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Turkish Medical Student Journal (TMSJ) is the first scientific journal in Türkiye to be run by medical students and to publish works of medical students only. In that respect, TMSJ encourages and enables all students of medicine to conduct research and to publish their valuable research in all branches of medicine.

Turkish Medical Student Journal publishes researches, interesting case reports and reviews regarding all fields of medicine. The primary aim of the journal is to publish original articles with high scientific and ethical quality and serve as a good example of medical publications for those who plan to build a career in medicine. TMSJ believes that quality of publication will contribute to the progress of medical sciences as well as encourage medical students to think critically and share their hypotheses and research results internationally.

The journal is published every four months. The language of publication is English.

The Editorial Board of TMSJ and the Publisher follows the principles of the International Council of Medical Journal Editors (ICMJE). Only unpublished papers that are not under review for publication elsewhere can be submitted. The authors are responsible for the scientific content of the material to be published. TMSJ reserves the right to request any research materials on which the paper is based.

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Turkish Medical Student Journal publishes original researches, interesting case reports, and reviews regarding all fields of medicine. All of the published articles are open-access and reachable on our website. The primary aim of the journal is to publish original articles with high scientific and ethical quality and serve as a good example of medical publications for stimulating students, doctors, researchers. Our mission is to feature quality publications that will contribute to the progress of medical sciences as well as encourage medical students to think critically and share their hypotheses and research results internationally.

The Editorial Board and the Publisher adheres to the principles of ICMJE Committee on Publication Ethics (COPE).

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TMSJ
TURKISH MEDICAL STUDENT JOURNAL

Contents

TMSJ
TURKISH MEDICAL STUDENT JOURNAL

Editorial

Farewell to a Remarkable Journey

Dear Readers,

It is with a bittersweet mixture of pride and nostalgia that I write this editorial, for this issue of the Turkish Medical Student Journal marks the end of my six-year journey as an editor. As I reflect upon the pages of this journal, I am reminded of the countless hours, tireless dedication, and unwavering passion that have gone into its creation. Today, I stand before you with immense gratitude for the opportunity to serve this remarkable publication and the medical student community at large.

When I first started as an editor six years ago, I aspired to create a platform that would inspire and nurture the voices of aspiring medical professionals across Turkey. Over the years, we have strived to uphold the highest standards of scientific rigor, integrity, and ethical practice. Now, as I prepare to pass the torch, it is with great confidence and enthusiasm that I entrust the role of Editor-in-Chief to a talented and dedicated individual who embodies the spirit of this journal. Eylül Şenödeyici has been an integral part of our editorial team, contributing tirelessly to our mission and vision. Her deep understanding of the medical field and her unwavering commitment to fostering the next generation of medical professionals make her the ideal successor.

Eylül Şenödeyici is no stranger to the values that this journal upholds. She has demonstrated a passion for medical research and writing that has not only contributed to the growth of this journal but has also inspired her peers and colleagues. I have every confidence that she will lead this publication to new heights, nurturing the talent of our contributors and steering the Turkish Medical Student Journal toward an even brighter future.

As I step down from my role as Editor-in-Chief, I carry with me the memories of the incredible journey we have undertaken together. I am immensely proud of what we have achieved and the community we have built. I am grateful for the support of our dedicated editorial team, the trust of our readers, and the unwavering commitment of our contributors. It has been an honor and a privilege to serve in this role, and I know that the Turkish Medical Student Journal is in capable hands.

In closing, I want to express my heartfelt thanks to all of you for being a part of this journey. The Turkish Medical Student Journal will continue to be a platform where knowledge and innovation flourish, where young minds share their unique perspectives, and where the future of medicine takes shape. I look forward to witnessing the continued growth and success of this journal.

With gratitude and anticipation for what lies ahead,

Beliz Koçyiğit
Editor-in-Chief, Turkish Medical Student Journal
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TURKISH MEDICAL STUDENT JOURNAL

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REVIEW

A BRIEF INSIGHT INTO STIMULANTS' EFFECTS: A REVIEW BASED ON STUDENTS

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ABSTRACT

Central nervous system stimulants of various levels of effectiveness are commonly used among students worldwide. These stimulants are a group of drugs that increase vigilance, alertness, and excitation. In the present study, three stimulants; caffeine, methylphenidate, and modafinil are compared in terms of their mechanisms of action, effects on memory, and addiction, especially in the younger population. Caffeine is the most widely used stimulant after methylphenidate and modafinil. Although the possibility of addiction due to caffeine is highly dose-dependent, there is a potential for abuse of methylphenidate and modafinil. These stimulants are used for a variety of reasons, such as staying awake to study, increasing alertness to complete assignments, or for recreational purposes among students. Also, since many stimulants are readily accessible to many individuals, such substances may be misused. The aim of the study is to show different aspects of caffeine, methylphenidate, and modafinil use on epidemiology, mechanism, addiction, and effect on electroencephalogram and long-term memory.

Keywords: Caffeine, methylphenidate, modafinil, attention, ADHD

INTRODUCTION

In circadian rhythm, the cortex moves around two states: Wakefulness and sleep (1). Wakefulness indicates an aroused state of mind (2). This arousal can either be achieved physiologically by the hypothalamus and reticular activating system or through certain central nervous system (CNS) stimulants (3). CNS stimulants are a group of drugs that increase vigilance, alertness, and excitation (4). Several compounds can be listed, but a few of them are more popular than others. One such compound is caffeine, which is found especially in coffee (5). Other chemicals that are classified as drugs can be used legally or illegally for many purposes, one of them being attention deficit hyperactivity disorder (ADHD) (6).

Despite their benefits in the treatment of disorders such as ADHD and narcolepsy, modafinil and methylphenidate can be misused due to their reputation as "brain juice" (3). Perceiving them as brain juice, students may show a tendency to use these drugs without a prescription (6). Half of non-prescribed drug users take them to complete school or work assignments and for entertainment. The route of administration depends on the desired effect, availability, and environment (7). According to the National Survey of Drug Use and Health, 1.5% of adolescents aged between 12 and 17 report using non-prescribed stimulants (8). The peak age of non-prescribed stimulant use has been recorded as 16 years (8). Additionally, 35% of college students reported using stimulants prior to college entrance (8).

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Students who started using non-prescribed stimulants before college were reported to be more likely to use them via non-oral routes and have worse health than those who started using non-prescribed stimulants at college (8). Informing students about effective study techniques might not be very beneficial since the students that tend to misuse these stimulants are appeared to implement study strategies on a regular basis (9). However, users expressed that stimulants help them "be competitive" and maintain a certain level in their field. Fear of losing their standards seems to be a great motivation for stimulant use (9). While academic purposes seem like the primary reason for non-prescribed stimulant use, studies show that other motivations include increasing the effect of alcohol, partying, and socializing longer (10). Additionally, amphetamines are used for enhancing physical performance, and these drugs are categorized as "doping" and fall under bans (5). Because of their affect on dopamine receptors, such drugs can be addictive (11). CNS stimulants may increase the levels of certain chemicals, alter vital signs, and cause changes in electroencephalogram (EEG) patterns (1).

An electroencephalogram is a device that measures the electrical activity of the brain. It was discovered in 1875 by Richard Caton, and Hans Berger made the first recording of EEG 50 years later (12). EEG is used for measuring the electrical potentials produced by neurons under the cerebral cortex. These measurements are made by electrodes that are positioned around the scalp and sometimes other parts of the head. These recordings are made based on a reference electrode (12).

