lesions; provide anatomical diagrams of glandular tissues; and identify normal and abnormal fetuses. It is widely used in ophthalmology, obstetrics and gynecology, the cardiovascular system, digestive system, and urinary system.[9]

Color Doppler ultrasound imaging (color Doppler ultrasound) is an advanced imaging examination method that adds Doppler blood imaging technology to B-ultrasound. Known as "non-invasive angiography," it uses three frequency probes: high, medium, and low. During the examination, the probe is in contact with the skin of the relevant part through an adhesive, and the scanning results form a two-dimensional cross-sectional sonogram on the monitor, which can be saved in the form of color photographs. This method is used to examine the heart, great blood vessels, cerebral arteries, liver, spleen, kidneys,

uterus, appendages, prostate, testicles, and other organs, accurately measuring blood flow, and the size, texture, and boundaries of stones and masses. In addition, it can be used in clinical interventional examination and treatment.

3.4. Nuclear medicine imaging

The nuclear medicine imaging system, also known as the radionuclide imaging system, detects rays emitted by radionuclides in the human body. The image signal reflects the concentration distribution of the radionuclide and displays both morphological and functional information. Nuclear medicine imaging is essentially different from other imaging modalities. Its images depend on factors such as blood flow, cell function, cell number, metabolic activity, and excretion and drainage of organs or tissues, rather than changes in tissue density. It is a functional image, and the clarity of the image mainly depends on the functional status of the organ or tissue. Since changes in functional metabolism during disease processes often occur before morphological changes, nuclear medicine imaging is considered one of the earliest diagnostic methods of significant value.

Nuclear medicine imaging technology is an imaging method that captures the radioactive differences between normal and diseased tissues within and outside the organ. Nuclear medicine imaging examination first involves selectively administrating certain radioactive drugs (trace, accurately targeted, safe, and harmless enhanced tracers) to the human body. These drugs accumulate in specific organs or participate in certain bodily processes. During these metabolic processes, radionuclides emit gamma rays, which then photographed by nuclear medicine imaging instruments photograph to get images on the concentration distribution and metabolic process of these radionuclides in organ tissues. Nuclear medicine imaging examination methods are widely used in medicine. The primary difference between it and XCT is that the ray source of XCT is outside the body, while the ray source of nuclear medicine imaging is inside the body. Nuclear medicine imaging technology can obtain not only anatomical images of human organs but also physiological, biochemical, pathological processes, and functional images. Using mathematical algorithms, a three-dimensional "transparent human body" image showing the density distribution of radioactive elements in the body can be reconstructed in a computer.

At present, the main technologies for nuclear medicine imaging include gamma photography, single-photon emission computed tomography (SPECT), and PET. The latter two are collectively called emission CT, with PET also becoming a primary technical means for studying brain function.

The gamma camera consists of three parts: a probe, an electronic circuit, and a display device. The gamma camera can observe all parts of an entire organ image simultaneously in a short period of time. It has high sensitivity, can image at 1 time, and can conduct continuous and dynamic observation of the organ. Cameras equipped with electronic computers are most suitable for quantitative analysis and rapid continuous dynamic analysis of organ and tissue imaging, significantly improving the diagnostic effect of *in vitro* imaging and being valuable for diagnosing cardiovascular diseases, tumors, and other conditions.

The SPECT probe is a gamma camera that rotates 360° around an organ of the patient. During rotation, it collects a frame of images at specific angles. After computer processing, the images are superimposed, and the filtered back-projection method is used to reconstruct cross-sectional images from a series of projections. From the three-dimensional information of transverse tomographic images, sagittal, coronal, and arbitrary oblique tomographic images can be obtained through image recombination. SPECT combines the characteristics of both gamma cameras and CT, allowing for stereotaxic positioning and dynamic observation of changes in local function and metabolism of organs. The detection rate of lesions and the clarity of imaging are better than those of gamma cameras.

PET[10] is a relatively advanced clinical examination imaging technology in the field of nuclear medicine. The general method involves labeling a substance necessary for the metabolism of biological life, such as glucose, protein, nucleic acid, or fatty acid, with a short-lived radionuclide (such as fluorine-18 or carbon-11), and then injecting it into the human body. The accumulation of this substance in metabolism reflects the metabolic activities of life, thereby achieving the purpose of diagnosis. The main substance used in hospitals is fluorodeoxyglucose, commonly abbreviated as FDG. Its detection mechanism relies on the fact that the metabolic status of different tissues varies. In high-metabolizing malignant tumor tissues, glucose metabolism is strong, and there is greater accumulation. These characteristics can be reflected through images, enabling the diagnosis and analysis of lesions.

3.5. Near-infrared imaging

The infrared thermal imaging device uses an infrared detector to detect infrared rays radiated from the surface of the human body and convert them into electrical signals. The infrared camera acquires the video signal, which is then amplified, filtered, and sent to a computer for imaging. This technology can be used to diagnose temperature-related diseases, such as superficial tumors,

early-stage breast cancer, peripheral vascular diseases, the viability of replanted limbs, and skin conditions. Medical infrared thermal imaging cameras display the temperature distribution of the human body (thermal image) to indicate changes in the temperature distribution, location, and extent. Various inflammations and malignant tumors cause higher temperatures in the affected areas on the heat map, while embolisms, decreased microcirculation, and effusions show lower temperatures. Near-infrared thermal imaging diagnoses diseases based on the difference in thermal radiation between normal and abnormal tissue areas. By receiving infrared thermal radiation during the metabolism of body cells, it measures the depth, shape, and intensity of the thermal radiation source. Based on the thermal radiation of the whole body, after sorting and quantifying the corresponding relationship between the distribution status and each tissue and disease, the image reconstruction algorithm is used to obtain the temperature image and then cross-sectioned. By analyzing the relationship between the thermal radiation differences in the body's cell metabolism and the health status, the location and nature of the body's lesions can be determined, providing a basis for diagnosis. Near-infrared thermography is non-invasive and can estimate the physiological and molecular processes of intact tissues of the body, providing corresponding chemical and physical information.[11] Near-infrared thermal imaging can effectively evaluate tumor markers and can be used clinically for diagnosing tumors, cardiovascular and cerebrovascular diseases, respiratory system diseases, digestive system diseases, and more. It can detect lesions earlier than other examination methods, making it especially valuable for the differential diagnosis of tumors.

3.6. Medical endoscopic imaging

A medical endoscope is a diagnostic instrument that is directly inserted into the inner cavity of human organs to observe the surface shape of the inner cavity in real time. The images it obtains are realistic and intuitive. There are more types of endoscopes, each designed for specific cavities of the human body, such as the esophagoscope, gastroscope, enteroscope, colonoscope, choledochoscope, mediastinoscope, bronchoscope, urethroscope, cystoscope, renal pelvis endoscope, colposcope, hysteroscope, laparoscope, and arthroscope. Recently, thin-diameter endoscopes with diameters as small as 0.07 mm have been introduced. An endoscope can be used for multiple purposes, including examining coronary arteries. Laser endoscopy and three-dimensional endoscopy are also under development. Laser endoscopy is a new generation of endoscope that combines diagnostic and treatment functions. Three-dimensional endoscopes can provide three-dimensional images, enabling difficult operations to be performed smoothly and significantly improving the safety of surgeries.

The invention of laser technology in the 1960s was one of the major scientific and technological achievements of the 20th century. The high directionality, brightness, monochromaticity, coherence, and biological effects of lasers have been widely used in medicine. Medical lasers are often used for surgical cutting, tissue cauterization, coagulation and hemostasis, light needle acupoint irradiation, laser blood cell counting, laser microspectroscopy analysis, laser holography diagnosis, and laser Doppler blood flow velocity measurement. Consequently, "laser medicine" has become an important branch of medicine.

3.7. Comparison of the medical imaging technology

The comparative analysis of CT, MRI, and endoscopy highlights the distinct advantages and limitations of each modality in medical imaging. CT imaging, with its high spatial resolution and rapid acquisition, is well suited for visualizing bone structures and detecting traumatic injuries, making it invaluable in emergency settings. However, its use is constrained by ionizing radiation exposure, which poses risks, particularly in sensitive populations.[5] In contrast, MRI offers superior soft-tissue contrast and multiplanar imaging capabilities, making it indispensable for evaluating neurological, musculoskeletal, and cardiovascular conditions without the use of ionizing radiation. Nonetheless, MRI is associated with longer acquisition times, higher costs, and contraindications in patients with certain metallic implants or claustrophobia. Endoscopy provides direct visualization and intervention capabilities, enabling real-time examination and tissue sampling for accurate diagnosis and targeted interventions in gastrointestinal and respiratory disorders. However, its invasive nature carries inherent risks such as perforation, bleeding, and infection and may require sedation or anesthesia, leading to patient discomfort and procedural complexity. Overall, understanding the strengths and limitations of CT, MRI, and endoscopy is essential for health-care professionals to effectively utilize these modalities in clinical practice, ensuring optimal patient care and outcomes.

4. Safety of the medical imaging technology

When evaluating medical imaging systems, the safety of the human body is a critical concern. The damage caused by ionizing radiation to the human body primarily includes both direct damage (such as local redness, hair loss, and a possible increase in the incidence of certain diseases) and indirect damage, mainly genetic damage (such as genetic mutations and deformities). In general speaking, CT scans account for about 2% of radiological examinations, but the radiation dose released exceeds the radiological average

and accounts for about 20% of the population's radiation exposure. The annual number of head CT examinations accounts for about 60% of total examinations. In addition, nearly half of the head CT examinations include both plain and enhanced scans, doubling the radiation dose. Therefore, the potential radioactive hazards of CT cannot be ignored. For example, if a pregnant woman is exposed to radioactive lead during the 3rd month of pregnancy, it may cause serious consequences such as fetal hydrocephalus, hematopoietic system defects, and skull defects. Therefore, when pregnant women undergo CT examination, they need to place an X-ray protection device on the abdomen to avoid and reduce the risk of fetal malformations.

During operation, the MRI instruments can generate strong static magnetic fields, ultra-low-frequency and highly variable electromagnetic fields, and radiofrequency fields. Radiofrequency fields are not an important part of MRI protection. At present, MRI protection mainly relies on shielding from static magnetic fields and ultra-low-frequency high-variable magnetic fields, especially static magnetic fields. In addition, MRI is an expensive and sophisticated equipment that usually requires an air-conditioning system, resulting in higher indoor radon concentrations.[12] To prevent the impact of magnetic fields on the human body, magnetic field shielding is commonly used, and methods such as copper mesh are used for degaussing. However, MRI is generally much less harmful to the human body than CT.

Nuclear medicine imaging, also known as radioisotope imaging, also causes ionizing radiation exposure in the human body. In X-ray photography, although the intensity of radiation is relatively high, the patient is only exposed for a short period of time. In contrast, the concentration of radioactive materials used in radioisotope imaging is very low, but the exposure of radiopharmaceuticals to the human body continues for a period of time until they are excreted from the body or decay. Therefore, an important factor to consider when selecting a radioactive material formula is the requirement for a short half-life.

When evaluating the ionizing radiation damage caused by X-ray and radioisotope imaging in the human body, attention should be given to both the intensity and duration of exposure. In X-ray imaging, the radiation intensity is relatively high, but the exposure time is short. Conversely, although the concentration of radioactive isotope materials is low, the exposure to the human body lasts for a longer period until it is excreted or decays. Therefore, during X-ray examinations, the dose to the human body should be minimized as much as possible. When selecting radioactive materials, their short half-life should be considered to reduce prolonged exposure.

Available statistics indicate that the levels of ultrasound exposure used in diagnosis do not cause harm to the human body. Due to its non-destructive and non-invasive nature, ultrasound imaging has been increasingly used in clinical practice. This is especially true for sensitive areas, such as fetal and eye examinations, where ultrasound is much safer than X-ray. However, even ultrasound imaging should be used with caution for embryos in the early stages of development.

5. Development trends in medical imaging technology

The future development trend of modern medical imaging aims to improve information transmission methods, improve information transmission efficiency, create new methods of information expression, and improve image display quality on the premise of ensuring personnel safety. The ultimate medical significance is to more accurately detect initial pathological changes in human tissues, providing a basis for early diagnosis and treatment. Medical imaging technology is evolving from analog to digital images; from two-dimensional images to three-dimensional images; from local images to overall images; from macro images to micro images; from static images to dynamic images; from morphological to functional images; and from single to integrated images.[3] This includes acquiring multi-temporal (dynamic) images, multi-dimensional images, multi-parameter images, multi-modal images, and multiple clinical indicators (including lesion examination, characterization, organ function assessment, and blood flow estimation), as well as treatment aspects (including three-dimensional positioning, volume calculation, and surgical planning) and multi-regional display observation. Improvements can be made from the following aspects:

(i) Developing ultra-high-resolution phenomenon systems
(ii) Enhancing the performance of imaging equipment and adding new functions. For instance:
    - MRI: Incorporating magnetic resonance spectroscopy imaging[13]
    - Ultrasound: Implementing color blood flow imaging, intracavity ultrasound imaging, digital processing for three-dimensional image display, and ultrasound CT
    - CT: Continuing to improve spatial resolution and scanning speed, focusing on studying changes in disease metabolism, and reducing costs.

Medical images can intuitively display the anatomical structure, form, and functions of various tissues and organs inside the body, making them indispensable in clinical diagnosis. However, existing imaging examinations cannot yet fully meet clinical diagnostic needs, as any single

imaging method can only obtain partial information about the body and cannot provide a sufficient basis for accurate diagnosis. At present, no imaging results can completely replace clinical examinations, and imaging needs to be combined with other examination methods to achieve accurate diagnosis.