Electrical activity is mainly generated by pyramidal cell bodies that are located in the third and fifth layers of the cerebral cortex. Secondary to this activity, excitatory and/or inhibitory postsynaptic potentials (EPSP/IPSP) are produced with the help of neurotransmitters. In a certain cortical region, EPSP and IPSP create an electric field with positive and negative poles, and that is recorded by EEG (13).

The normal EEG is diverse, with a wide range of variability (13). Pathologies may appear differently in the EEG, and these patterns may be altered by the intake of stimulants (14). Frequencies that are recorded by the EEG are important for describing the pathology since different waves are involved in different states and pathologies (15). Waves are named in terms of their frequencies; waves with a frequency of 1-3 Hz are delta waves, 4-7 Hz are theta waves, 8-12 Hz are alpha waves, and 13 Hz and over are beta waves (12, 15). Although gamma waves with frequencies over 25 Hz are not commonly recorded, they can be encountered during intracranial recordings. Gamma waves of 25-70 Hz are called low gamma, and waves over 70 Hz are high gamma waves (15). Frequencies over 100 Hz are generally referred to as ripples, and they can be seen in EEGs with epileptiform activities (15).

This review aims to show the epidemiology, mechanism of action, addictive aspect, EEG changes, and long-term memory effects of caffeine, methylphenidate, and modafinil use among students.

CAFFEINE

Epidemiology

According to a survey of 37602 people from different age groups in the United States (US) 85% of the people consume at least one cup of caffeine-containing beverage per day (16). Coffee is the main source of caffeine in all age groups (16). In another study involving 1248 university students in the US, it was revealed that 92% of the students consume caffeine in various forms. The two most common reasons for caffeine consumption are caffeine's effect on feeling awake and liking the taste of caffeine (17). Caffeine consumption was also common among high school students in Delhi, India (18). Of the 300 high school students, 291 of them regularly consumed caffeine (18).

In a survey conducted on tertiary students in New Zealand, it was determined that 99.1% of the participants consume caffeine (19). These students consume products involving caffeine, such as tea and chocolate, for their taste and coffee to stay awake (19).

Mechanism of Action

The blood level of caffeine peaks approximately 1 hour after oral consumption (20). Almost all caffeine is metabolized in the liver by the CYP1A2 isoenzyme (21, 22). Its half-life is 3-5 hours, and it can easily cross the blood-brain barrier (21). Its excitatory effect on the brain starts when caffeine inhibits the non-specific inhibitory A1 and A2 adenosine receptors (23). The effects of caffeine vary depending on age, gender, and demographics (24). This difference is thought to be caused by the variations in CYP1A2 enzyme activity of the individuals (22).

Effects on Memory and Attention

Caffeine, the most widely used psychostimulant, has important effects on memory and attention (25). Kahathuduwa et al. (26) stated that caffeine significantly improved the cognitive simple visual reaction time. It also has a positive effect on sustained attention and long-term memory as a cognitive enhancer (27, 28). In addition, it has been shown to improve memory performance in people who are sleep-deprived or elderly (29). The fact that it increases concentration in sleep-deprived people confirms that it may have a stimulant effect, but a statistically significant result was not found in healthy people, which makes it difficult to accept caffeine as a pure stimulant (30). Additionally, it is thought to reduce the impairment of memory in neurological diseases such as Alzheimer's (29).

Effects on EEG

The electroencephalograms of ten healthy young men were recorded before and after caffeine consumption in a randomized, controlled, double-blind trial (31). According to the EEG results, it was determined that the amplitude in the fronto-parieto-occipital and central electrodes were reduced after caffeine intake (31).

In another study, EEG waves were measured during the caffeine withdrawal period, and it was found that there was a significant increase in the amplitude of alpha and theta waves during this period (32). In the clinical trial conducted by Sigmon et al. (33), it was observed that caffeine increased the amplitude of theta waves in the EEG and decreased the amplitude of the beta 2 (25-40 Hz) waves in the acute withdrawal period. Additionally, the acute effects of caffeine were detected in several parameters, such as cerebral blood flow and EEG, while the effect of chronic caffeine consumption was only seen in beta 2 waves in the EEG (33).

Another study revealed that people who took citicoline-caffeine drinks exhibited higher attention and learning speeds, considering their EEG results (34). Citicoline, which is formed by the combination of cytidine and choline, has a positive effect on the cognitive functions of the brain in addition to the effects of caffeine on attention and neurocognitive functionality (34).

In addition to the effect of caffeine on EEG, it can be said that caffeine reduces the reaction time and the number of mistakes made in event-related potentials (ERP) (35). In this case, it is seen that the stimulating effect of caffeine is also supported by the ERP (35).

Caffeinated Energy Drinks: An Important Health Issue for Adolescents

Energy drinks are high-caffeine beverages formulated to improve mental and physical stimulation (36). In recent years, they have become very popular among adolescents, despite their various side effects (37). Caffeine intoxication is one of the main side effects of energy drinks (36). It has been determined that side effects are more common in those who drink more than 5-7 energy drinks per week (36).

According to a study, 67% of high school students consume these drinks to stay awake, 65% to increase their energy, and 54% to drink with alcohol at a party (38). As side effects, headaches were reported in 22% of young people, and tachycardia was observed in 19% (38).

Addiction

Although it is not recommended for children and adolescents to consume caffeine, up to 6 mg/kg of daily consumption may be suggested for young adults because of its positive effects (20). However, consuming more than 300 mg of caffeine at a time or more than 1000 mg daily may lead to caffeine intoxication, which may cause arrhythmia, tachycardia, muscle tremors, or psychomotoric distress (20, 21).

Students under stress tend to consume more coffee, which increases the risk of developing caffeine addiction (39). Especially among senior students, excessive coffee intake has been associated with anxiety and depression symptoms (40). A randomized controlled trial also found that caffeine delays rapid eye movement (REM) sleep (40).

METHYLPHENIDATE

Epidemiology

Methylphenidate is one of the primary drugs for the treatment of ADHD, and it is used as a secondary treatment for narcolepsy (41, 42). It was proven that it is used by the majority of patients diagnosed with ADHD (41). Male gender and comorbidity of neuropsychiatric disorders increase the tendency to use methylphenidate (43). Additionally, males tend to misuse cognitive enhancers more (44). Hunter et al. (45) examined the prescription trends in 2009-2018 and reported that methylphenidate accounts for 15% of the 356,548 pediatric psychotropic drug prescriptions. In the US, non-medical utilization of stimulants on prescription is common and increasing among college students, as is the consumption of methylphenidate in the world (44). Increased awareness of ADHD and extended treatment duration may have caused an increase in the prescription of methylphenidate (44).

Mechanism of Action

Methylphenidate interrupts catecholamine metabolism, stops the reuptake of norepinephrine and dopamine in synapses, and enhances the stimulant effect on the CNS, mainly at the prefrontal cortex (11). Norepinephrine and dopamine transporters have particularly high affinities for methylphenidate. It blocks dopamine and norepinephrine transporters by competing with catecholamines, resulting in higher concentrations of dopamine and norepinephrine (11). In addition to the stimulation, because of the impact on dopamine and other catecholamine mechanisms, it increases the motivational willingness of the individual (11). It gets metabolized primarily in the liver by re-esterification to ritalinic acid, and 78-98% of the drug is excreted by urination (46).

Effects on Memory and Attention

In healthy subjects, methylphenidate increases attention and cognition by increasing dopamine and norepinephrine in many parts of the CNS, such as the dorsolateral prefrontal cortex, posterior parietal cortex, and striatum of the subcortical basal ganglia (47). Baseline working memory capacity is a parameter that is positively correlated with the striatal synthesis of dopamine (48). Van der Schaaf et al. (48) reported that methylphenidate improves the performance of cognitive tasks in high-working memory subjects or impairs it in low-working memory subjects.

Repantis et al. (27) stated that methylphenidate affects long-term declarative memory positively. Another study by Rostami Kandroodi et al. (49) showed that the effects of methylphenidate depend on the individual differences of the subjects, the drug improves cognitive task performance while impairing learning in participants with higher-working memory capacity. It has also been shown that language processing was better with lower-working memory capacity after methylphenidate was administered (50).

Effects on EEG

In acute usage, it is found that the alpha and beta activites increase in the frontal areas, and the delta and theta activites decrease in the parieto-occipital and occipital areas (51). It is also reported that P3, an evoked response potential identification component on EEG, can be used to differentiate people with ADHD from healthy individuals for visual and auditory tasks by ERP technique (52). It is also proven that the theta/beta ratio was more sensitive in continuous performance tests in parieto-occipital areas of the right hemisphere when methylphenidate was administered (52).

Addiction

Based on clinical experience, a clinical trial stated that methylphenidate is said to be beneficial for substitution therapy for cocaine (53). Duka et al. (54) reported that genetic variants of the *GABRA2* gene may be associated with methylphenidate addiction, which may explain why some people are more prone to addiction. In another study, it was suggested that a combination of fluoxetine and methylphenidate mimics cocaine activity both on behavioral effects and gene regulation in the striatum, indicating a potential risk of substance abuse (55). Recently, misuse of pharmaceutical cognitive drugs has increased, with the prevalence varying from 6% to 20% among university students (6). Several cases of toxicity and fatalities have been reported due to methylphenidate misuse, and its effects on the heart, such as palpitations, tachycardia, hypertension, and endocarditis, seem to be important for methylphenidate analogs' toxicity (56). It is suggested that high-risk groups, such as students and addicts, should be educated about the dangers and consequences of using such substances (56). From a legal perspective, methylphenidate and its analogs may be systematically controlled (56).

MODAFINIL

Epidemiology

Modafinil, a stimulant that is used to treat ADHD, is also a wakefulness-promoting agent used for the treatment of excessive daytime sleepiness associated with disorders such as narcolepsy, sleep apnea, and shift-work sleep disorder. Modafinil was found to improve attention and memory while helping to maintain wakefulness in well-rested individuals (57). However, some studies note that, particularly in healthy, non-sleep-deprived college students, modafinil does not have positive effects on sustaining studying except for non-demanding tasks (58). This evidence points out that modafinil has limited potential as a cognitive enhancer if the individual is not sleep-deprived (59).

In an online poll managed by Nature, 20% of the 1400 responding readers reported non-medical use of modafinil or beta-blockers, 62% reported taking modafinil, and 44% reported taking modafinil for non-medical reasons (57). The main reasons for the non-medical use of modafinil were improving focus, preventing jetlag, and overcoming sleep deficiency (57). It is also known that modafinil is misused by college students for academic purposes (57). The indirect evidence for the misuse of modafinil can also be proved by comparing the sale numbers of modafinil to the patients suffering from the disorders from which these substances are used (57). Modafinil is also used by military personnel during long missions, as depicted in the Memorandum of the United States Air Force "Modafinil and management of aircrew fatigue" (2nd December 2003), which approves the use of modafinil for missions of great duration (57).

Mechanism of Action

Modafinil is a stimulant that is better tolerated than conventional stimulants such as methylphenidate, which has a selective site of action in the brain (60). Modafinil is associated with improved attention, vigilance, memory, and learning as it affects the frontal lobe (61). One study shows that modafinil affects cortical areas of the frontal lobe and has minor activity in subcortical regions. It increases extracellular catecholamine levels through the inhibition of dopamine and noradrenaline transporters like methylphenidate and indirectly activates the hypocretinergic system. However, modafinil's exact mechanism is not yet clear. It is important to note that modafinil is also believed to affect other neurotransmitter systems, including serotonin, histamine, and glutamate pathways (62, 63).

In healthy male volunteers, the duration of modafinil activity was investigated in two double-anonymous crossover studies (64). These studies indicated that modafinil has a long duration of action while predominantly exhibiting alerting properties from dopaminergic activity. The mode of action was explored by using a model about the relationship between total sleep time and duration of REM sleep. While the model showed no evidence of direct suppression of REM sleep, it revealed that the increase in REM sleep was because of the alerting activity of dopaminergic activity. Enhanced performance with modafinil during overnight work varied with dose. However, when the next-day performance was evaluated, cognitive enhancement was the least at the highest dose (300 mg) due to the disturbance of prior sleep (64).

Effects on Sleep, Memory, and Attention

According to a systematic review, modafinil helps healthy individuals maintain wakefulness and improve memory after one night of sleep deprivation (63). Another study showed that the administration of psychotropic medications such as modafinil to long-term cocaine users may be beneficial in improving memory (65). For non-sleep-deprived healthy individuals, modafinil may have stimulating effects in maintaining relatively difficult and monotone tasks and improving memory (63). In a study, modafinil significantly enhanced performance on digit span tests, visual pattern recognition memory, spatial planning, and stop-signal reaction time. There were no significant effects of the drug on spatial memory span, spatial working memory, rapid visual information processing, or attentional set-shifting (66).

Effects on EEG

Modafinil is a wakefulness-promoting agent that affects hypothalamic structures involved in the homeostatic and circadian regulation of vigilance. In the case of sleep deprivation, the administration of modafinil reduces the need for recovery sleep and decreases the rebound in EEG slow wave activity (67). Modafinil administration during continuous positive airway pressure (CPAP) withdrawal increased awake EEG activation, which is associated with an improvement in neurocognitive performance. This study presents supporting neurophysiological evidence that modafinil may be a potential short-term treatment option during acute CPAP withdrawal (68).

Addiction

Modafinil was originally indicated as a cognitive enhancer that has a low risk of addiction with a few side effects. It is becoming clearer that the drug is acting on dopaminergic transmission, and, like other psychostimulants, it has a risk of addiction. Yet, the long-term effects are still not completely explored (69). It has been reported that modafinil and armodafinil, which is an oral non-amphetamine wake-promoting agent, improve excessive daytime sleepiness symptoms and have little abuse potential (70).

Dextroamphetamine is authorized for use by the aircrews of all US military services, but its potential for abuse and subsequent addiction is of concern. Finding an alternative stimulant like modafinil, which has a low affinity for dopamine uptake binding sites, would be beneficial, as it does not have the potential for abuse, unlike dextroamphetamine (71). Another study suggests that modafinil does not have a high potential for abuse in cocaine abusers. With the increased cocaine abstinence and reduced craving results in some studies, it may be a promising medication (72).

CONCLUSION

Students may use different stimulants for several reasons, such as staying awake to study, increasing alertness to complete assignments, or for recreational purposes (73). With a lifetime prevalence of 6.9% among American college students, stimulant use seems to be perceived as a "physically harmless" and "morally acceptable" act (42, 74). Students are often inclined to justify the use of stimulants by comparing them to synthetic drugs, expressing that they are using stimulants to achieve better grades or study more effectively, and arguing that no physical or cognitive side effects exist (74). One such substance, methylphenidate, is widely used for ADHD and has a high potential for abuse (75). A study by Barrett et al. (75) reported that while 70% of methylphenidate abusers used the substance for recreational purposes, the remaining 30% used it solely for academic purposes. This may challenge the justifications regarding stimulant use to increase academic performance. In addition, since many intoxications were reported due to the misuse of methylphenidate, high-risk populations, such as students and current users, should be educated about the negative side effects of such drugs (56).