In recent years, advancements in medical imaging technology have significantly enhanced its role in clinical diagnosis. However, several challenges remain, such as avoiding interference from various factors during the imaging process and improving imaging quality. In multi-modal imaging, there is a need for better integration of images from different sources after certain transformations to obtain comprehensive information about the anatomy and function of the body from a single image. Some imaging methods still require improvement. For instance, MRI primarily relies on hydrogen molecular imaging, which poses challenges for imaging areas like the alveoli, which are mainly gas filled and contain almost no water. From a safety perspective, although the concentration of radioactive tracers used in nuclear medicine imaging is very low, these radioactive tracers cause radiation exposure in the patient's body until they are eliminated or decay. Therefore, when selecting a radioactive tracer, its half-life and radiation dose should be considered.[14]

In medical image analysis, convolutional neural networks (CNNs) achieve over 90% accuracy, aiding in lesion identification.[15] They efficiently extract features, expediting diagnosis and reducing physician workload, thereby improving diagnostic consistency. CNNs find broad applications, revolutionizing medical imaging across domains such as radiology and pathology.[16] CNNs distinguish themselves from other artificial neural networks primarily through their incorporation of convolutional layers. This addition significantly enhances the performance of neural networks, spurring the creation of numerous convolutional models and techniques aimed at further optimization and innovation.[15] A novel approach, termed the differential CNN, coupled with simultaneous multidimensional filter implementation, has enhanced the performance of CNNs while mitigating the computational cost associated with conventional methods.[17] Techniques such as deep learning and machine learning algorithms, including CNNs, have demonstrated remarkable success in tasks such as tumor detection, lesion localization, and disease classification, substantially augmenting the precision and effectiveness of image interpretation. Furthermore, AI leverages extensive data analysis and pattern recognition capabilities to unveil intricate patterns and features within images, aiding in disease risk assessment and prediction.[18] By analyzing vast repositories of image data alongside clinical information, AI facilitates the identification of disease risk factors, enabling proactive interventions and personalized medical management. Moreover, AI-driven image reconstruction and enhancement methodologies refine image quality and resolution, empowering physicians with clearer and more informative visuals for precise diagnostic and therapeutic decision-making.[19,20] In essence, the fusion of medical imaging technology and AI signifies a paradigm shift in medical practice, underpinning enhanced diagnostic accuracy, personalized care, and elevated standards of health-care delivery.[21]

6. Conclusion

The development of medical imaging technology will accelerate, with applications becoming more mature, image quality becoming clearer, and the advantages of imaging increasingly integrated. This progress brings new hope to countless patients and will contribute significantly to the prevention, early diagnosis, and treatment of diseases.

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

A historical perspective on clonidine as an alpha-2A receptor agonist in the treatment of addictive behaviors: Focus on opioid dependence

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Abstract

Clonidine operates through agonism at the alpha-2A receptor, a specific subtype of the alpha-2-adrenergic receptor located predominantly in the prefrontal cortex. By inhibiting the release of norepinephrine, which is responsible for withdrawal symptoms, clonidine effectively addresses withdrawal-related conditions such as anxiety, hypertension, and tachycardia. The groundbreaking work by Gold *et al.* demonstrated clonidine's ability to counteract the effects of locus coeruleus stimulation, reshaping the understanding of opioid withdrawal within the field. In the 1980s, the efficacy of clonidine in facilitating the transition to long-acting injectable naltrexone was confirmed for individuals motivated to overcome opioid use disorders (OUDs), including physicians and executives. Despite challenges with compliance, naltrexone offers sustained blockade of opioid receptors, reducing the risk of overdose, intoxication, and relapse in motivated patients in recovery. The development of clonidine and naltrexone as treatment modalities for OUDs, and potentially other addictions, including behavioral ones, underscores the potential for translating neurobiological advancements from preclinical models (bench) to clinical practice (bedside), ushering in innovative approaches to addiction treatment.

Keywords: Behavioral addictions; Clonidine; Opioid use disorder; Substance use disorder; Naltrexone; Locus coeruleus

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1. Introduction

Clonidine is a well-researched molecule patented in 1961 but was not used medically until 1966.[1] Catapres is a brand name for clonidine, which is used to treat high blood pressure, as well as, off-label, attention deficit hyperactivity disorder (ADHD),[2,3] withdrawal from substances such as opioids, alcohol and nicotine, menopausal flushing, and selected painful conditions.[2,3] Administration of clonidine can be oral, transdermal, or by injection with an onset of action within 1 h. Most side-effects are reversible when withdrawn.[4]

To design a comprehensive review, our team searched PubMed, MEDLINE, Cochrane Library, and references from relevant articles for publications dating from June 1, 2014, to August 1, 2020. We searched for the Medical Subject Headings terms "Opioid-Related Disorders," or "Analgesics, Opioid" and "Substance Withdrawal Syndrome." This work is worthy of a thorough review as current reviews of clonidine often overlook much of the laboratory and clinical discovery work focusing on 2014 – 2020.[5]

2. Pharmacokinetics and pharmacodynamics of clonidine

Importantly, clonidine crosses the blood-brain barrier.[6] Gold *et al.*[7] demonstrated that clonidine's molecular mechanism of action occurs due to its agonism at the alpha-2A receptor, a subtype of the alpha-2 adrenergic receptor, found primarily within the prefrontal cortex (PFC). Alpha-2A adrenergic receptors inhabit the presynaptic cleft of the neuron and, when activated by an agonist, inhibit downstream neurons. The stimulation of alpha-2 receptors arrests the secretion of the neurotransmitter norepinephrine (NE).

While usually not severe, common side effects of clonidine include dry mouth, dizziness, headaches, and sleepiness. However, in rare cases, severe adverse effects include heart arrhythmias, confusion, and even hallucinations. Clonidine should be avoided during pregnancy or breastfeeding because it crosses the placental barrier and is present in breast milk. Moreover, if abruptly stopped, withdrawal reactions could occur. Clonidine, first patented in 1961, was the 79th most commonly prescribed pharmacologic agent in the United States and by 2017, with over 10 million prescriptions.[8]

Following oral ingestion, the drug is absorbed into the bloodstream very promptly and almost completely, with peak concentrations in human plasma within 60 – 90 min.[9] It is important to emphasize that clonidine is lipid-soluble to some extent. The partition coefficient logarithm (log P) is equal to 1.6.[10] It is well-known that the optimal log P for a drug to enter the central nervous system through the blood-brain barrier is 2.0.[11]

Approximately one-fifth of an oral dose will not be absorbed and is excreted in the feces, while under half of the absorbed dose will be metabolized by the hepatic tissue into inactive metabolites, with the rest excreted unchanged

by the kidneys. Moreover, the half-life of clonidine varies widely, between 6 and 23 h, depending on kidney function.[11]

2.1. The off-label use of clonidine to treat opioid withdrawal syndrome

The off-label use of clonidine to ease symptoms associated with abrupt withdrawal from long-term use of opioids, alcohol, benzodiazepines, and nicotine[12,13] is the main topic of this review. Clonidine can alleviate opioid withdrawal symptoms by reducing the sympathetic nervous system response, including tachycardia, hypertension, sweating, hot and cold flashes, anxiety, and general restlessness. These sedating effects of clonidine may also aid smokers in quitting. However, side effects can include insomnia, exacerbating an already common feature of opioid withdrawal.[14] Clonidine induces a reduction in blood pressure in both normotensive and hypertensive patients but may also induce hypotension and postural hypotension during opioid withdrawal. Notably, clonidine may also reduce the severity of neonatal abstinence syndrome for infants with maternal substance use disorder.[15,16] Although off-label clonidine has been replaced clinically by buprenorphine and other treatments,[16] it may improve the Network Neurobehavioral Score in neonatal intensive care units for neonatal withdrawal syndrome.[17]

2.2. Better outcomes for impaired health professionals: Why?

Opioid use disorder (OUD) is common and generally untreated. Medications and medication-assisted recovery have gained support as it is evidence-based, safe, and useful.[18] Nevertheless, most adults with OUD do not receive outpatient treatment to address their addiction and remain untreated.[19] At present, outcomes for impaired health professionals[20] and others with OUD are markedly different, even when receiving the same treatments. Relapse to OUD and treatment discontinuation are common among most patients but not among impaired physicians.[21] The fear of overdose, slip, or relapse, which can result in death, may differ due to the fear of losing licensure requirements as professional and mandated requirements to be drug-free.[22] The treatment procedures, follow-up, and case management available to physicians through impaired health professional programs, including group therapy, caduceus meetings, medication, and particularly random urine testing, contribute to their successful recovery.[22] The usual goal of treatment for OUD is being alive and taking the opioid agonist or antagonist medication. Urine testing confirmed OUD outcomes for impaired physicians at 80%, with most tested drug-free and functioning at premorbid levels at 5-year follow-ups.[23] These outcomes are significantly better than those reported for non-health professionals, who are rarely studied for at least 6 months. The characterization of OUD treatment outcomes includes treatment discontinuation, dropouts, relapses, overdoses, and numbers of hospital visits.[24] Return to premorbid functioning socially, jobwise, and in other spheres are not investigated as thoroughly as it is for physicians. Physician outcomes focus on full recovery and return to work.[25] Thus, OUD treatment tends to replace opioids with medications such as buprenorphine and methadone.

Physicians and other health professionals are likely to opt for detoxification from opioids and placement on long-acting injectable naltrexone.[26] The frequent choice of clonidine may be related to licensure and drug-free job regulations. Clonidine's choice may also relate to changes in perception and cognitive functioning felt on chronic opioids compared to the effects of detoxification and abstinence or detoxification and naltrexone. In the highest-risk group of physicians with OUDs, such as anesthesiologists, the decision to treat with naltrexone may be directed by the physician health program itself.[27] In this case, clonidine may be incorporated in post-assessment detoxifications.

Clonidine is widely used today as an adjunct treatment for opioid withdrawal, OUD-related craving, and anxiety, and in the transition to naltrexone for treating physicians, executives, and other patients with OUDs.[28] The neurochemical mechanisms of clonidine, especially related to catecholaminergic activity involving NE and dopamine, provide promising treatments to reverse opioid-induced changes in the locus coeruleus (LC) and boost dopaminergic recruitment across this brain region to attenuate NE hyperactivity.[28,29]

Although the United States Food and Drug Administration (FDA) approved lofexidine,[30] which has a higher affinity and specificity for alpha-2A adrenergic receptors, induces less hypotension and other serious side effects than clonidine, and does not reinforce opioid dependence, the high cost of lofexidine has kept clonidine ahead of lofexidine prescriptions for OUD detoxification.[31]

2.3. Opioid withdrawal: Clinical syndrome and pathophysiology

Despite effective treatment for opioid addiction, including buprenorphine (suboxone) and methadone, most patients still relapse into opioid misuse, often resulting in overdoses during these slips and relapses. Acute precipitants, such as stress, exposure to drug-associated cues, or the use of an initially small amount or priming dose of a drug, can trigger relapses, shorter lapses, and episodes of craving. Treatments that buffer the effects of these acute triggers might improve buprenorphine maintenance outcomes.

2.4. Definitions of withdrawal

Withdrawal from a substance is characterized in the Diagnostic and Statistical Manual for Mental Disorders, 5th edition (DSM-5), as "a substance-specific problematic behavioral change, with physiological and cognitive concomitants, that is due to the cessation of, or reduction in, heavy and prolonged substance use." The International Classification of Diseases, 10th edition, defines withdrawal as "a group of symptoms of variable clustering and severity occurring on absolute or relative withdrawal of a psychoactive substance after persistent use of that substance." The characteristic clinical signs of opioid withdrawal syndrome include hypertension, tachycardia, mydriasis, piloerection (goosebumps), lacrimation, rhinorrhea, yawning, insomnia, nausea, vomiting, and diarrhea.[32] The progression of opioid withdrawal is primarily influenced by the half-life of the specific opioid involved. Opioids characterized by short half-lives, such as heroin (with a half-life of 3 – 5 h), prompt the onset of withdrawal symptoms within approximately 12 h after the last dose. Conversely, discontinuation of opioids with longer half-lives, such as methadone (with a half-life of up to 96 h), may lead to withdrawal symptoms emerging 1 – 3 days following the last dose. Moreover, the duration of the withdrawal syndrome typically aligns with the half-life of the opioid. For instance, heroin withdrawal typically spans 4 – 5 days, while methadone withdrawal can extend from 7 to 14 days and, in certain cases, persist for several weeks. Other than alpha-2 receptor agonists, other agents, such as pro-dopamine regulators, may be useful adjuncts to intervene in heightened NE activity during opioid withdrawal.

2.5. Development of the locus coeruleus noradrenergic hyperactivity theory

In 1977, Gold's group tested clonidine in humans with OUD after opioid discontinuation and the emergence of withdrawal signs and symptoms as a test of the LC noradrenergic hyperactivity theory.[33,34] These clinical scientists chose clonidine over other available alpha-2 adrenergic agonists because clonidine was widely used worldwide and considered safe, effective, and approved by the FDA for hypertension. During that period, the WHO was concerned about any medication that reversed opioid withdrawal in laboratory investigations. In the experiments, clonidine acutely reversed opioid withdrawal, including neonatal opioid withdrawal distress, reduced naloxone-precipitated withdrawal distress, facilitated rapid and ultra-rapid opioid detoxification, and provided an option for impaired health professionals and others interested in a drug-free treatment. Clonidine also improved the transition from opioid agonist to naltrexone and enhanced treatment outcome success rates for both naltrexone and buprenorphine.[35-38]

Gold's group conducted a series of studies on LC stimulation and ablation in rodents at the College of Medicine, University of Florida, in the early 1970s, and later at Yale in the late 1970s and early 1980s. These studies led Gold and his associates to hypothesize that the nucleus LC might be responsible for some opioid withdrawal syndrome symptoms. They continued this work with rats and non-human primates at Yale in the Aghajanian and Redmond laboratories. Specifically, they stimulated the LC and produced hypertension, tachycardia, and other signs of opioid withdrawal, including piloerection (bristling) in animals that had never been exposed to opioids.[39] This LC electrical stimulation produced signs, symptoms, and behaviors similar to those induced by the alpha-2 adrenergic antagonists yohimbine and piperoxane.[40] They could reverse the effects of electrical stimulation with morphine, and this effect could be reversed again with the opioid antagonist naloxone.[41] Moreover, these researchers could reverse the effects of yohimbine and piperoxane with clonidine.[42] They were also able to pre-empt the effects of these agents by lesioning the nucleus LC.[39]

As a known alpha-2 adrenergic receptor agonist, clonidine was first tested in rodents and non-human primates and ultimately in humans in cases of both precipitated and naturally occurring opioid withdrawal by Gold's group in the 1970s. This work (Gold *et al*., 1982), recognized by the American Psychiatric Association with the Foundations Fund Annual Award and Prize, represents the first true translation of basic science into discoveries that help patients in psychiatry.[43]

2.6. Mechanisms in withdrawal symptomatology

The noradrenergic hyperactivity theory for opioid withdrawal changed the field in many ways.[34,42] First, it provided the first neuroanatomy of opioid withdrawal, which could be tested, and identified the roles of alpha-2 adrenergic and opioid inputs. This allowed for a better understanding of both opioid and non-opioid treatments or withdrawal reversal methods. Second, it enabled physicians to explore and develop new opioid, mixed opioid, and non-opioid treatments, including pharmaceuticals and nutraceuticals.[44,45-50] Third, it introduced a new class of treatments, such as lofexidine, guanfacine, and others, which use the same mechanism of action–alpha-2 adrenergic agonist stimulation and inhibition of the nucleus LC.[31,43,51] These treatments could potentially exhibit better side effect profiles and other advantages.[36,52]

2.7. Summary of empirical research

Several behavioral and biochemical studies[53] support Gold's hypothesis that naloxone-precipitated withdrawal can be attenuated by targeting the LC. Subsequent studies demonstrated that clonidine reduced morphine withdrawal-induced increases in regional cerebral metabolic rates for glucose, irrespective of the distribution of alpha-2 adrenergic receptors. Clonidine acts primarily at the LC and central amygdala, and it may also have importance in other regions.[54]

Research conducted on non-human primates has revealed that the noradrenergic LC may play a role in various aspects of the brain's alarm function, encompassing attentiveness, arousal, anxiety, fear, and terror, along with their physiological manifestations. These investigations involved comparing the outcomes of electrically stimulating the LC with minute electrodes to the effects induced by other agents or conditions capable of modulating LC activity. The findings suggested that endogenous morphine-like substances and opioids serve to inhibit the activation of the LC system, and the onset of opioid withdrawal syndrome arises from the reactivation of this LC-noradrenergic system.[35] Clonidine, which suppressed noradrenergic LC activity in low doses, was therefore postulated to suppress opioid withdrawal signs and symptoms. Many signs of opioid withdrawal produced through electrical or chemical stimulation of the nucleus LC increase noradrenergic activity and the concentration of the noradrenergic metabolite 3-methoxy-4-hydroxy-phenyl glycol within the brain. Clonidine, an alpha-2 adrenergic agonist, can inhibit signs of opioid withdrawal in animals and humans.[55]

Clonidine likely attenuates opioid withdrawal syndrome due to the reduction of noradrenergic neuronal activity originating in the LC. However, alpha-2 adrenergic receptors located throughout the body and other mechanisms may also play a role. In a series of studies, Gold's group explored the LC alpha-2 adrenergic receptor selectivity and the neuroanatomical and pharmacological anti-withdrawal action of clonidine (Table 1). Confirmation of this hypothesis in rats, monkeys, and human subjects has added to the understanding of the mechanisms of opioid action and withdrawal.