Increased delta and theta activity in the EEG is generally linked to inattentiveness and poor task performance. A study by Lubar et al. (76) revealed that increased beta activity was correlated with increased delta and theta activity when participants were taking methylphenidate, which contrasts with the positive relationship between improved task performance and increased beta activity. However, it does align with previous reports suggesting that long-term use of methylphenidate may not lead to significant cognitive or academic improvements (57). It is possible that increased slow activity while under the influence of methylphenidate impedes cognitive processing, despite reducing hyperactive behavior in individuals with ADHD (57).

The effects of another cognitive enhancer, modafinil, have been found to be inconsistent and varying among sleep-deprived and non-sleep-deprived individuals (59). Fernández et al. (58) reported that non-sleep-deprived, healthy students do not benefit from modafinil, which is often used for its enhancing effects on studying and focus. Since no significant difference between 100 mg and 200 mg doses of modafinil was reported and the safety of the drug remains unclear in healthy and non-sleep-deprived individuals, its effectiveness as a cognitive enhancer seems to be lacking (59). Modafinil reduces sleepiness and attenuates theta wave activity in the EEG during wakefulness. However, it does not affect EEG activity during REM sleep. Furthermore, it does not alter slow-wave sleep or slow-wave activity in the EEG during non-REM (NREM) sleep following sleep deprivation, suggesting that modafinil does not interfere with the compensatory increase in NREM sleep after prolonged wakefulness (60).

Caffeine is the most widely used stimulant in all age groups, and its benefits include a longer attention span, enhanced long-term memory, and improved visual reaction time (17, 27-29). Despite its many favorable effects, taking more than 1000 mg daily may lead to caffeine intoxication, causing tachycardia and muscle tremors. Since caffeine is a well-studied food component and is consumed by 85% of the US population daily, individuals should be well-informed about the benefits, ideal doses, and unfavorable side effects of this substance (17). It has been reported that headaches during caffeine withdrawal and an increase in alpha and theta amplitudes in the EEG are a result of cerebral vasodilation. However, caffeine intake seems to reverse these effects by causing vasoconstriction (32). Interestingly, a study by van Oosterhout (77) revealed that caffeine may have no effects on the alpha frequency amplitudes in resting-state EEG, highlighting the need for more studies in the area. It would perhaps be a correct approach to investigate whether different doses of caffeine intake affect the amplitude of the alpha waves.

Since many stimulants are readily accessible to many individuals, such substances have the potential to be misused. Although it is important to monitor the prescription and distribution of cognitive-enhancing drugs, it is crucial for individuals who use cognitive enhancers and those contemplating doing so to be

fully aware of the benefits, risks, side effects, and unfavorable outcomes of using such substances.

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REVIEW

STEROID THERAPY FOR CHILDREN WITH IDIOPATHIC NEPHROTIC SYNDROME: IS A SHORT-TERM OR LONG-TERM TREATMENT PLAN MORE EFFECTIVE?

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ABSTRACT

Idiopathic nephrotic syndrome is one of the most common glomerular diseases in childhood. Corticosteroids are the first line of treatment for this disease. Although the majority of patients respond to steroids, recurrences of idiopathic nephrotic syndrome are quite common. Therefore, it is important to determine the most appropriate duration of treatment because of relapses and possible steroid side effects. In this review, the literature is summarized by evaluating the difference between the short (2 to 3 months) and long (>3 months) durations of steroid therapy in terms of relapses and steroid side effects. In most of the studies, it has been seen that the two-to-three-month treatment protocol is sufficiently efficient. Nowadays, most prefer 2-3 months of steroid treatment to achieve good disease control and avoid steroid-related side effects. Yet, studies with larger patient groups on this subject need to be carried out.

Keywords: Nephrotic syndrome, children, steroids, therapy

INTRODUCTION

Idiopathic nephrotic syndrome is a kidney disease that is common in childhood and occurs because of a damaged glomerular filtration barrier (1). Although it may differ depending on ethnicity and region, it affects between 1.15 and 16.9 per 100,000 children worldwide each year (2). Genetic ancestry is also important in the incidence of the disease and the patient's response to treatment (3). This disease is defined by nephrotic-level proteinuria, hypoalbuminemia, and generalized peripheral edema (2). At the same time, due to increased hepatic lipoprotein synthesis, hyperlipidemia is also observed (4).

The two most common histopathologic findings of idiopathic nephrotic syndrome in children are minimal change disease and focal segmental glomerulosclerosis (5). Minimal change disease accounts for 70-90% of idiopathic nephrotic syndrome cases in children older than one year (6, 7). Most of the cases are idiopathic (primary nephrotic syndrome) and have a very good prognosis (7, 8). However, it can develop secondary to conditions such as infection, neoplasia, allergy, drug use, human immunodeficiency virus, systemic lupus erythematosus, and type 1 diabetes mellitus (8).

Focal segmental glomerulosclerosis, on the other hand, is less common than minimal change disease. However, it may have further negative long-term consequences and can progress to end-stage renal disease due to its resistance to treatment (4).

Corticosteroids, which are the most important and first-line drugs in the treatment of idiopathic nephrotic syndrome, can cause various side effects with long-term use. It increases susceptibility to infection due to immunosuppression. It may also cause many side effects, such as central obesity, moon face (a round face), buffalo hump (due to unusual fat accumulation on the back), hyperglycemia, and hypertension. In addition, if steroid treatment is stopped suddenly, a serious clinical picture called "adrenal crisis" may occur due to the lack of cortisol production in the adrenal gland (9).

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This review aims to determine the duration of treatment that will be most effective and cause the fewest side effects, considering the recurrence rates of the disease and possible side effects of corticosteroid use.

In this literature review, we will discuss the pathogenesis of idiopathic nephrotic syndrome and try to evaluate randomized controlled trials (RCTs) and meta-analyses on this subject between 2012 and 2022. The results of the studies were evaluated in terms of the number of relapses and side effects of steroids after short-term (2-3 months) and long-term (>3 months) steroid use in children with idiopathic nephrotic syndrome.

Pathogenesis

The exact pathogenesis of childhood nephrotic syndrome is not fully understood, but recently, immune system dysregulation has been the focus, and many other diverse factors, such as genetic factors and environmental factors, are thought to play a role in podocyte dysfunction (10). Infections have also been suggested to play a role in disease onset or recurrence (11).

In recent years, it has been stated that the presence of autoantibodies against the protein called nephrin, which is expressed on the surface of podocytes and has a critical role in preventing proteinuria, is important in minimal change disease (12, 13). It is thought that "diffuse podocytopathy," which is seen frequently in children with nephrotic syndrome, is caused by the damage of podocytes due to an autoimmune response (14, 15).