Moreover, a double-blind, placebo-controlled, and cross-over trial from Taylor *et al.*[53] found that clonidine eliminated the symptomology of opioid withdrawal for 240 – 360 min in 11 hospitalized OUD subjects. In the longer term, the same patients, in an open pilot study of the effects of clonidine taken for 1 week, also experienced the elimination of opioid abstinence symptoms. These data suggest that opioid withdrawal is due to increased neuronal activity in areas regulated by alpha-2 adrenergic and opioid receptors, like the LC. The early clinical studies, combined with more direct observations in rodents and non-human primate studies, are consistent with the hypothesis that in humans, brain NE systems become hyperactive during opioid withdrawal and that clonidine suppresses this hyperactivity of NE systems.[29]

3. Modern architectural analysis of treatment for OUD: Inducing "dopamine homeostasis" to treat protracted withdrawal

In the United States, a national opioid epidemic[4] has prompted the recommendation of three FDA-approved medications for the prevention and treatment of OUD: methadone, buprenorphine, or naltrexone. There is ample evidence of their efficacy; however, these medications are under-prescribed.[18] The objective here is to briefly review and synthesize data from the available medical literature on these FDA-approved medications and provide a framework to demonstrate the optimal approach for outpatient management of OUD.

Clonidine and lofexidine have improved and refined the medical approach to opioid withdrawal states while transitioning opioid-dependent adults to extended-release injection naltrexone.[56] Opioid agonists like methadone, mixed agonists like buprenorphine, and the combination of buprenorphine with naltrexone and clonidine are now used to treat OUD.[57] The authors assessed the efficacy of two outpatient opioid detoxification methods and relapse prevention in a trial transition induction to extended-release (XR)-naltrexone. A 7-day detoxification regimen utilizing naltrexone with a single day of buprenorphine administration was followed by a gradual increase in oral naltrexone doses, supplemented with clonidine and other medications. Similarly, a buprenorphine-assisted detoxification protocol involved a 7-day tapering of buprenorphine, followed by a week-long interval before initiating XR-naltrexone, in accordance with official prescribing guidelines. The combination of naltrexone treatment and adjunctive clonidine facilitated complete withdrawal for 38 out of 40 methadone-dependent patients within a span of 4 – 5 days. Naltrexone dosing typically commenced at 1 mg/day and was incrementally raised to 50 mg/day over a 4-day period for most patients. Clonidine administration helped mitigate the intensity of naltrexone-induced withdrawal symptoms. Significant reductions in blood pressure were observed without instances of syncope, and although certain symptoms persisted, including anxiety, anorexia, insomnia, restlessness, and muscular aching, they were either substantially alleviated

Table 1. Key facts

Clonidine.
Locus coeruleus stimulation promotes the release of norepinephrine.
Reduces the severity of withdrawal from opioid use.
Narcotic replacement therapy
Methadone-synthetic opioid and buprenorphine (Subutex) are agonists.
Suboxone; buprenorphine/naloxone is an agonist/antagonist.
Buprenorphine and methadone maintenance are equally effective in retaining patients in substance abuse treatment and in reducing illicit opioid use.
Narcotic replacement therapies have high treatment compliance.
Reduce overdoses and Emergency Department treatment-seeking.
Subutex and suboxone induction and maintenance are available in outpatient or physician offices.
Disadvantages buprenorphine
Chronic blockade of opioid receptors has anti-reward effects, increasing relapse potential when coupled with a narcotic antagonist
Does not activate the areas of the brain associated with relapse in some studies.
Possible lack of effectiveness in patients who require high methadone doses.
Locks people into addiction and also causes a "zombie" like effect.
There is evidence of potential suicide ideation and accompanied depression.
Even in the injectable form of delivery, there is poor compliance.
Naltrexone
Naltrexone is an opioid antagonist.
Provides chronic opioid receptor blockade and prevents overdose and opioid intoxication.
Agonists are better at this than antagonists unless in mandated impaired physician programs with monitors.
The main issue with naltrexone is poor compliance, but it can be assisted with Pro-dopamine regulation like KB220 variants or other modalities like rTMS.
Positive effects of treatment
All forms of treatment are significantly less costly and more effective than no treatment.
Reduction or abstinence in illicit opioid use.
Reduction in the severity of withdrawal from opioid use.
Retention in treatment for persons enrolled in opioid withdrawal or opioid cessation programs.
Harm reduction
Summary Points
• The adverse effects of OUD include (fatal overdose, infectious disease transmission, elevated health care costs, public disorder, and crime) and the available treatments.
• The alpha-2-adrenergic receptor a subtype of the alpha-2-adrenergic receptor secretes norepinephrine (NE).
• NE causes the symptoms of withdrawal.
• Withdrawal-symptoms include hypertension, tachycardia, and anxiety.
• Clonidine is a molecular agonist of the alpha-2A receptor.
• Gold *et al.*[7] found that clonidine can reverse the effects of locus coeruleus stimulation.
• This noradrenergic hypothesis for opioid withdrawal changed the field.
• Successful comprehensive treatment programs that used clonidine to transition to long-acting injectable naltrexone for impaired physicians and other very motivated patients with OUDs were confirmed in the 1980s.
• The naltrexone, despite poor compliance, provides chronic opioid receptor blockade that prevents overdose, opioid intoxication, and subsequent re-addiction in recovered in motivated patients.
• The development of clonidine and naltrexone as treatment agents for OUD demonstrates that neurobiological advances could be translated from rodents to non-human primates to man into new effective clinical approaches.
• The traditional narcotic substitution therapies, like methadone maintenance, provide agonistic activity but do not target or block delta or mu receptors. The combination treatment of narcotic antagonism and mu receptor agonist therapy (even at minimal doses of naloxone) seems parsimonious but may induce anti-reward
• Clinical studies indicate that buprenorphine maintenance is as effective as methadone but less cardiac adverse effects maintenance in retaining patients in substance abuse treatment and in reducing illicit opioid use.
• Clinical studies indicate that buprenorphine maintenance is as effective as methadone maintenance in retaining patients in substance abuse treatment and in reducing illicit opioid use.
• The negative effect on reward circuitry is that chronic blockade of opioid receptors, even with partial opioid agonist action, may ultimately block dopaminergic activity, causing anti-reward effects and increasing relapse potential.
• Based on initial results with large populations receiving D2 agonist therapy with KB220, a safe, non-addicting, natural dopaminergic receptor agonist that potentially up-regulates instead of down-regulating dopaminergic receptors could be a co-therapy for long-term treatment to prevent relapse rather than the combination of buprenorphine/naloxone alone.
• Futuristic frontline modalities should include genetic addiction risk testing, which could lead to precision medicine by matching polymorphisms in risk alleles with medications or nutraceuticals.

Abbreviations: rTMS: repetitive transcranial magnetic stimulation; OUD: Opioid use disorder.

or resolved entirely by the time of discharge. The use of clonidine for opioid detoxification may pave the way for naltrexone maintenance in many clinical settings and might also succeed with patients receiving methadone doses up to 50 mg/day.[56]

This development of clonidine and naltrexone as a treatment for opioid addiction demonstrates the translation of neurobiological advances into new and effective clinical approaches. Naltrexone provides a chronic opioid receptor blockade, which prevents opioid intoxication and subsequent re-addiction in recovery. This sequential use of naltrexone for opioid receptor blockade, in conjunction with clonidine to treat withdrawal symptomatology during rehabilitation, represents a viable and effective treatment for opioid addiction in motivated patients.

3.1. Summary of the clonidine/naltrexone approach to opioid withdrawal

Gold *et al.*[58] summarized experiences with the clonidine/naltrexone approach in motivated OUD patients. Clonidine hydrochloride, an alpha-adrenergic agonist, is a non-opioid medication that, when used in detoxification from opioids, exhibits rapid suppression of the signs and symptoms associated with opioid withdrawal. Studies have demonstrated that clonidine is useful in detoxifying for withdrawal from methadone maintenance patients, achieving zero dosage in <14 days with a high success rate, compared to the usual 3 – 6 months. In a clinical investigation, clonidine suppressed opioid withdrawal symptomatology in patients on doses of up to 75 mg of methadone daily, and shorter-acting narcotics withdrawn in less than a week. To prevent relapse, post-detoxification counseling and the use of the narcotic antagonist, naltrexone, are recommended.[37]

Clonidine's ability to reverse opioid withdrawal syndrome in acute withdrawal and anti-craving studies supported the NE hypothesis and suggested a new use for clonidine.[32,55,59-63] The effectiveness of lofexidine provided further validation for the noradrenaline (NA) hypothesis. Clonidine has been demonstrated to be a potent emergency intervention for acute opioid withdrawal, facilitating detoxification from methadone, heroin, and other opioids. By reversing cognitive, affective, and physiological manifestations of withdrawal, clonidine not only alleviates immediate symptoms but also maintain suppression of their reoccurrence when administered over a period of 10 – 14 days within a detoxification regimen.[55,59]

Clonidine appears most appropriate for clinical application as a transitional intervention bridging opioid dependence and naltrexone therapy. A 10-day outpatient detoxification regimen involving clonidine has proven highly successful in enabling patients to cease opioid use abruptly and maintain abstinence long enough to commence naltrexone treatment. However, the sedative and hypotensive side effects associated with clonidine have constrained its clinical utility, particularly among outpatients, prompting exploration into alternative alpha-2 noradrenergic agonists that may offer similar anti-withdrawal efficacy without the undesirable side effects of clonidine. Initial outpatient evaluations of lofexidine, a structural analog of clonidine, suggest that it could be equally effective for opioid detoxification and potentially more suitable for outpatient management if it lacks the sedation and hypotension occasionally observed with clonidine.[64]

Blum *et al.*[65] developed a protocol that included the neuronutrient KB220Z and other anti-withdrawal agents, such as clonidine, to investigate initial detoxification from OUD in treatment centers, with particularly heavily dependent OUD subjects. Among the 17 subjects in the study, only three were administered buprenorphine/naloxone (Bup/Nx) alongside KB220Z. Initially, in this pilot phase, five patients received 6 days of KB220Z at a dosage of 2 oz twice daily before meals, in conjunction with clonidine, benzodiazepines, and other adjunctive medications such as gabapentin to manage nausea and sleep disturbances. Subsequently, the second protocol involved 12 patients receiving a higher dose of 4 oz every 6 h for 6 days. Only three individuals experienced relapse within the initial 2 weeks, while the remaining 14 subjects remained on KB220Z without requiring additional Bup/Nx for periods ranging from 120 to 214 days.

Due to the inclusion of standard detoxification agents, definitive conclusions regarding the effects of KB220Z cannot be drawn. However, the fact that only three out of 17 subjects needed Bup/Nx is notable. If corroborated by larger, more comprehensive studies, this opioid/opioid detoxification approach could offer a novel strategy for managing withdrawal without relying on addictive opioids. Combining alpha-2 agonist therapy with KB220Z, a pro-dopamine regulator, may emerge as a frontline option alongside other treatment modalities. Notably, neuroimaging studies comparing KB220Z and placebo have demonstrated robust and specific blood oxygen level-dependent dopamine activation in animal models[66] and abstinent heroin addicts,[67] suggesting putative induction of "dopamine homeostasis."

Previously, Blum *et al.* published several articles arguing against the long-term utilization of opioid agonists such as methadone and buprenorphine, except for harm reduction, but did not favor their prophylaxis use.[66-90]

In terms of post-withdrawal treatment options, many articles discuss opioid agonists and narcotic antagonism,

including alpha-2 stimulation with agents such as clonidine and lofexidine. While some of these articles may be somewhat cryptic, they expand understanding of this important topic.[60-62,91,92] Other important novel therapeutic modalities include repetitive transcranial magnetic stimulation,[93-101] exercise,[102-107] and precision addiction management, which couples genetic addiction risk testing[67,69,73,73,76-79,81,82,88,89,108-113] with pro-dopamine regulation.[65,72,72,82,111-121]

4. Long-term use of opioid agonists engendering antireward

Physicians treat opioid-dependent patients with an office-based maintenance program using buprenorphine, a partial mu-opioid receptor agonist. Basic science predicted[122] and clinical experiences have confirmed that buprenorphine effectively controls opioid withdrawal in OUD treatment, especially in fentanyl use disorders. Patients often prefer opioid replacement with detoxification and abstinence or detoxification and naltrexone. Buprenorphine is more effective than abstinence or placebo for managing opioid addiction; however, if high doses are needed, it may not be superior to methadone. Treatment phases include induction, stabilization, and maintenance. The treatment outcome is comparable to lower doses of methadone. However, the current "standard of care" necessitates the initiation of buprenorphine therapy at the onset of withdrawal symptoms, adjusted to address symptoms and craving severity. The advantages of buprenorphine include some reversal of anhedonia, good availability for office use, and somewhat lower abuse potential. Disadvantages include lack of effectiveness and high cost in patients who would require high methadone doses.

However, as a cautionary note, while short-term therapy with buprenorphine appears very appropriate, this may not be the case for prolonged maintenance therapy. The Bup/Nx combination has acute benefits for the treatment of heroin use disorder (HUD) but not for relapse prevention and may increase the probability of relapse.[123-125] Specifically, opioid agonists, such as methadone and buprenorphine, are clinically effective in reducing withdrawal and craving during heroin detoxification but fail to reduce the likelihood of relapse after detoxification.

Neuroimaging studies have significantly enhanced our comprehension of why methadone or buprenorphine often fall short in reducing the likelihood of relapse. These findings, widely recognized for their reliability, shed light on the neurobiological mechanisms underlying relapse and aid in the development of more effective therapeutic strategies. Mei *et al.*[126] conducted research investigating the immediate impacts of buprenorphine on neurological responses to cues associated with heroin. The functional magnetic resonance imaging (fMRI) investigation provided insights into the neurobiological mechanisms underlying addiction and relapse, as well as the therapeutic effects of buprenorphine. While under the influence of buprenorphine, neurological responses to cues associated with heroin diminished notably in regions including the amygdala, hippocampus, ventral tegmental area, and thalamus. However, no significant changes were observed in the ventral striatum, orbital-prefrontal-parietal cortices, or the cingulate gyrus. This absence of response in the cingulate gyrus underscores its partial role in the process of relapse.

Neuropsychological and functional neuroimaging evidence converges to indicate that the dorsal anterior cingulate cortex (dACC) is dysfunctional in substance abuse. Yücel *et al.*[127] investigated the biochemical and physiological properties of the dACC. Using fMRI and proton magnetic resonance spectroscopy (^{1}H-MRS), researchers investigated the biochemistry and physiological activity of the dorsal anterior cingulate cortex (dACC) during a behavioral control task in 24 individuals with opioid dependence. This group was compared to 24 gender-, intelligence-, age-, and performance-matched healthy subjects. While both groups exhibited comparable levels of activation in the dACC during the task, the opioid-dependent group showed heightened task-related activation in frontal, parietal, and cerebellar regions, alongside reductions in concentrations of N-acetyl aspartate and glutamate/glutamine in the dACC. Moreover, the opioid-dependent group failed to demonstrate the anticipated correlations between dACC activation and behavioral measures of cognitive control. These findings suggest that long-term opioid dependence may result in biochemical and physiological abnormalities in the dACC.

Individuals with OUD may necessitate increased activation of the frontoparietal and cerebellar networks involved in behavioral regulation to achieve normal levels of task performance and behavioral control. Tailoring treatment to the specific needs of patients who are most susceptible to the effects of chronic opioid administration appears prudent. In addition, Mei *et al.*[126] observed an unaltered fMRI response to heroin-related cues in various brain regions, including the ventral striatum, orbital, parietal, lateral, and PFC, indicating a lack of modulation by buprenorphine. This lack of buprenorphine effect on these key brain regions linked to relapse may explain its limited therapeutic effects on relapse.[128] For a review of the effects of opioid agonists on dACC function, see Lin *et al.*,[129] who found positive effects on emotional reactivity

but not reward activity in treatment-resistant mid- and late-life depression. Verdejo-García *et al.*[130] demonstrated a beneficial role of high-dose methadone on dACC biochemistry and linked elevated myoinositol levels to depressive symptoms following buprenorphine treatment. Seah *et al.*,[131] showed in a small sample (N = 4) that group-level analyses revealed buprenorphine significantly activated brain regions, including the thalamus, striatum, frontal, and cingulate cortices, compared to a saline vehicle in awake non-human primates. It is noteworthy that animal studies involving the incubation of cocaine craving have indicated that a novel target for withdrawal is the GluR2-lacking AMPA receptors in the ventral striatum.[132] This notion has received support in humans, whereby Hermann *et al.*[133] revealed a positive correlation between glutamate levels and previous withdrawals, and an increase in glutamate/glutamine with age in contrast to a decrease in controls, indicating a destabilization of the glutamate system in opioid-dependent patients and supporting the glutamate hypothesis of addiction.

There are several limitations to the long-term utilization of methadone and buprenorphine (with and without naloxone) and their associated side effects.[90,109,134-137] Moreover, Chalhoub and Kalivas[138] reviewed the limitations and challenges of the current maintenance and medication-assisted withdrawal strategies commonly used to treat OUD. Using animal models of opioid addiction, they noted the roles of endocannabinoid, orexin, and glutamatergic signaling in the expression and maintenance of addiction-like behaviors and suggested these systems as potential targets to expand therapeutic options for treating OUD. One important aspect related to the effects of chronic buprenorphine use concerns brain glucose metabolism. Walsh *et al.*[139] compared the effect of buprenorphine to a placebo and found that buprenorphine significantly reduced the cerebral glucose metabolism rate and regional cerebral metabolic rate for glucose in 19 of 22 bilateral and four midline regions by up to 32%.

4.1. Locus coeruleus: Beyond drug withdrawal

The locus coeruleus is a compact nucleus situated deep within the brainstem, serving as a pivotal hub for the extensive noradrenergic neurotransmitter system of the brain. The seminal work of Dahlströem and Fuxe[140] in 1964, which unveiled the presence of monoamine-containing neurons in the central nervous system, laid the foundation for subsequent systematic investigations into the structure and functionality of the LC. Recent research has harnessed an impressive array of advanced neuroscience techniques to delve into and understand the intricacies of this enigmatic nucleus, unearthing novel layers of organization and function, particularly pertaining to human behavior. Although all neurons within the LC receive inputs associated with autonomic arousal, subsets of these neurons can encode distinct cognitive processes, potentially through more specialized inputs originating from forebrain regions. As highlighted by Poe *et al.*,[141] the LC exhibits specific patterns, diversity in receptor distributions, and innervation of target areas, suggesting that stimulation (activation) of the LC can exert more nuanced influences on target networks than previously thought.

4.2. Stress

Stressors activate the locus coeruleus-NA (LC-NA) system through corticotropin-releasing factor (CRF), leading to an inclination toward high-tonic activity in LC neurons while reducing their responsiveness to discrete stimuli.[142] Chemogenetic LC activation might mimic acute stress, increasing brain-wide functional connectivity, especially in salience and amygdala networks. Moreover, activation initiates reduced exploratory and enhanced anxiogenic behavior.[143] Interestingly, enkephalin-containing axon terminals converge on some of the same LC dendrites as CRF-containing axon terminals.[144] Furthermore, these enkephalin-type neurons have opposing effects on LC discharge during stress,[145] implying that enkephalin afferents to the LC (acting at mu-opioid receptors) are part of the stress coping and recovery from the opioid system.[146] Importantly, gender is a determinant of LC sensitivity to stress. In animals, the LC neurons of females are more sensitive to CRF and less sensitive to enkephalin than males.[147] Indeed, Brady *et al.*[148] recommended that the higher prevalence of stress-induced psychiatric disorders in females may be partly due to the molecular effects of sex hormones. The interaction of CRF and other neurotransmitters like dopamine may yield anti-stress effects due to the blocking effect on NE. This interaction may have particular relevance for both substance and non-substance behavioral addictions.

4.3. Summary

Clonidine has played a pivotal role in the history of addiction medicine for many reasons. It was the first medication-assisted treatment (MAT) to be discovered and translated from science to practical use in rats, monkeys, and humans. The discovery of clonidine's anti-opioid withdrawal efficacy resulted from understanding LC hyperactivity or release from LC chronic opioid inhibition.[42] Kleber *et al.*[37] demonstrated that clonidine is the first non-opioid medication to reverse opioid withdrawal. Clonidine reduced detoxification distress to the point that naltrexone[149] became a viable alternative to methadone and, ultimately, buprenorphine.

Today's treatment of OUDs often begins with an overdose intervention in an emergency or hospital department, followed by a rapid transition to buprenorphine. Although treatment algorithms for OUD have been well described,[18] they are often one-size-fits-all. Many patients not engaged in this transition from active use to treatment are lost to follow-up, drop out, or continue receiving buprenorphine or methadone for years. Some patients who want to detoxify or switch to monthly naltrexone injections can benefit from using non-opioid medications, such as clonidine or lofexidine, to treat withdrawal symptoms. Non-opioid treatment options are essential for physicians and those at risk for OUDs. Clonidine is important in the transition of physicians from OUDs to naltrexone and the transition of thousands of patients maintained on methadone and buprenorphine to naltrexone. MAT discontinuation is an important overdose risk factor, and clinicians often recommend naltrexone after long-term agonist maintenance for OUDs. Clonidine may have additional roles in reducing withdrawal distress from other drug cravings during MAT maintenance and in neonates.

An intriguing concept is that receptor tolerance entails the enhancement of receptor regulation mechanisms, such as desensitization and internalization. Furthermore, as suggested by Christie,[150] the adaptations leading to cellular tolerance are multifaceted, involving several significant processes, including upregulation of cAMP/PKA and cAMP response element-binding signaling, as well as mitogen-activated protein kinase cascades in opioid-sensitive neurons. These mechanisms have implications not only for tolerance and withdrawal but also for synaptic plasticity during cycles of intoxication and withdrawal. Such adaptations could potentially impact the likelihood of relapse.

It is also important to point out that some early experiments suggested that the LC might not be a primary site for opioid-induced withdrawal. However, a complete lesion of catecholaminergic nerve cell bodies in the LC, achieved by intracerebroventricular injection of 6-hydroxydopamine, resulted in the total abolition of SS14-specific binding in the structure. Specifically bound [^{125}I][Tyr^{0},D-Trp^{8}]SS14 and TH+ cell density overlapped with SS14. Furthermore, it is known that tyrosine hydroxylase is the rate-limiting enzyme involved in the synthesis of catecholamines, especially dopamine. Gagne *et al.*[151] revealed that somatostatin binding sites are uniformly localized on all noradrenergic neurons of the LC. There is abundant evidence supporting the role of catecholamines, especially in opioid-induced withdrawal and LC.[152]

The shift to long-term or perpetual use of powerful and addictive opioids such as buprenorphine and methadone is a logical response to an OUD crisis and opioid overdose epidemic. Detoxification and abstinence are associated with more deaths, overdoses, and medical problems. It is of interest that the combination of clonidine and long-acting naltrexone maybe as effective and comparable in some cases to just using buprenorphine alone, to detoxify patients for opioid treatment (X: BOT). However, work be Lee *et al.* in an attempt to determine the potential effectiveness of naltrexone versus buprenorphine did not provide definitive results. As suggested by Lee *et al.*[153] except for health and other professionals, successful outcomes are not generally the case. This prompts the question: What are the logical short- and longer-term outcomes to be achieved for OUD patients? Typically, a positive OUD outcome is defined by not dying, attending clinics to receive opioid maintenance medication, or avoiding overdoses and emergency room visits. In physicians, outcomes are distinctly different, focusing on returning to full premorbid function. These include negative urine tests, attending Caduceus meetings, following a detailed psychosocial post-evaluation treatment plan, and achieving positive social, job return-performance, and spouse-partner ratings.

While extensive research is required, it is necessary to revisit the issues of depression, suicide, and despair associated with chronic iatrogenic opioid administration using MATs. Treatment without a focus on recovery and without addressing "dopamine homeostasis" may contribute to a revolving door, where many patients with OUD relapse and overdose, repeatedly receiving the same treatment without long-term success.[70,154]

5. Locus coeruleus therapeutics: Applications to other areas – behavioral addictions

As discussed in the current article, dysfunction of the LC-NA system affects many neuropsychiatric and neurological diseases, including opioid and other drug withdrawal symptomatology, Parkinson's disease, depression, anxiety, post-traumatic stress disorder, ADHD, and Alzheimer's disease. It has become evident that even in cases where the LC is not directly involved in the disorder, manipulating LC activity could improve health outcomes. Disruption of the feedback loop supporting the dysfunction could re-establish a healthy physiological response, moving the patient toward normal daily activity.[141] There are selective NA reuptake inhibitors, such as atomoxetine, used for opioid withdrawal.[155] NA agonistic agents are used for ADHD,[156] and for Parkinson's disease, the alpha-2 adrenergic receptor antagonist lofexidine[157] is used for cognitive dysfunction[158,159] and reboxetine for depression.[160]

Emerging evidence suggests the possibility of minimally invasive procedures for manipulating the LC, such as regulating the circuit from the suprachiasmatic nucleus to the LC through a relay in the dorsomedial hypothalamus.[161] Another potential method is transcutaneous vagus nerve stimulation, a non-invasive procedure reported to possess positive effects on psychiatric and neurological disorders, such as depression.[162]

Despite the potential benefits, noradrenergic compounds are not frequently administered as a frontline therapeutic modality. NA dysfunction contributes to many aspects of brain disorders, but many human clinical trials have not distinguished specific NA effects from dopamine effects. Indeed, more is known about the specific effects of dopamine and associated neuron degeneration and other physiological and psychiatric effects.[163]

To investigate the involvement of the noradrenergic system in pathological gambling (PG), Pallanti *et al.*[164] measured the neuroendocrine growth hormone (GH) response to the alpha-2 adrenergic receptor agonist clonidine and placebo in PG individuals and controls. One hypothesized mechanism, as proposed by these authors, is that clonidine's net effects entail reducing neurotransmission by suppressing LC activity and stimulating GH secretion through activation of post-synaptic alpha-2 adrenergic receptors in the hypothalamus. The area under the curve for GH response to clonidine was found to be significantly lower in the PG group compared to controls. Notably, individuals with PG exhibited significantly blunted GH responses relative to controls at 120 and 150 min post-clonidine administration. These findings support the notion that the diminished sensitivity of post-synaptic alpha-2 receptors may be linked to elevated noradrenergic secretion in PG. This peripheral noradrenergic dysfunction aligns with attenuated corticofrontal noradrenergic function observed in positron emission tomography (PET) studies of PG.[17]

In an interesting study, Saddichha *et al.*[165] demonstrated that clonidine was effective in reducing compulsive soap eating, known as sapophagia, but not feeding behavior, suggesting an effect on compulsive behavior rather than on eating disorders. Another study by Cazala[166] demonstrated that clonidine specifically stimulates alpha-noradrenergic receptors and has two distinct effects on intracranial self-stimulation (ICSS) behavior: it acutely depresses ventral hypothalamic ICSS at low doses, while it causes a discrete increase in dorsal ICSS. In addition, evidence indicates that chronic clonidine administration affects conflict behavior in rats, increasing punished responding in the conflict test. The authors suggest that clonidine may have some potential as an anti-panic drug.[167] Experiments in Blum's laboratory clearly pointed out that a reduction in serotonin levels in the brains of rodents resulted in an enhanced fear reaction, potentially implicating clonidine in serotonergic transmission.[168]