There are various theories regarding immune system dysregulation in the pathogenesis of idiopathic nephrotic syndrome. According to the "T-cell theory," upregulation of CD4+ T helper cells and downregulation of CD8+ cytotoxic T-cells creates an imbalance resulting in increased Th2, and this plays a role in the pathogenesis of the nephrotic syndrome. The fact that immunosuppressants (corticosteroids, cyclosporine, and cyclophosphamide) that suppress T-cell function are beneficial in the treatment and that patients go into remission due to impaired T-cell function in some cases of measles supports this hypothesis. The demonstration that anti-CD20 B-cell targeted therapies (e.g., rituximab) are effective in children with frequent relapses or steroid-dependent nephrotic syndrome supports the "B-cell theory." It has also been suggested that circulatory factors play a role in the pathophysiology, especially in patients with focal segmental glomerulosclerosis (10).

Genetic factors also play an important role in the development of nephrotic syndrome. There is a single gene mutation associated with podocytes in 30% of steroid-resistant cases (10). This is mostly seen in the steroid-resistant condition that emerges in the first three months of life, which is called congenital nephrotic syndrome (CNS) rather than idiopathic nephrotic syndrome cases. For example, the absence of nephrin protein due to the *NPHS1* gene mutation causes Fin-type nephrotic syndrome, which is an important subtype of CNS (12). In addition, it has been suggested that the human leukocyte antigen-DQ and HLA-DR regions are associated with steroid-sensitive nephrotic syndrome (SSNS) cases (10).

Clinical and Laboratory Findings

Idiopathic nephrotic syndrome is clinically characterized by generalized edema, nephrotic-range proteinuria (>40 mg/m^2/h or urine protein to creatinine ratio (Up/Uc) >2 mg/mg in the first-morning specimen or urine protein 3-4+ with dipstick/boiling test), hypoalbuminemia (<3 g/dL), and hyperlipidemia with an increase in total and low-density lipoprotein (16, 17). Thromboembolic events may also accompany the disease (18).

Generalized edema is the most important finding of idiopathic nephrotic syndrome in children, especially in minimal change disease (19). It increases with standing, especially during the day, and is in the form of soft edema that leaves pitting. In these patients, acid accumulation in the abdomen, scrotal, penile, or labial edema may develop (5).

Two hypotheses, namely "underfilling" and "overfilling," have been proposed for edema formation. In both of these hypotheses, proteinuria resulting from kidney damage lowers oncotic pressure by causing a decrease in serum albumin. Therefore, intravascular fluid shifts into the interstitial space and the increase in interstitial fluid causes edema. According to the "underfilling" hypothesis, blood pressure decreases due to decreased plasma and blood volume, and the renin-angiotensin-aldosterone system is activated. There is an increase in the plasma volume, which supports the increase of interstitial fluid and, therefore, the formation of edema. According to the "overfilling" hypothesis, there is an increase in sodium retention due to kidney damage and, therefore, in plasma volume. As a result, increased plasma volume combined with the loss of albumin leads to increased edema (20).

Although edema is the main finding of nephrotic syndrome, patients may present with complications of nephrotic syndrome, such as thromboembolic events (pulmonary embolism, deep vein thrombosis, etc.), spontaneous bacterial peritonitis, cellulitis, and abdominal pain due to intestinal wall edema or hypoperfusion (5).

Even though some patients with nephrotic syndrome may have nephritic features (hypertension, hematuria, and decreased kidney function), these findings are not expected in minimal change disease (5, 16). Therefore, persistent hematuria suggests different diseases , such as infection-related glomerulonephritis, complement C3 glomerulopathy, or systemic lupus erythematosus, and additional investigations are required (16).

On physical examination, edema is the predominant finding. During the examination, syndromic conditions and complications of nephrotic syndrome (thromboembolism, peritonitis, and cellulitis) should be investigated (5).

Diagnosis and Kidney Biopsy

According to International Pediatric Nephrology Association (IPNA) recommendations, depending on the age and clinical

characteristics of the cases, different approaches are used for diagnosis (2).

Kidney biopsy is not routinely performed in the diagnosis of SSNS in children aged 1-12 years with typical symptoms because of its limited prognostic and clinical utility. However, there are several situations where a biopsy is indicated. If the patient has atypical features such as macroscopic hematuria, low C3 levels, acute kidney injury not related to hypovolemia, sustained hypertension, arthritis, and/or rash, which are not routinely seen in nephrotic syndrome, a kidney biopsy is initially performed to elucidate the etiology (2).

In patients presenting with typical nephrotic syndrome findings, an age-based approach is preferred for the diagnosis. If the patient is older than 12 years of age, two different strategies are considered on a case-by-case basis: performing a kidney biopsy or initiating corticosteroid therapy directly without a biopsy. Steroid treatment is started initially in the patient group between the ages of 1 and 12 years. In patients aged 3-12 months, there are three different options for diagnosis: Genetic testing (the primary choice), starting treatment without a biopsy, or performing a kidney biopsy. If the patient is younger than three months of age, has extrarenal features, or has a family history that suggests hereditary and syndromic steroid-resistant nephrotic syndrome, genetic testing is primarily preferred to clarify the CNS (2).

As mentioned in IPNA recommendations, kidney biopsy is also suggested in patients with persistent microscopic hematuria in populations where glomerular diseases such as IgA nephropathy are prevalent (2).

Treatment

Steroids have been the cornerstone of treatment, as most children with idiopathic nephrotic syndrome achieve remission after 4-6 weeks of daily prednisone or prednisolone therapy (21, 22). Particularly, minimal-change disease responds favorably to corticosteroid therapy (7).

To summarize the treatment algorithm generally, corticosteroid therapy (prednisone/prednisolone) is started at the first stage (2, 21). If relapses are frequent (≥2 relapses in the first 6 months following remission of the initial episode or ≥3 relapses in any 12 months) or if significant steroid toxicity has occurred, low-dose alternate-day steroids or steroid-sparing drugs (levamisole, mycophenolate mofetil, etc.) can be given to reduce dependence on steroids and minimize the side effects of steroid therapy (16, 21). In addition, it is also crucial to refer the patient to a pediatric nephrologist who can provide individualized treatment recommendations (21).

There are two different treatment protocols specified in the IPNA recommendations for patients with the first episode of idiopathic nephrotic syndrome. The first of these suggestions is four weeks at 60 mg/m^2 or 2 mg/kg (maximum dose of 60 mg/day), followed by alternate-day prednisone/prednisolone at 40 mg/m^2 or 1.5 mg/kg (a maximum dose of 40 mg on alternate days) for four weeks. The other suggestion, which is stated to be a "grade A strong recommendation", is six weeks at 60 mg/m^2 or 2 mg/kg (a maximum dose of 60 mg/day), followed by alternate day prednisone/prednisolone at 40 mg/m^2 or 1.5 mg/kg (maximum dose of 40 mg on alternate days) for six weeks (2).

Since the Kidney Disease: Improving Global Outcomes 2021 Guidelines, 8-12-week treatment plans of prednisone/prednisolone are recommended instead of a 24-week regimen in minimal-change disease. It is recommended to establish a treatment plan in which patients are given 60 mg/m^2/day or 2 mg/m^2/day in the first half and 40 mg/m^2/day or 1.5 mg/kg/day in the second half of the treatment period (23).

Although minimal change disease is steroid-sensitive, relapses are very common in these cases (7). Approximately 70-80% of the patients experience at least one relapse during follow-up (2). Repeated steroid therapies can cause toxicity after a while (24). Due to the side effects of glucocorticoid therapy such as obesity, hypoglycemia, cataracts, and hypertension, a treatment plan should be considered to minimize steroid toxicity (25, 26). Therefore, it is important to investigate the efficiency of long- and short-term treatments for nephrotic syndrome.