Some researchers consider smoking behavior, not just nicotine dependence, to be related to oral fixation and potentially a behavioral addiction.[169] Moreover, combined data from nine double-blind placebo-controlled trials (N = 813) revealed that the smoking quit rate with clonidine was significantly greater than with placebo. Moreover, the in-depth analysis suggested that clonidine potentiates the effect of individual behavior therapy and may be more beneficial for female smokers compared to male smokers.[170] Current standards of care for medically supervised withdrawal include treatments with mu-opioid receptor agonists such as methadone, partial agonists like buprenorphine, and alpha-2 adrenergic receptor agonists such as clonidine and lofexidine. Newer agents also utilize these pharmacological mechanisms, including tramadol for mu-opioid receptor agonism and tizanidine for alpha-2 agonism.[5] To explore the initiation of detoxification in individuals addicted to opioids/opioids, Blum's laboratory developed a protocol for use in treatment centers, particularly for heavily dependent opioid/opioid subjects.[171,172] Moreover, future research endeavors encompass managing withdrawal while stabilizing patients with OUD on extended-release naltrexone, transitioning patients from methadone to buprenorphine for OUD treatment, and tapering opioids in patients with chronic, non-cancer pain. However, compliance remains a challenge that could potentially be addressed through the addition of a pro-dopamine regulator.[173]

6. Limitations

While this article takes a narrative approach rather than a systematic review, we acknowledge the potential for bias in our overall perspective on this topic. It is important to recognize that some studies present alternative views, suggesting that regions proximal to the LC, such as the periaqueductal gray, as well as other brain structures independent of the LC noradrenergic system, may play a more significant role in the manifestation of opioid withdrawal syndrome.[174]

In a study by Christie,[174] intracellular recordings of membrane potassium current were conducted from rat LC *in vitro*. The researchers observed tolerance to the opioid-induced increase in potassium conductance, with a more pronounced effect observed for normorphine compared to [Met^5]enkephalin and [D-Ala^2,MePhe^4,Gly^5-ol]enkephalin. Experiments using the irreversible receptor blocker beta-chlornaltrexamine indicated that normorphine exhibited

lower intrinsic efficacy than [Met[5]]enkephalin and [D-Ala[2], MePhe[4], Gly[5]-ol]enkephalin. This adaptation was not attributed to any changes in the properties of the potassium conductance mediated by mu-receptors, as both full and partial agonists at alpha-2 adrenergic receptors, which are linked to the same potassium conductance, remained unchanged in their effectiveness. In addition, no association was found between this adaptation and any alterations in the affinity of mu-receptors for the antagonist naloxone.

We believe that other sites besides the LC are certainly involved in opioid-induced withdrawal. However, the preponderance of available literature supports the role of the LC, as evidenced by a plethora of clinical data, with at least 80 articles suggestive of the LC's role in opioid withdrawal.[175]

7. Conclusion

To assist the readership's comprehension, a summary schematic is provided (Figure 1). Clonidine operates through agonism at the alpha-2A receptor, a subtype of the alpha-2 adrenergic receptor predominantly located within the PFC. In the PFC, it inhibits the release of NE, which is implicated in withdrawal symptoms. Consequently, clonidine is effective in alleviating withdrawal-related anxiety, hypertension, and tachycardia. Gold *et al.* demonstrated the ability of clonidine to reverse the effects of LC stimulation, thereby propelling the noradrenergic hypothesis for opioid withdrawal into the forefront of research. In the 1980s, the efficacy of clonidine in facilitating the transition to long-acting injectable naltrexone was confirmed for physicians, executives,

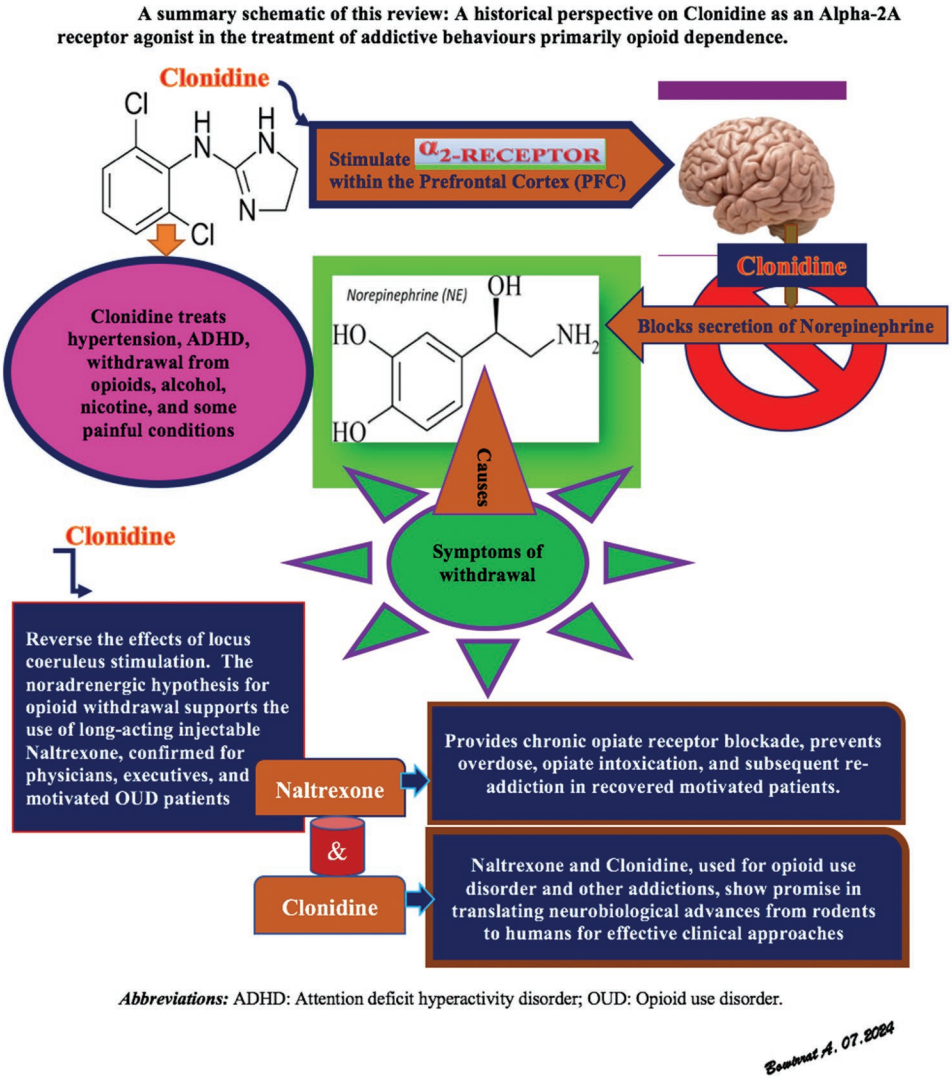

Figure 1. A summary schematic of this review
Abbreviations: ADHD: Attention deficit hyperactivity disorder; OUD: Opioid use disorder.

and other motivated individuals with OUDs. Despite its challenges with compliance, naltrexone offers sustained blockade of opioid receptors, mitigating the risk of overdose, intoxication, and subsequent re-addiction in motivated patients. The development of clonidine and naltrexone as treatment modalities for OUDs, as well as other addictions, underscores the potential for translating neurobiological advancements from rodent models (bench) to non-human primates and ultimately to humans (bedside), leading to novel and efficacious clinical interventions (Table 1).

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Conflict of interest

Dr. Blum is the inventor of GARS® and Pro-dopamine Regulation (KB220Z™). There are no other conflicts of interest.

Author contributions

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Writing – original draft: All authors
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ACCSCIENCE PUBLISHING

INNOSC Theranostics and Pharmacological Sciences

PERSPECTIVE ARTICLE

Recommendations on the management and prevention of spinal cord injury in children following backbend dance

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Abstract

Spinal cord injury (SCI) is a kind of disease that indiscriminately affects all age groups, although the number of SCI cases in children is far lower than that in adults. In this paper, we discuss the appropriate diagnostic methods and prevention methods for SCI caused by repetitive hyperextension movement. Case study reports available in the published literature concerning SCI due to hyperextension movement, which were categorized using the American Spinal Injury Association (ASIA) grades, were gathered. Moreover, the age, gender, lesion length on magnetic resonance image (MRI), time of symptoms appearance, initial spinal cord atrophy region, neurological level of injury, and initial and final ASIA grades were analyzed. A total of 144 cases with SCI after backbend dance were included in our analysis, with some cases with an incubation period ranging between 15 min and 4 h showing no symptoms. Most of the collected cases were young girls of <11 years old. Early MRI showed that the pathological changes had extended toward cephalocaudal regions. In summation, the number of SCI cases, which are disabling for many children, is rapidly accumulating in China. Thus, SCI following repetitive hyperextension movements requires further research.

Keywords: Spinal cord injury; Repetitive hyperextension; Backbend dance; Children; China

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1. Introduction

Spinal cord injury (SCI) is a devastating ailment to the affected children, given that they need to endure the implications and burdens posed by this disorder for a long time from a young age.[1] This ailment is financially burdening for the affected individuals and their families owing to the costly treatment and rehabilitation services, in addition to the need for personal assistance, lost productivity due to disability, and social isolation. Thus, it is conceivable that the lifelong treatment cost of SCI for children is much higher than that for adults.[2,3]

SCI in children is far less common than in adults, accounting for 2 – 5% of all spine traumas.[4] An epidemiological study in the United States of America found that the

incidence of SCI in children is 8 – 13/million annually.[5] To date, much of the management and treatment of pediatric SCI has been extrapolated from the adult literature, given the low incidence of pediatric SCI and the limited number of cases in children.[4]

China has a significant number of SCI cases. However, there is no national or regional SCI registry system in China. As a result, the incidence of SCI in all age groups, especially among children, remains unclear.[6,7] Public education, improved safety features in motor vehicles, and the stricter enforcement of seat belt laws, driving-under-the-influence regulations, protection of minors, and prohibition of child labor may have contributed to a decline in SCI rates among children. In China, the number of SCIs in children caused by backbend movements during dance training is increasing and accounts for 50% of all SCIs in children in the past two decades.[8,9] SCI caused by these movements, which have been left unaddressed and unresearched for a long time, brings a heavy psychological and economic burden to children and their families.

2. Case studies and findings

More than 200 cases of SCI have been reported in China over the past 5 years.[8-11] In this paper, we reviewed the data gathered from six articles, which included 144 Chinese children with SCI that happened after repetitive hyperextension movement (backbend movements) during dance training.[8-10] Other cases of SCI that was not related to repetitive hyperextension movement were excluded.

Almost all of the cases occurred in young girls under 11 years old. The age distribution of this injury ranges from 3 to 11 years old (Figure 1), and 99% of cases were under 10 years old with an average age of 6 ± 2 years. All of them presented hyperacute onset of SCI within the first 4 h after the injury, and many cases showed no symptoms during the first few minutes after injury. However, during the incubation period (15 min to 4 h) from accidental injury in dance training to paraplegia, no interventional approaches or effective treatments were implemented, such as training cessation, immobilization or supine rest, and early medical treatment. However, no obvious fractures or dislocations were found in the imaging examination after the injury. Only magnetic resonance imaging (MRI) showed a longitudinally diffuse intramedullary high-intensity signal and spinal cord edema, while the extent of edema was not consistent with the neurological level of injury (Table 1 and Figure 2). The final follow-up showed poor prognosis in patients with complete SCI (American Spinal Injury Association [ASIA] grade A)[8-10] (Table 2). The final follow-up MRI conducted 1 – 4 months after intervention showed distal spinal cord atrophy (Figure 3). Moreover, the long-term follow-up also revealed that the patients with complete SCI were grappling with serious complications, such as scoliosis, hip dysplasia or dislocation, bedsore, urinary tract infection, lower limb neurogenic fracture, and muscle atrophy.[1,10]

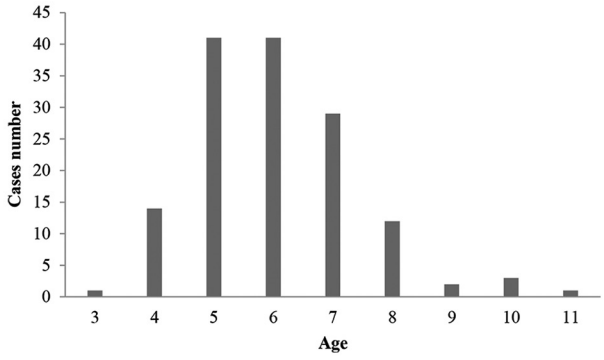

Figure 1. The age distribution of spinal cord injury after hyperextension movement. Data used to derive this figure are taken from reference.[8-10]

3. Practical implications

With the continued enhancement of health consciousness, many children are becoming involved in recreational activities, such as yoga, gymnastics, ballet, cheerleading, acrobatics, pilates, backbends, surfing, and swimming, which often entail a certain degree of risk to continuous spine hyperextension movements. However, SCI resulting from backbend dance remains largely neglected by the public, dance training institutions, as well as sports centers in China.[8-11] Such negligence will not put a halt to the occurrence of SCI due to hyperextension movement.[10] At present, there are no relevant recommendations to prevent SCI occurrence in children during dance training.

According to China's seventh census, there are more than 250 million children aged 0 – 14 in the country, accounting for 17.95% of the total national population.[12] Following a shift to China's fertility policy[13] – replacement of the one-child policy with the three-child policy – expectedly China will usher in a new round of baby boom, which potentially increase the proportion of children population. At the same time, as part of the government's education reform efforts, measures are being taken to reduce the excessive burden of homework and after-school tutoring for primary and middle school students. The policy change and the implementation of new homework burden-lessening measures would spur the increased participation of children in recreational activities,[14] which will inevitably raise the incidence of SCI in this young-age population.

Wang *et al.* hypothesized that the effect of internal organs on the inferior vena cava may lead to impaired blood perfusion, primarily at the T9-T10 level, ultimately resulting in ischemic SCI.[10] At present, magnetic resonance

Table 1. The distribution of intramedullary lesion length with high-intensity signal based on MRI

	Number of vertebral levels with high-intensity signal based on MRI															
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**17**
Complete injury	0	0	1	3	5	5	10	19	15	9	9	13	7	5	4	1
Incomplete injury	4	0	3	0	9	11	18	24	22	10	10	15	0	6	0	0

Notes: Complete injury: ASIA grade A; Incomplete injury: ASIA grades B, C, and D. Data used to derive this table is taken from reference.[8-10]
Abbreviation: MRI: Magnetic resource imaging.