DURATION OF TREATMENT, EFFECTIVENESS, AND CORTICOSTEROID SIDE EFFECTS

In an RCT conducted by Yoshikawa et al. (27) in 2015 involving 246 children from 90 centers in Japan, patients were treated with corticosteroids for 2 or 6 months, then followed for 24 months for relapse. The time to first relapse and the side effects that may be seen due to steroids are similar for both groups receiving 2 or 6 months of treatment. Therefore, it was recommended that the prolongation of the treatment period was not clinically significant.

A similar result was found in another RCT in England, which was published in 2019 and included 237 children aged 1-14 years during the first episode of SSNS. In this study by Webb et al. (28), the efficiency of 8- and 16-week treatment plans was compared in patients divided into two groups. Afterwards, they were followed for 24 months to observe recurrence and side effects, and no significant difference was found between the two groups in terms of time to first relapse. Concerns about side effects were also not supported in the long-term steroid group. A possible weakness of this study is the possible exclusion of young children who cannot take the trial drug, which is provided as a crushable tablet (28).

In the study conducted by Sinha et al. (29) in 2015, the effectiveness of the 3- and 6-month treatment processes was investigated. A total of 181 patients were included in this study conducted in northern India, 92 of whom received 6-month treatment and 89 of whom were in the 3-month treatment group. At the end of the one-year follow-up, the number of relapses was 1.54 in the 3-month treatment group and 1.26 in the 6-month group. Since these values were not statistically significant, it was concluded that the 3-month treatment provided sufficient efficiency. The strengths of the study are

that it was designed as a anonymous, placebo-controlled, multicenter study and has a low risk of selection, performance, and selective reporting bias. The main limitation is that it is not stratified for key variables that may affect disease severity, such as age and gender (29).

In another study implemented by Kainth et al. (30) in 2021, the duration of continued steroid treatment after remission was compared. A total of 117 patients participated in this prospective study, 55 of whom were on the short regimen and 62 on the standard treatment regimen. Both randomly allocated groups received prednisolone 60 mg/m^2 until remission. One of the groups was given a short regimen of 40 mg/m^2 for two weeks after remission, while the other group was given a 4-week standard regimen. As a result of the study, the relapse rates in the short regimen group were similar to those in the other group; therefore, the short treatment was not inferior. Although the number of participants in the two groups is not equal, which is a limitation of the study, it has been stated that this situation did not cause a statistical difference (30).

One hundred twenty patients were included in the study conducted by Al Talhi et al. (31) in 2018, and prednisolone treatment was given for 3 months to one group and 7 months to the other. The follow-up period was two years. As a result of this study, it was observed that the risk of relapse was significantly lower in the patient group that received 7 months of treatment (31). The reason why the result of this study conducted in Saudi Arabia differs from the others may be due to the limited number of patients participating in the study and the differences in patients due to factors such as genetic predisposition and living conditions.

In an RCT involving 69 centers in the Netherlands, it was investigated whether the duration of steroid treatment or the cumulative dose was important. Hundred and fifty children aged 9 months to 17 years were included in the study. In this study, prednisolone treatment was given to one group for 3 months and to the other group for 6 months, but the total cumulative doses received by both groups were kept equal. As a result, the fact that there was no significant difference between the two groups in terms of relapse rate showed that the important factor was the cumulative dose (32).

In the study conducted by Geng et al. (33), a similar result was obtained. This prospective, non-RCT included 89 new-onset primary nephrotic syndrome cases between December 2017 and May 2019. One of the groups was given 2 mg/kg/day prednisone treatment for 4 weeks and the other for 6 weeks. Then, both groups were treated every other day with 2 mg/kg prednisone for 4 weeks, and the doses were gradually reduced until drug withdrawal. When the recurrence rates in the two groups were evaluated, it was observed that the amount of recurrence was significantly higher in the first 3 months in the group that received a total of 8 weeks of treatment. At one-year follow-up, there was no significant difference between the 8- and 12-week regimens. As a result of this study, it was recommended to give a 12-week regimen in total, especially considering the recurrence difference in the first 3 months (33). For this reason, considering various studies, treatment of nephrotic syndrome in children should be planned for at least three months (34, 35).

Studies on the duration and efficiency of corticosteroid therapy in patients with idiopathic nephrotic syndrome are summarized in Table 1, along with their aims and results.

According to the meta-analysis published by Schijvens et al. (36), when the two- and three-month treatment regimens were compared, the group who received steroid treatment for two months experienced 51% more recurrences than those who received three-month treatment.

Table 1: Studies on the duration and efficiency of corticosteroid therapy and their results.

Study, year	Country	Types of studies	Number of participants	Duration of the follow-up period	Aims of studies	Results of studies
Yoshikawa et al. (27), 2015	Japan	RCT	246	24 months	Evaluation of 2 and 6 months of corticosteroid treatment in terms of relapse and side effects.	Since the relapse rates were similar in both groups, it is recommended that there is no need to extend the treatment.
Webb et al. (28), 2019	England	RCT	237	24 months	Evaluation of 8- and 16-week treatment plans in terms of time to first relapse in patients with their first episode of SSNS.	There was no significant difference between the two groups in terms of time to first relapse.
Sinha et al. (29), 2015	North India	RCT	181	12 months	Evaluation of recurrence rates of 3 and 6-month corticosteroid treatments.	Since the difference between the two groups was not statistically significant, it was stated that the 3-month treatment regimen was sufficient.
Kainth et al. (30), 2021	India	RCT	117	12 months	Comparison of recurrence rates in groups receiving short-term therapy (2 weeks) and standard therapy (4 weeks) after remission.	It has been demonstrated that the short-term regimen is not inferior to the standard regimen.
Al Talhi et al. (31), 2018	Saudi Arabia	RCT	120	24 months	Comparison of recurrence rates in patients treated with prednisolone for 3 or 7 months.	It was found that the relapse rate was lower in the group that received 7 months of treatment.

Table 1: Continued.

Study, year	Country	Types of studies	Number of participants	Duration of the follow-up period	Aims of studies	Results of studies
Teeninga et al. (32), 2013	Netherlands	RCT	150	32-60 months (median: 47 months)	Investigation of the difference between the relapse rates of the 3- or 6-month treatment regimens given by keeping the total cumulative dose constant.	Since there was no significant difference between the recurrence rates of the 3- and 6-month regimens, it was mentioned that the important parameter in terms of relapse was the cumulative dose.
Geng et al. (33), 2022	China	Non-RCT	89	12 months	Comparison of relapse rates in the first 3 months and after 1 year of 8- or 12-week corticosteroid regimens.	A higher rate of recurrence was seen in patients who received an 8-week treatment regimen in the first 3 months. Based on this, it is recommended that the treatment be arranged for at least 12 weeks.

RCT: Randomized controlled trial, SSNS: Steroid-sensitive nephrotic syndrome

Although long-term treatment is recommended in some studies, the Cochrane Database showed that three months of corticosteroid treatment was sufficient for children with nephrotic syndrome (35, 37).

CONCLUSION

On this subject, the lack of stratification such as ethnicity, gender, and genetic predisposition in RCTs causes limitations. In addition, standardized studies involving more patients will be useful in determining the most efficient protocol for the treatment plan.

We tried to evaluate the current literature for ideal duration of steroid treatment for idiopathic nephrotic syndrome. Nowadays, a 2- to 3-month steroid regimen seems sufficient for disease control and to avoid potential steroid-related side effects. On the other hand, it should be noted that the duration and intensity of steroid therapy in childhood NS should be weighed against the benefits and risks. The intensity of the disease, response to initial treatment, risk of recurrence, and presence of complications should be determined, and the patient should be monitored in close contact with a pediatric nephrologist.