Table 2. ASIA grades during initial and final follow-up

ASIA grades during initial follow-up		ASIA grades during final follow-up and number of cases				
ASIA grade	**Number of cases during initial follow-up**	**A**	**B**	**C**	**D**	**E**
Grade A	104	101	2	1	0	0
Grade B	26	0	11	6	7	2
Grade C	7	0	0	2	4	1
Grade D	7	0	0	0	6	1

Note: Data used to derive this table are taken from reference.[8-10]
Abbreviation: ASIA: American Spinal Injury Association.

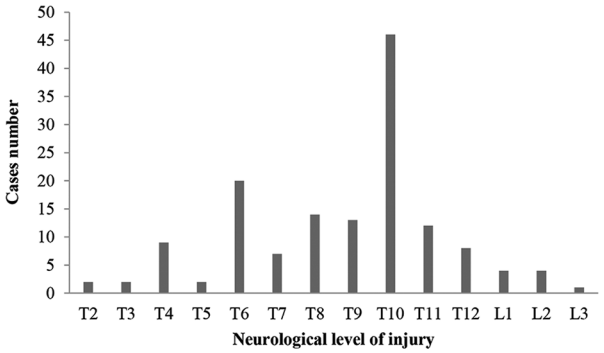

Figure 2. The distribution of neurological level of spinal cord injury. Data used to derive this figure are taken from reference.[8-10]

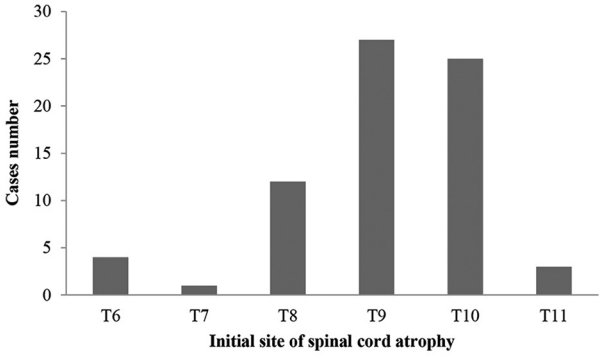

Figure 3. The extent of spinal cord atrophy. Data used to derive this figure are taken from reference.[8-10]

angiography remains the gold-standard technique for confirming and predicting the prognosis of repetitive hyperextension-induced SCI.

After confirming the diagnosis and assessing the severity of the injury, it is crucial to monitor the patient's vital signs. Conservative treatment can yield significant results in stable cases but may not improve the condition in unstable cases. Overall, the prognosis of SCI depends on the severity of the initial injury, the timing and accuracy of diagnosis, and the implementation of appropriate treatment.[15-17] At present, a limited range of therapeutic treatments is available for both adults and children affected by SCI, and the lack of treatment would lead to severe and persistent neurological abnormalities and disabilities that can persist for decades until death.[15-17] Therefore, to expand the variety of SCI treatments for children, further and extensive research is required. Badhiwala *et al.* advocate that "time is the spine," but whether early surgical intervention is necessary for this specific SCI is still unknown.[16] Therefore, preventing SCI during dance training is currently the best feasible strategy.

According to the literature, we propose the following several recommendations on the management and prevention of SCI for children engaging in backbend dance:

1. Children under the age of 10 should refrain from practicing spine hyperextension movements
2. Coaches and parents of children participating in hyperextension activities should be informed that these spine movements may lead to SCI. Furthermore, they should take careful measures to protect the children and prevent accidental injuries during training sessions that involve hyperextension movements
3. At the time of injury, training should be stopped immediately, and children should be immobilized and placed in a supine resting position to prevent injury deterioration and the occurrence of secondary injury. Medical help should be sought immediately if any neurological symptoms appear
4. The potential occurrence of SCI due to backbend dance should be brought to the attention of the general public, guardians, sports institutions, and education departments, as part of the awareness-

raising effort. It is also necessary to issue proper guidelines highlighting the potential hazards of backbend dance and providing recommendations on the effective measures to prevent SCI arising after repetitive hyperextension movement

5. Legislations should be in place to prohibit children under the age of 10 from participating in this type of sport.

4. Conclusion

SCI resulting from backbend dance differs from other types of SCI in terms of the mechanism of injury, symptoms, and imaging abnormalities. Deserves in-depth investigations of this devastating injury are warranted to widen our understanding of its underlying pathogenic mechanisms and pathways as well as to explore effective treatments that can minimize the injury's perennial complications.

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Conflict of interest

The authors declare that they have no competing interests.

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Not applicable.

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ACCSCIENCE PUBLISHING

INNOSC Theranostics and Pharmacological Sciences

MINI-REVIEW

Innovative approaches and challenges in antibody-based therapeutics for Alzheimer's disease

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Abstract

Neurological conditions, such as Alzheimer's disease (AD) and tau-related pathologies, represent a substantial worldwide health dilemma. This review explores recent advancements and challenges in single-domain antibody (VHH)-based strategies targeting beta-secretase 1 (BACE1) inhibition and tau-related therapeutics. The methodology adopted in this study involved a comprehensive search of databases (e.g., Scopus, PubMed, and Google Scholar), with recent articles cited to provide an up-to-date overview. The research examines the limitations of current AD treatments, emphasizing the need for innovative techniques. We, further, discuss potential gene transfer methods using adeno-associated virus-based vectors for central nervous system delivery of single-domain immunoglobins/antibodies (VHHs) to inhibit BACE1. In addition, insights into anti-tau therapeutics, including passive immunization and active vaccines, offer novel insights into research. These findings hold implications for the redesign of therapies for neurodegenerative disorders and the advancement of clinical understanding.

Keywords: Neurological condition; Single-domain antibodies; BACE1 inhibition; Tau-related therapeutics

1. Introduction

Neurodegenerative diseases, such as Alzheimer's disease (AD) and tau-related pathologies, present a growing global health issue. AD predominantly afflicts the

elderly, causing dementia characterized by abnormal protein deposits leading to plaques and tangles. These manifestations result in gradual nerve cell degeneration and impaired communication.[1] Clinical studies focusing on immunotherapy targeting amyloid beta (Aβ) face challenges due to the observed lack of correlation between Aβ levels and dementia severity, which raises questions about the capabilities of Aβ-focused interventions.[2] Current United States Food and Drug Administration-approved drugs, including acetylcholinesterase inhibitors (e.g., donepezil and rivastigmine) and aducanumab, which target Aβ plagues, offer temporary relief by delaying the progression of AD. However, they fail to reverse neuronal loss, brain atrophy, or cognitive decline.[3] Aβ, derived from amyloid precursor protein cleavage, is a central focus of therapeutic research, with drugs targeting β- or γ-secretase and Aβ aggregation.[4-6] However, these approaches provide limited benefits and may lead to adverse effects. Recent findings suggest that Aβ immunotherapy not only reduces Aβ but also lowers tau levels in animal models. This discovery has prompted researchers to explore tau immunotherapy as a promising avenue, particularly in early-stage AD, given the evident interaction between Aβ and tau.[7-9] The development of single-domain antibodies (VHHs) stemmed from the limitations of conventional antibodies, such as their larger size, limited tissue penetration, and susceptibility to denaturation and aggregation, which hindered their effectiveness in targeting specific epitopes, particularly in the central nervous system (CNS).[7]

Antibody-based therapies offer a promising alternative to small-molecule drugs, especially when comparing inhibitors of beta-secretase 1 (BACE1) to VHHs targeting BACE1. However, the development of BACE1 inhibitors has faced challenges leading to their failures, highlighting the need for further exploration and understanding of these therapies. Despite recent developments in monoclonal antibody research and the emergence of disruptive therapeutics such as variable heavy chain domains, which are VHHs (Table 1), their potential application in CNS disorders remains underexplored due to the inherent challenge of these agents crossing the blood–brain barrier (BBB).[10] Recent setbacks, such as the termination of the variable heavy chain domains B (VHH-B) clinical trial, underscore the critical need for innovative systems and a comprehensive understanding of the molecular mechanisms involved in targeting key proteins such as BACE1 and tau.[11] The proposed gene transfer method using adeno-associated virus (AAV)-based vectors offers a novel avenue for effectively delivering therapeutic antibodies into the CNS. Moreover, investigating the system of anti-tau therapeutics, including passive immunization, active tau-targeted vaccines, and tau aggregation inhibitors, is imperative for advancing the field and addressing the challenges posed by tau-related disorders. Therefore, this review aims to address these gaps by elaborating on current studies exploring gene transfer methods to deliver therapeutic agents directly into the CNS, with the goal of offering insights into therapies inhibiting BACE1 and advancing our understanding of their therapeutic potential. This article holds significant implications for both preclinical and clinical studies. For instance, it could contribute to the broader understanding of antibody-based therapies in neurodegenerative disorders, shedding light on the challenges and opportunities associated with VHHs in applications. In addition, discussions on tau-related therapeutics offer a comprehensive overview, laying a foundation for refining existing scientific models and developing innovative methods.

Table 1. Studies and clinical trials on VHH-based therapeutics for CNS disorders

Study/ Clinical trial	Focus	Findings	Implications
Singh *et al.*[10]	Monoclonal antibody research and VHHs in CNS disorders	Underexplored potential due to BBB challenge	Highlights the need for innovative approaches
Das and Yan[11]	Molecular intricacies of targeting BACE1 and tau	Termination of the VHH-B trial emphasizes the need for understanding	Calls for a comprehensive approach in targeting key proteins
Marino *et al.*[14]	Gene transfer method using AAV-based vectors to deliver VHHs into CNS	Promising results in inhibiting BACE1	Offers novel avenue for effective CNS delivery
Yadav *et al.*[15]	Infusion of anti-BACE1 antibodies via i.c.v. administration in primates	Significant reduction in Aβ peptides in CSF and brain parenchyma	Highlights potential of direct CNS injection for uniform antibody distribution
Burns *et al.*[16]	VHH-B clinical trial termination due to safety concerns	Emphasizes the need for further refinement of VHH-based therapeutics	Caution in translating to human CNS disorders

Abbreviations: AAV: Adeno-associated vector; Aβ: Amyloid beta; BACE1: Beta-secretase 1; BBB: Blood–brain barrier; CNS: Central nervous system; CSF: Cerebrospinal fluid; i.c.v.: Intracerebroventricular; VHH: Single-domain antibody.

2. Innovative approaches and challenges in variable heavy chain domains-based therapeutics for CNS disorders

Monoclonal antibodies have emerged as an important field in research, offering targeted interventions against specific molecules such as lipids and proteins.[12] Within this realm, VHHs, known for their potential as revolutionary treatments, are gaining scientific recognition.[13] Despite their versatility in addressing medical challenges, these antibodies have seen limited application in neurological disorders due to the formidable obstacle posed by BBB.[14] In a recent study, researchers devised a gene transfer strategy utilizing vectors equipped with BBB-crossing capabilities to deliver therapeutic molecules directly into the CNS of mammals. Specifically, the study aimed to inhibit a key enzyme in the brain using a developed VHH named VHH-B9.[14] The results indicated a high selectivity of VHH-B9 for BACE1, with a single systemic dose demonstrating enduring benefits such as improved cognition in mice lasting up to 12 months.[14] This innovative protocol holds promise for exploring therapeutic interventions in neurological disorders.

In another investigation by Yadav *et al.*,[15] anti-BACE1 antibodies were infused into primate subjects through intracerebroventricular administration, resulting in a substantial and sustained reduction of up to 70% in Aβ peptides within the cerebrospinal fluid (CSF). Notably, direct CNS injection achieved uniform antibody distribution (20 – 40 nM) in the brain parenchyma, leading to a 50% reduction in Aβ in cortical parenchyma. However, intravenous administration in nonhuman primates yielded non-significant changes in cortical or CSF Aβ levels, accompanied by a low brain concentration (approximately 0.6 nM) of anti-BACE1 antibodies.[15]

Despite promising results in preclinical studies, the translation of VHH-based therapies to clinical trials has faced challenges. The VHH-B clinical trial, terminated in 2021, was deemed unsafe for human subjects due to unforeseen changes in cognitive function, brain volume loss, and body weight loss.[16] This finding highlights the imperative for further investigation and refinement of VHH-based therapeutic methods before their application in human CNS disorders.

3. Advancements and strategies in anti-tau therapeutics

Tau, a microtubule-associated protein crucial for stabilizing axonal microtubules, becomes problematic when deposited abnormally, leading to various diseases, including dementia.[17] Figure 1 illustrates the pathogenesis of tau, highlighting the complexities involved. Current therapeutic approaches target intracellular and extracellular tau to hinder pathological tau formation, accumulation, and spread.[18] The strategies involved encompass protein kinase inhibition, inhibition of tau aggregation, antisense oligonucleotides, as well as active and passive immunotherapies.[18] Notably, numerous small molecules directly or indirectly modify tau aggregation.[19]

The efficiency of tau immunotherapies was initially demonstrated in the Janus kinase- and nucleotide-binding oligomerization domain-like receptor protein 3 mouse model, also known as JNPL3 in 2007, demonstrating reduced tau pathology in various brain regions.[21] At present, the majority of tau-targeted therapies in preclinical

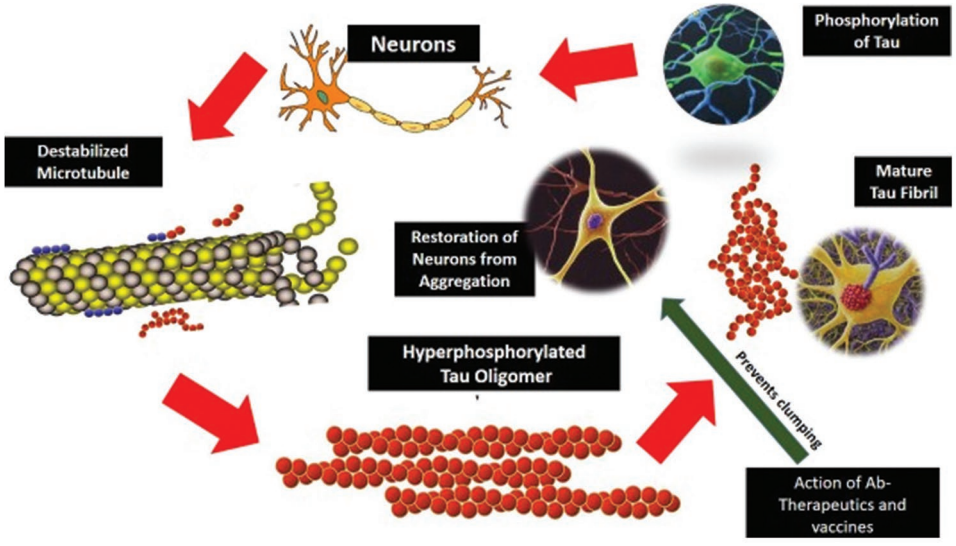

Figure 1. Proposed mechanism of pathogenesis of Tau.[20]

investigations revolve around immunotherapies.[17] In addition, passive immunization, which involves administering monoclonal immune-proteins, has marked a significant growth.[17] The development of passive immunization techniques such as the JNJ-63733657 and UCB0107 marks a significant improvement in the quest for effective anti-tau treatment, offering hope in the fight against tau-related neurodegenerative diseases.

JNJ-63733657 is a humanized IgG1 monoclonal antibody that targets the temperate region of tau and exhibits potential to prevent tau's cell-to-cell propagation and aggregation.[22] Janssen Research and Development conducted two phase 1 clinical trials, demonstrating safety and tolerability, pharmacokinetics, and pharmacodynamics of a drug candidate in a small group of healthy volunteers or patients.[23] In a 2019 phase 1 trial, dose-dependent increases in exposures were reported, with CSF concentrations of approximately 0.2% of serum levels, and dose-dependent reductions in p217+ tau in CSF were observed.[24] A phase 2 study commenced in January 2021, involving 420 patients with the early AD symptoms and a positive tau PET scan, set to conclude in 2025.[25] Similarly, UCB0107 is a humanized monoclonal IgG4 antibody that exhibits an affinity for binding to paired helical filaments of tau, recognizing amino acids 235–250 near tau's microtubule-binding domain.[22] Union Chimique Belge (UCB) completed a phase 1 trial in March 2019, assessing adverse events and pharmacokinetics in 24 healthy Japanese men who received a single, unspecified UCB0107 dosage or placebo.[26] The ongoing phase 2 trial aims to randomize 450 individuals with mild cognitive impairment or mild AD dementia to different doses or a placebo for 80 weeks, concluding in 2025.[25]

3.1. Active tau-targeted vaccines

At present, ACI 35 and AADvac-1 are two active anti-tau vaccines undergoing clinical research. ACI 35, a liposome-based vaccine, consists of 16 copies of synthetic tau fragments phosphorylated at S396 and S404. These fragments are anchored into a lipid bilayer to stimulate the immune system and generate antibody proteins.[17] In July 2019, AC Immune and Janssen initiated a small phase 1b/2a trial to assess the safety and immunogenicity of ACI-35 in individuals with early AD. By July 2020, the lowest-dose cohort had completed the trial, revealing favorable safety, tolerability, and immunogenicity data.

In February 2022, interim data from the trial confirmed the consistent safety and potent immunogenicity of the p-Tau Alzheimer's vaccine in a high-dose cohort in early AD. AADvac-1, an active vaccine, is designed to provoke an immune response against a pathologically altered form of tau pathology.[17] This first-generation active immunotherapy targets 12 amino acid sequences in the microtubule-binding region of the tau protein.[27] Axon neuroscience initiated a phase 1 trial in May 2013 with 30 patients with mild-to-moderate AD. The results from the trial indicate high immunogenicity, with IgG antibodies against tau peptides induced in 29 out of 30 patients. Overall, AADvac-1 demonstrated encouraging safety and tolerability profiles. The subsequent phase 2 trial involved 196 patients with mild-to-moderate AD, meeting the major objectives and secondary objectives, including the evaluation of immunogenicity and treatment efficacy.

3.2. Tau aggregation inhibitor

The primary direct tau binder is methylene blue, also known as methylthionine chloride.[26] Variants of methylene blue have demonstrated particular promise in blocking tau clumping.[26] TRx0237 (LMTM), a second-generation tau aggregation inhibitor for AD and frontotemporal dementia, is the purified form of methylene blue. Wilcock and colleagues explored the effectiveness of LMTM as monotherapy in a non-randomized cohort during an 18-month phase 3 trial in mild AD. The results supported the hypothesis that it could be effective as monotherapy, particularly when administered at a dose of 4 mg twice a day. Although the phase 3 trial did not slow cognitive or functional decline in mild-to-moderate AD, re-investigation of the data revealed a significantly lower brain atrophy rate in patients receiving monotherapy compared to those receiving placebo. A phase 3 trial with a lower dose has been conducted and concluded in December 2022.[25]

4. Implications of findings

The ever-evolving nature of research addressing neurological conditions presents promising avenues and challenges for future exploration. The change from a singular focus on Aβ in AD to an exploration of alternative targets such as tau highlights the need for novel advancements in therapeutic methodologies. Innovations in VHH-based therapies, such as the gene transfer method using AAV-based vectors, offer the potential to overcome challenges associated with BBB penetration. However, setbacks in clinical studies, such as the termination of the VHH-B trial, indicate the imperative for rigorous preclinical investigation and improvement of methodology. In addition, utilizing VHH-based therapy is accompanied by limitations, including potential immunogenicity, limited epitope diversity, and challenges in large-scale production and formulation for therapeutic use. In addition, their small size may lead to rapid renal clearance, necessitating modifications to improve their pharmacokinetic properties. Therefore, this comprehensive review of anti-

tau interventions, encompassing passive immunization, active vaccines, and aggregation inhibitors, suggests that current drawbacks may soon be transformed into research advantages, driving progress in this field. Future directions may encompass personalized therapies, multi-modal systems, advancements in gene editing, and innovative tactics for BBB penetration. Longitudinal studies, biomarker identification, and considerations of neuroinflammation and immune modulation will be crucial. As the field advances, a patient-centric focus and global collaboration for data sharing will be paramount for accelerating advancement in understanding and treating neurodegenerative disorders.

5. Conclusion

The detrimental impact of tau phosphorylation on neurons underscores the urgent need for effective therapies targeting this pathway. Antibody-based therapeutics, particularly those utilizing VHH antibodies, hold immense promise for future research and applications, due to their potential to overcome BBB limitations. As research progresses, further investigation into VHH-based therapies and their efficacy in clinical trials will be crucial for developing successful antibody-based therapies for neurodegenerative disorders. The advancement of these therapies will pave the way for personalized treatment options and improved patient outcomes.

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The authors declare that they have no competing interests.

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SHORT COMMUNICATION

Transcriptomic signature of CD4-expressing T-cell abundance developed in healthy peripheral blood predicts strong anti-retroviral therapeutic response in HIV-1: A retrospective and proof-of-concept study

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Abstract

CD4-expressing T-cells (CD4Ts) play a crucial role in maintaining the normal functioning of the mammalian immune system and overall systemic health. Diseased individuals, such as those infected with the human immunodeficiency virus type I (HIV-1), experience progressive and eventual depletion of CD4T leading to uncurable conditions and ultimate death if left untreated. Although much is known about the role of CD4T-mediated immunity, the understanding of CD4T-related transcriptomic patterns remains incomplete. This proof-of-concept study aims to identify a transcriptome-wide gene signature for CD4T abundance by Least Absolute Shrinkage and Selection Operator (LASSO) regression modeling in 340 healthy peripheral blood samples. The optimized LASSO model demonstrated computational robustness (tenfold average Pearson's r = 0.89) and biological relevance evidenced by four significant Gene Ontology terms (all odds ratio [OR] ≥ 4.5 and false discovery rate ≤0.05). Subsequently, in an independent cohort with 24 HIV-1-infected men who received anti-retroviral therapies, there is a significant, positive association between the gene signature and a strong anti-retroviral response before (OR = 13.6, $P < 0.05$) and after adjusting for subject age, sex, and race (OR = 14.4, $P < 0.05$). Taken together, the gene expression pattern associated with CD4T abundance is predictable, generalizable, and biologically relevant, shedding new light on the importance of CD4T abundance.

Keywords: CD4T; Gene signature; HIV-1; Immune cells; Transcriptomics

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1. Introduction

CD4-expressing T lymphocytes (CD4Ts) are crucial players in the human immune system. The CD4T plays a role in the modulation of antiviral immune responses primarily by enhancing the function of other immune cells. During the initial stages

of a viral infection, CD4 helper T-cells activate B-cells and cytotoxic lymphocytes to enable the proper immune response against the invading pathogen.[1]

In the case of human immunodeficiency virus (HIV) infection, CD4Ts are specifically targeted, exploited, and destroyed, resulting in compromised immunity.[1,2] The progressive depletion of CD4T, as seen in HIV-infected patients without anti-retroviral treatment, is responsible for the development of acquired immunodeficiency syndrome, which renders an individual vulnerable to even the most commonplace opportunistic pathogens.[1,2] Among HIV patients receiving anti-retroviral treatment, CD4T abundance is associated with favorable clinical outcomes and serves as a prognosis indicator.[3]

The most recent meta-analysis of transcriptomes validated the hypothesis that HIV infection induces characteristic changes in CD4T gene expression and biological pathways.[4] Nevertheless, an in-depth understanding of CD4T biology in both the healthy and diseased states remains limited. Thus, this study aims to explore the possibility of building a transcriptome-wide gene signature using clinically measured CD4T abundance as the outcome in blood samples of non-diseased human subjects. Subsequently, this study investigates the potential prognostic utility of the gene signature in a cohort of HIV-1-positive subjects receiving anti-retroviral therapies to offer insights into the role of CD4T abundance.

2. Materials and methods

2.1. Study populations and datasets

All datasets used in this study are publicly available and obtained from Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo). The "discovery population" (accession GSE58137) consists of 340 subjects with both transcriptome-wide gene expression data (measured by the Illumina Human HT 12 v3.0-4.0 array) and the putative CD4T proportions calculated from the matched whole-genome methylation profiles.[5] The "application population" (accession GSE19087) consists of 24 HIV-1 positive men with both gene expression (Illumina Human WG 6 v3.0 array) and CD4T counter before and after a 48-week anti-retroviral therapeutic regimen.[3] Covariates including age and race were available in both populations. Subject demographics are summarized in Table S1.

Some genes measured on the Illumina array had multiple transcript variants. In addition, the expression platforms were of two different versions. To ensure comparability between the datasets, each population's expression values were aggregated by unweighted mean across all available transcript variants of a common gene so that there would be only one unique gene symbol. Next, each population's gene expression matrix was restricted to a common set of 12,549 genes. To remove platform-related artifacts or experimental noise, each expression matrix was mean-centered and standard deviation-scaled and then constrained within values ±5.0 as previously described.[6-8]

2.2. Gene signature discovery from the transcriptomes of healthy peripheral blood

The CD4T abundance gene signature was identified using Least Absolute Shrinkage and Selection Operator (LASSO; *glmnet* R package v.4.1.8), an established approach with the ability to reduce model coefficients to zero, thereby nullifying the effect estimate of a given feature unless it is very strong.[9,10] LASSO was built on the shared 12,549-gene set against the min-max scaled CD4T proportions in the discovery population, as follows:

$$MinMax(CD4T) \sim gene_{k} + R \quad \text{(I)}$$

where R is the LASSO penalty term defined by setting the *glmnet* hyperparameter alpha to 1.0. *MinMax* is a scaling function that transforms the input vector into a distribution between 0 and 1:

$$MinMax(x_{k}) = (x_{k} - min(\mathbf{x}))/(max(\mathbf{x})-min(\mathbf{x})) \quad \text{(II)}$$

This procedure initially yielded 334 (2.7%) gene features with non-zero regression coefficients. Given some coefficients had low magnitudes, the LASSO-selected genes were further filtered by a coefficient threshold of 1.25e-3, yielding 207 (114 positive and 93 negative) gene features, hereafter referred to as the "CD4T gene signature" (Table S2).

2.3. *In silico* validation of the gene-signature final model with *K*-fold method

The identified gene signature was validated with a *K*-fold cross-validation experiment. Specifically, the entire dataset was divided into K=10 partitions (i.e., folds, each with n = 34). The final, optimized version of the LASSO model was built on K-1=9 training partitions and evaluated on the remaining test partition. For each round of cross-validation, several metrics for model evaluations were calculated on the test partition: Root mean-squared error, Pearson's correlation coefficient (r), and R-squared. The entire procedure was repeated K times so that every fold participated in at least one round of training and testing. Each evaluation metric was averaged across the K trials to yield a final performance indicator (Table S1).

2.4. Biological interpretation of the CD4T gene signature

Separate *Gene Ontology: Biological Processes no-redundant* analyses (WebGestalt implementation, www.webgestalt.org)[11] were performed on the gene sets with positive and

negative LASSO coefficients against the 12,549 genes used as the input for gene-signature discovery. Raw *P*-values were adjusted by the Benjamini–Hochberg false discovery rate (FDR) method.

2.5. Gene signature-based stratification of an independent cohort of HIV-1-positive men

The CD4T gene signature was subsequently applied to the application population consisting of 24 peripheral blood samples from HIV-1-infected men receiving anti-retroviral therapies. All study participants had the absolute CD4T cell count before and after treatment. The percent change is defined as:

$$\text{Percent change} = 100 \times \frac{\text{Change in CD4T}}{\text{Baseline CD4T}} \quad \text{(III)}$$

Unsupervised hierarchical clustering with Euclidean and *Ward D* hyperparameters (*pheatmap* R package v.1.0.12) followed by a dendrogram-tree split at the first node was used to stratify the application population into two groups for downstream statistical analyses.

2.6. Statistical analysis

Unless otherwise specified, the computational environment used was R 4.3.1 (https://www.r-project.org) with data analysis and visualization packages *base* (4.3.1), *ggplot2* (v.3.4.3), and *matrixStats* (v.1.0.0). All statistical tests used were two-sided. The univariate (unadjusted) association between two binary variables was determined by a Fisher's exact test with odds ratio (OR) and 95% confidence interval (CI) estimates. To address potential confounding, the multivariate association was determined by multivariate logistic regression (a generalized linear model with a family "Binomial") with adjusted ORs estimated by exponentiating the model coefficients. The mean difference between any two groups was determined by Welch's *t*-test. A generalized linear model with family "Gaussian" was the generalization of the *t*-test to control for potential confounding variables. All R code is deposited to GitHub (https://github.com/ydavidchen/cd4t_pilot_signature).

3. Results

3.1. Identification and characterization of a CD4T abundance signature in the transcriptomes of healthy peripheral blood samples

A transcriptomic gene signature was identified by supervised modeling of gene expression against CD4T proportions using LASSO regression, a conservative statistical approach for selecting the most important features from high-dimensional data space.[9,10] LASSO is well-known for its ability and tendency to shrink the model coefficients to zero, thereby revealing input features (i.e., genes) most strongly correlated with the outcome (i.e., CD4T abundance). A tenfold cross-validation experiment demonstrated the robustness of the final model (tenfold average Pearson's $r = 0.89$ and $r^2 = 0.79$; Table S1). The initial CD4T gene signature consisted of 334 (2.7%) genes with non-zero LASSO coefficients (Figure 1A). The final version of the signature with 207 genes (1.6%) was obtained by coefficient thresholding (Figure 1A and Table S2).