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REVIEW

METABOLIC PERSPECTIVE OF CANCER: KETOGENIC DIET AND METABOLISM ANTAGONISTS

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ABSTRACT

Abnormal cancer metabolism, a trending topic in recent years, has given rise to various studies and promising results for some cancer types. Ketogenic diets with metabolism antagonists, hyperbaric oxygen, and hyperthermia constitute part of the treatment options that were derived from the metabolic perspective of cancer. Most of them exploit the glucose, glutamine, and fermentation dependence of cancer cells. In addition, they are known to increase the efficacy of current therapies. Ketogenic diet aims to decrease available glucose and increase non-fermentable ketone bodies. In this review, we aim to inspect the abnormal cancer metabolism, starting with the Warburg effect, current advancements, and promising therapeutic uses of these metabolic pathways by primarily focusing on the ketogenic diet and metabolism antagonists.

Keywords: Cancer, glucose, ketogenic diet

INTRODUCTION

Cancer presents a major public health threat worldwide and is known to be the second leading cause of death in the United States of America (USA). Approximately 600.000 people died per year due to cancer between 2015 and 2020 in the USA, and it is estimated that 609.000 people will die from cancer in 2023 despite the efforts made by states, healthcare industry, and non-governmental organizations (1, 2). The majority of these deaths are predicted to result from cancers of the lung, prostate, and colorectum in men, whereas lung, breast, and colorectal cancers are the leading causes in women (1). The primary risk factor for lung cancer is tobacco use, which has been known for many years (3). We have been getting promising results for the treatment of cancer types including but not limited to breast, thyroid, and prostate. However, we cannot say the same for glioblastoma multiforme (GBM) or lung cancer (1, 4). Even though a remarkable effort was put in to improve the prognosis of these groups of cancers, significant outcomes have not yet been seen. It is understood that current treatment modalities need revisions and improvements. These updates should be made to acknowledge the importance of the pathological metabolic processes seen in cancer cells, which have been known since the 1920s but have not been utilized enough in treatment approaches (5).

Even though the first observations of metabolic abnormalities in cancer cells were made almost a century ago, they have not been the focal point of cancer treatment research (5). Utilizing one of the hallmarks of cancer, the abnormal metabolism, in treatment approaches is only a recent focus of researchers and is still debated (6-8). We have seen a massive surge in published papers about this topic in recent years (9).

For many years, abnormalities in cancer metabolism have been used for prognosis prediction and diagnosis in an orthodox paradigm via fluorodeoxyglucose-positron emission tomography (PET). Excessive glucose dependence of tumors is utilized in PET (8). Developing a metabolic perspective suggests the use of this metabolic abnormality not only for diagnosis and prognosis but also for treatment strategies that can be combined with current therapies (7, 8). In this review, we aim to explore the unique and altered metabolism of cancer cells and how it can be utilized primarily via the ketogenic diet. We will also mention some therapy options that can be combined with ketogenic diet therapy.

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Cancer Metabolism

Studies revealed the consequences of abnormal metabolism of cancer at various stages of tumorigenesis. Some changes, such as the modification of metabolite influx and reprogramming of the assignment of nutrients to metabolic pathways, are seen to meet the bioenergetic, biosynthetic, and redox demands of cancer cells. Also, alterations of the tumor microenvironment components and differentiation of cancer cells create long-ranging effects on cellular fate. Reprogrammed metabolism is considered a hallmark of cancer because some of the metabolic changes are observed across various types of cancer (5, 10).

With increased energy usage by cancer cells due to reasons such as increased proliferation, cells must increase nutrient uptake. Two major nutrients that mammalian cells use to support biosynthesis and survival are glucose and glutamine (5). Cells use glucose and glutamine not only as energy sources but also as carbon sources since the catabolism of these monomers produces a variety of carbon intermediates for biosynthesis.

1. Glucose uptake

In the 1920s, Warburg et al. (11), who were working on the metabolism of tumors, described the increased glucose consumption by tumorous cells compared to normal cells. Further studies showed that increased glucose consumption correlates with a poor prognosis of cancer (12, 13). Cancer cells must increase their glucose uptake to match this increased glucose consumption. In mammalian cells, glucose uptake occurs via the glucose transporter (GLUT) family of membrane proteins. In many types of cancer, upregulation of GLUT1 and GLUT3 meets an increased need for glucose (14-16). Based on previous research, an increase in GLUT expression seems relevant to neoplastic transformation. However, another interpretation suggests that increased GLUT expression is caused by decreased intracellular glucose levels (17).

Various mechanisms regulate GLUTs. A study showed that hypoxia-inducible factor-1 (HIF-1) increases the expression of GLUT1 in hypoxic conditions, which is an important regulatory mechanism because of the presence of hypoxic areas in a tumor, described as tumor hypoxia (18). Hyperbaric oxygen therapy (HBOT) can decrease the expression of HIF-1 and reverse the Warburg effect in cancer cells, which is explained later in the text (19). Since various mechanisms regulate GLUT expressions, it is yet unknown whether HBOT directly affects GLUT expression. A study has shown that HBOT promotes GLUT4 expression in streptozotocin-induced type 2 diabetes mellitus mouse models (20). Further research is required to understand the association between HBOT and GLUT expression.

2. Warburg effect

The metabolic perspective of cancer utilizes the Warburg effect, which argues that, unlike normal cells, some cancer cells do not use the citric acid cycle and oxidative phosphorylation (OxPhos) for energy production. Instead, they predominantly use glycolysis followed by lactic acid fermentation to produce ATP even in the abundance of oxygen (11). This phenomenon, the Warburg effect, is also known as aerobic glycolysis (9). Glycolysis is ineffective in terms of the amount of ATP produced when compared to OxPhos, but it is 100 times faster than OxPhos. So, the exaggerated energy demand of rapidly proliferating cancer cells can be countered by an accelerated ATP-producing system. Also, this enhancement in the glycolysis pathway could provide sufficient NADH that is needed to sustain biosynthesis (7, 9). However, not all cancer cells fit into this perspective. Tumors are mostly heterogeneous, so there are Warburg-like and oxygen-consuming phenotypes (7).

3. Reactive oxygen species

The production of reactive oxygen species (ROS) naturally occurs with the reactions of oxygens via electrons. All reactive oxygen species types have unpaired valence electrons and unstable bonds. It has been known for years that reactive oxygen species damage all types of cells because of their highly reactive and unstable status. However, recent research has shown that ROS' implications can extend beyond their damage. While chronic and high-degree exposure to ROS can damage nucleic acids, lipids, and proteins, low to intermediate amounts of ROS can play a significant role in cell signaling cascades, even promoting cell survival (21, 22).

According to a research, OxPhos disruption leads to carcinogenic ROS accumulation (23). Genomic instability and mutations in cancer cells can be a downstream effect of ROS production (24). ROS can be thought of as a double-edged sword. Increased or tumor-promoting ROS can increase cell proliferation, cell cycle progression, survival signaling, genomic instability, epithelial-mesenchymal transition, and motility. If this threshold is exceeded, fatal effects are seen even on cancer cells. Cell cycle arrest, cell senescence, and cell deaths emerge secondary to excessive ROS levels, which are targeted by chemotherapeutics (22). Recent research has shown that injection of mitochondria isolated from healthy mouse livers into melanoma mouse models with lung metastasis has increased ROS levels compared to the control tumor group. This increase is more prominent in mitochondria isolated from young mouse liver rather than from aged mouse liver. Mitochondria replacement increased the survival days of the melanoma mouse models and delayed the growth of their tumors. In addition to these, it reduced glycolysis and reversed the Warburg effect (25). This paper shows that prophylactic treatment and acute treatment should be different in terms of ROS. ROS itself can both promote and inhibit carcinogenesis. More importantly, mitochondrial function and the Warburg effect are valuable for cancer prognosis.