To explore the biological relevance of the identified gene signature, *Gene Ontology: Biological Processes* analysis was performed on the gene features in the positive and negative directions, separately. The gene features positively associated with CD4T abundance strongly enriched for cellular adhesion (OR = 4.7, FDR = 0.01; Table 1). Notably, the members comprising this ontology term included high-profile immune genes involved in HIV-1 pathogenesis: CD28 and CTLA-4. The gene set negatively associated with CD4T abundance strongly enriched for metabolic processes of macromolecules (all OR ≥ 4.5 and all FDR = 0.05; Table 1). The genes encoding CD8 subunits, *CD8A* and *CD8B*, showed strong, negative association with CD4 abundance and were selected by the LASSO procedure (Table S3).

Hierarchical clustering of the identified gene signature segregated the discovery population into two major clusters: *Cluster 1* and *Cluster 2* (Figure 1B). The distribution of subject demographics, including race/ethnicity, sex, and age, appeared balanced across the gene-signature clusters (Figure 1B**,** horizontal tracking bars).

3.2. Application of CD4T gene signature to an HIV-1-positive cohort for biomedical knowledge discovery

The next objective was to assess the clinical relevance of the CD4T gene signature in human disease. Given the well-known role of CD4T in HIV-1 infection and recovery on anti-retroviral treatment,[3] the CD4T gene signature was evaluated in this disease context. Dataset GSE19087 has 24 HIV-1 positive men treated with an anti-retroviral regimen for approximately 1 year.[3] Hierarchical clustering of the CD4T gene signature stratified the HIV-1 positive, anti-retroviral treated men into two major groups (Figure 2). The cluster structure, indicated by the pattern of dendrogram branching, showed striking similarity to that of the discovery population. On stratification of cluster membership, the subject demographics present no significant differences between the two clusters (Table 2). However, the HIV-1-positive men in *Cluster 1* had an average of 122.7% CD4 increase at the end of the anti-retroviral therapy treatment, compared to

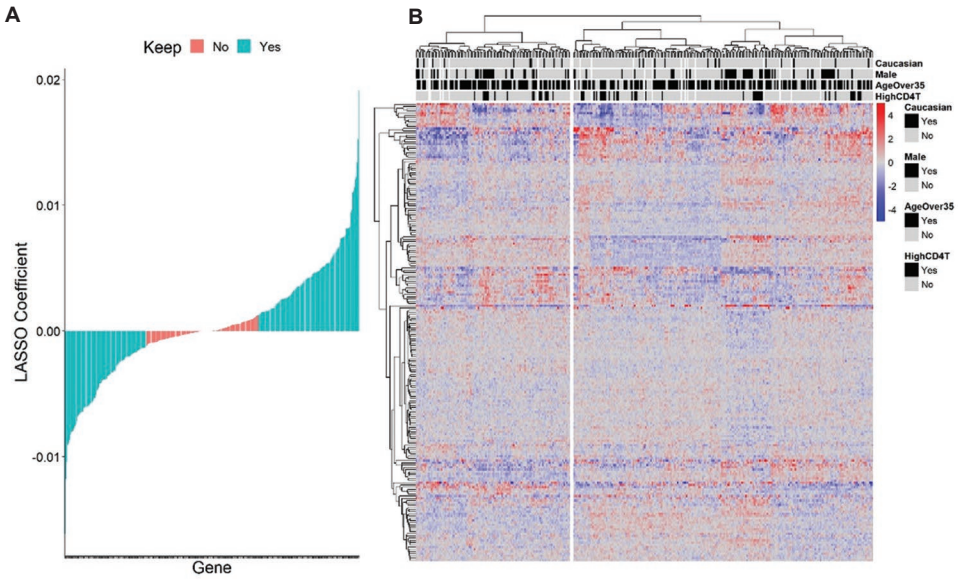

Figure 1. Discovery of the gene signature. (A) Distribution of 334 non-zero LASSO coefficients of the detected genes, with 207 of which meeting a 0.0015 cutoff included for downstream analyses. (B) Heat map showing the normalized expression of the 207 genes as rows and the 340 samples as columns. Horizontal tracking bars indicate clinical covariates. The dendrogram denotes the result of unsupervised hierarchical clustering.
Note: "HighCD4T" indicates whether a sample has an estimated CD4T proportion over 25%.

Table 1. ***Gene Ontology: Biological Processes*** **for the gene signature members, either positively or negatively associated with CD4T abundance in the discovery population**

Association with CD4T	ID	Process name	Number of genes observed	Number of genes expected	OR	*P*-value	FDR
Positive	GO: 0045785	Positive regulation of cell adhesion	11	2.3	4.7	1.7e-5	0.01
Negative	GO: 1901615	Organic hydroxy compound metabolic process	9	2.0	4.5	1.5e-4	0.05
Negative	GO: 0046486	Glycerolipid metabolic process	9	1.9	4.8	8.6e-5	0.05
Negative	GO: 0008202	Steroid metabolic process	7	1.2	5.8	1.7e-4	0.05

Abbreviations: OR: Odds ratio; FDR: False discovery rate.

Cluster 2 which had only 32.7%. On average, the CD4T percent increase was 90% higher in *Cluster 1* than in *Cluster 2 (*95% CI = 34.9 – 145.1%, *P* = 0.003, Welch's *t*-test; Table 2). This difference remained consistent and statistically significant after adjusting for subject age and race (estimated mean difference 81.9%, 95% CI = 4.6 – 159.2%, *P* = 0.05, multiple regression; Table 3A).

In the application population, an HIV-1 positive man was defined as having a "strong antiviral response (SAR)" if he showed a minimum of 50% increase in CD4T cell count by the end of the treatment regimen. All except one SAR subject were assigned to *Cluster 1*, the majority cluster (Figure 2 – horizontal tracking bars, and Table 2). On average, the SARs were 13.6 times more likely to adopt a *Cluster 1* gene signature pattern (95% CI = 1.2 – 751.3, *P* = 0.027, Fisher's exact test; Table 2). To rule out the possibility that known confounders drove the observed association, a multivariate logistic regression model was built. Adjusting for age and race, the association between the strong response and *Cluster 1* assignment remained significantly positive (adjusted OR = 14.4, 95% CI = 1.7 – 337.4, *P* = 0.03; Table 3B).

4. Discussion

This study provides evidence that transcriptome-wide gene expression captures biological and clinical insights into an infectious disease based on healthy CD4T abundance

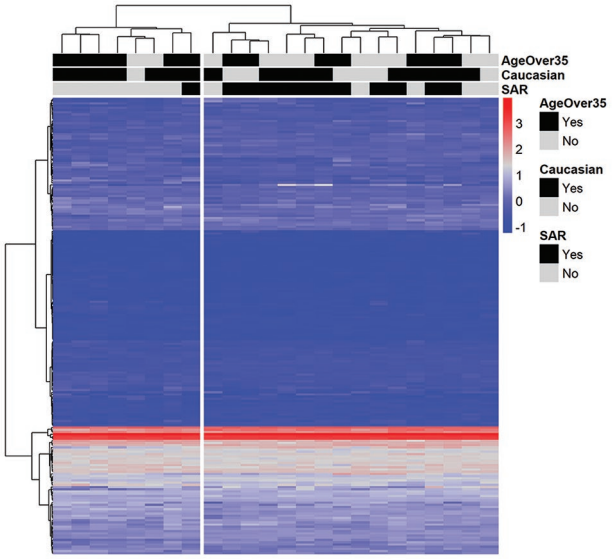

Figure 2. Application of the gene signature to an independent population of HIV1-positive, anti-retroviral therapy-treated men. Each row and column represents a gene and a sample, respectively. Horizontal tracking bars at the top indicate clinical covariates, which were used in univariate and/or multivariate analyses.
Note: "SAR" indicates a strong anti-retroviral response.

profiles. Using a statistically robust and conservative approach, LASSO, a panel of CD4T genes capable of stratifying high- versus low-abundance samples, was identified. The Gene Ontology analysis revealed that regulation of cell adhesion was positively associated with CD4T abundance. This result was unsurprising. During an inflammatory response, immune cells must be able to migrate and localize to the target tissue. *CD28* was among the genes accounting for this Gene Ontology term and was also top-ranked among all genes with positive LASSO coefficients. CD28 is a cell-surface glycoprotein essential for T-cell survival and proliferation. The implication of CD28 in HIV-1 pathogenesis has long been recognized. In HIV-1 patients, CD4 and CD28 coexpression is a predictor of progression to acquired immunodeficiency syndrome (AIDS).[12] Mechanistically, CD28 expressed on the T-cell surface can be targeted by two HIV-1 accessory proteins *Nef* and *Vpu* for degradation.[13] Meanwhile, the CD4T gene signature was depleted for metabolic processes of bio-macromolecules including glycerol-lipids and steroids. Unsurprisingly, both subunits of the CD8 molecule (*CD8A* and *CD8B*) were downregulated since the machine-learning model was optimized against characteristics associated with CD4 abundance. In a healthy tissue context, CD4T cells undergo metabolic programming and adaptations during an inflammatory response. Moreover, T-cell activation requires extensive metabolic reprogramming and elevated energy expenditure involving

Table 2. Stratification of application population by the gene-signature clusters

	Cluster 1	Cluster 2	*P*-value
n (%)	16 (66.7)	8 (33.3)	-
Age (mean±SD)	34.25±9.59	41.88±10.09	0.10
Race (%)			0.35
Caucasian	10 (62.5)	7 (87.5)	
Other	6 (37.5)	1 (12.5)	
CD4% change (mean±SD)	122.66±94.21	32.67±34.31	0.003**
SAR (%)			0.027*
Yes	11 (68.8)	1 (12.5)	
No	5 (31.2)	7 (87.5)	

Notes: *P* values were determined by two-sided Fisher's exact and Welch's *t*-test for discrete and continuous variables, respectively. *$P<0.05$, **$P<0.01$.
Abbreviation: SAR: Strong anti-viral response.

Table 3. Multivariate-adjusted analyses

(A)	Adjusted OR (95% CI)	*P*-value
SAR		
No	1.0 (reference)	-
Yes	14.4 (1.6 – 337.4)	0.03*
Age in years	0.94 (0.82 – 1.1)	0.32
Caucasian race		
No	1.0 (reference)	-
Yes	0.33 (0.01 – 4.3)	0.43
(B)	**Mean percent increase in CD4T level (95% CI)**	***P*-value**
Gene signature cluster		
#2	0.0 (reference)	-
#1	81.9 (4.6 – 159.2)	0.05*
Age in years	−0.51 (−4.1 – 3.1)	0.79
Caucasian race		
No	0.0 (reference)	-
Yes	−16.71 (−93.8 – 60.4)	0.68

Notes: Part (A) represents the association between strong antiviral response (SAR) with Gene Signature Cluster 1 estimated by multivariate logistic regression. Adjusted ORs were determined by exponentiating the model coefficients. Part (B) represents the mean difference between Gene Signature Clusters 1 and 2 estimated by multivariate linear regression. *$P<0.05$.
Abbreviations: OR: Odds ratio; CI: Confidence interval.

the aforementioned bio-macromolecules.[14] This finding, which appears contradictory, might be explained by a negative feedback loop due to overabundant CD4T.

When applied to an HIV-1-positive patient cohort receiving anti-retroviral therapies, the identified CD4T gene signature stratified the study population into clusters

significantly related to clinical response. The near-perfect stratification of diseased subjects here is consistent with the prior knowledge that CD4T abundance is essential to a favorable prognosis of anti-retroviral treatment response in HIV-1 patients.[1,2] In addition, formal statistical testing revealed a significant association between the gene signature and anti-retroviral response. This observed effect remained consistent in direction and significant statistically after accounting for clinical covariates. Taken together, these results support the generalizability of CD4T abundance-based gene signature, identified in the healthy context, to the diseased context, and reinforce the centrality of CD4T-based immune modulation during HIV-1 pathogenesis.

Several limitations of this study should be acknowledged. First, despite having high-quality gene-expression data, the application population used for biological and clinical inference is small. In particular, the LASSO model performance might improve if model training was conducted in a sex-stratified manner, a known risk-modifying factor in HIV/AIDS.[15] Due to the limited training sample size, model development on sex-stratified data was unlikely empowered to achieve robust performance. Second, there were variations in the study designs, and many clinical variables of the study populations used were not consistently collected or reported. Importantly, the gene-signature identification process leverages only gene expression without clinical covariates such as subject age and race. Finally, a broader limitation is the availability of datasets and samples with high consistency (i.e., low heterogeneity). Future confirmatory and follow-up studies with comprehensive and robust designs will be crucial to the understanding of the molecular mechanisms of diseases related to CD4T abundance.

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Conflict of interest

The author declares no competing interests.

Author contributions

This is a single-authored article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

As described in the Materials and Methods section, the datasets and code used in this study can be publicly accessed through Gene Expression Omnibus and GitHub, respectively.

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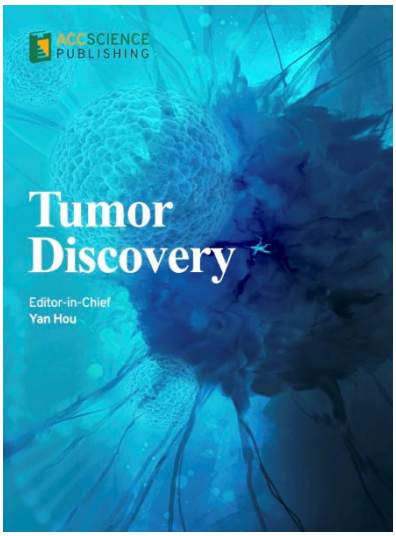

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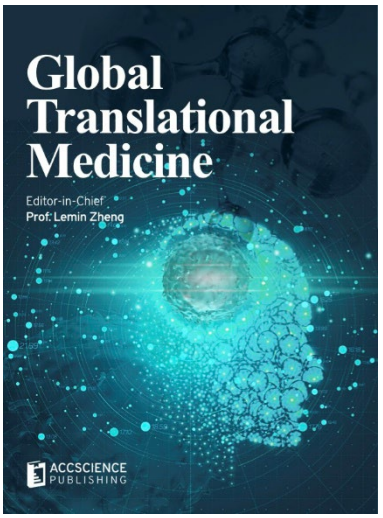

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