Ketogenic Diet

Dietary regimens and fasting have been used for more than 2000 years to treat epilepsy. The ketogenic diet is one that had been used in the 1920s, but with the development of antiepileptic drugs, it has fallen into disrepute (26). Researchers have recently started to pay attention to the ketogenic diet in terms of efficacy, safety, mechanism of action, therapeutic

actions, and its potential effect on chronic diseases such as diabetes and cancer (27). In terms of cancer management, different studies revealed that ketogenic diet reduced tumor growth and improved survival in animal models with malignant glioma, colon cancer, gastric cancer, and prostate cancer (28).

There are many types of ketogenic diets including the mediumchain triglyceride ketogenic diet and the modified Atkins diet. Generally, ketogenic diets are characterized by their low carbohydrate (20-50 g) content, which approximately composes 5-10% of the total daily calorie consumption. Fat becomes the major calorie source. Ketone bodies are synthesized when carbohydrate sources are limited. Ketone bodies are organic compounds that are mostly derived from the free fatty acid breakdown process in the liver. Ketogenesis is also seen in the heart, brain, gut, and kidneys to some extent. Free fatty acids released from adipose tissue that enter the mitochondria of hepatocytes are used to form acetyl-CoA by β-oxidation. If glucose levels are high, acetyl-CoA is further oxidized through the tricarboxylic acid cycle and electron transport chain. If glucose levels are low, ketogenic enzymes such as thiolase and hydroxymethylglutaryl-CoA synthase contribute to the production of acetoacetate, β-hydroxybutyrate (BHB), and acetone, which are the main ketone bodies (29).

Insulin and glucagon are key regulators of ketogenesis. While glucagon stimulates ketone body synthesis, insulin inhibits this process via the inhibition of hormone-sensitive lipase, which is responsible for the release of free fatty acids from adipocytes, thus withdrawing the substrate from ketone body enzymes (30).

Some studies suggest that a ketogenic diet can facilitate cancer cachexia by lowering the blood glucose level. However, there are papers contrasting this view, showing that a ketogenic diet can mitigate cachexia. These incoherent results likely occur due to the absence of standardization of the ketogenic diet composition, length of treatment, number of consumed calories, and to what extent nutritional ketosis is achieved (31). Also, deficiency of micronutrients is documented in ketogenic diets in some cases (32). In addition, the use of a ketogenic diet in refractory epilepsy cases can negatively affect the developing skeleton. Medicalization and control are important for the therapeutic use of a ketogenic diet to avoid its potential side effects (33). The most reported symptoms are constipation and asthenia. Hypoglycemia is the most anticipated adverse effect; however, mild hypoglycemia can be intended for therapeutic interventions (34). Because cancer cells lack metabolic flexibility due to their mitochondrial mutations and abnormalities, this hypoglycemic state can aggravate oxidative cellular stress. However, healthy cells in the same situation can compensate for the lack of glucose via ketone bodies. Mild hypoglycemia can also reverse the Warburg effect by reducing the amount of glucose (35).

Glucose transporter overexpression is associated with carcinogenesis. One study shows that a calorie-restricted ketogenic diet (KD-R) can promote GLUT expression, and this expression is likely to arise from the hypoglycemia caused by the KD-R (36).

Both ketogenic diets and insulin-like growth factor (IGF-1) reduce blood glucose levels. As IGF-1 is a biomarker of tumor progression and angiogenesis, circulating amounts are important. It is shown that the decrease in blood glucose levels is almost equal to the decrease in IGF-1 when brain tumor mouse models are put on a ketogenic diet (37).

Being aware of the glucose and fermentation dependence of cancer cells can offer some therapeutic interventions. There is a growing interest in the literature on ketogenic diet use both *in vitro* and *in vivo* (38). The main point is to make glucose-dependent cancer cells starve using their inability to entirely utilize the non-fermentable ketone, in contrast to the healthy cells that can (23, 38).

As mentioned above, while some types of cancer depend on aerobic glucose fermentation, the same phenomenon may not be valid for other cell lines and cancers. Therefore, the success of ketogenic diet therapy may vary depending on the different properties of cells since tumors are mostly heterogeneous (7).

Expression of ketolytic enzymes can provide predictive information about the response of a tumor to ketogenic diet regimens. An *in vitro* experiment showed that BHB supplementation to hypoglycemic groups PANC-1 cell line does not affect their proliferation, whereas BHB supplementation significantly promotes cell proliferation in HeLa cells. When researchers intentionally knocked down ketolytic enzymes 3-hydroxybutyrate dehydrogenase 1 (BDH1) and succinyl-CoA:3-oxoacid CoA transferase 1 (OXCT1) by infecting HeLa cells with lentivirus, BHB supplementation stopped promoting proliferation. In addition, mouse models of the PANC-1 cell line showed that mice fed a ketogenic diet had decreased tumor volume and weight and an increased percentage of survival when compared to mice in an approximately isocaloric standard diet group. HeLa mouse models showed that mice put on a ketogenic diet had a decreased survival rate. In mouse models including BDH1 and OXCT1, the knockdown of HeLa cells showed that mice fed a ketogenic diet had less tumor volume and weight when compared to the standard diet group. Interestingly, BHB supplementation in a high glucose medium did not affect the proliferation of HeLa cells *in vitro* (39).

In order to sustain ROS levels in tumor-promoting space, some antioxidant biomolecules, such as glutathione, can be required in the tumor. As the production of glutathione requires glycolysis and pentose phosphate pathways, which need glucose, a ketogenic diet can promise some treatments by limiting the availability of glucose (23-25, 38).

Also, it was shown that a ketogenic diet can be used to sensitize cancer cells to both radiotherapy and chemotherapy (38). This is the reason why researchers are investigating whether a ketogenic diet can be combined with current therapies.

The first case report of confirmed GBM treatment consisting of standard therapy (radiation with temozolomide chemotherapy)

with the combination of a ketogenic diet has shown rapid unusual regression of GBM (40). The relatively positive outcome of the ketogenic diet is attributed to its role in preventing high blood glucose levels, which promotes angiogenesis and prevents apoptosis via GF-1/phosphoinositide-3-kinase (PI3K)/Akt/HIF-1α signaling pathways (37, 41). Also, a decrease in inflammatory status is likely to occur via a ketogenic diet. The paper also notes that ketone bodies can be considered alternative metabolic fuels that can be utilized by healthy cells but not by cancer cells because of their mitochondrial dysfunctionality.

One case report that presents a human epidermal growth factor receptor 2 negative breast cancer that metastasized to the lungs, brain, mediastinum, liver, abdomen, and bones includes ketogenic diet use as well as hyperbaric oxygen and hyperthermia (HT) in combination with standard chemotherapy treatment. HT has a direct cytotoxic effect against cancer cells by increasing the treated tissue temperature up to 42 °C or higher and therefore exploiting the heat sensitivity of cancer cells. HT may also sensitize cancer cells to radiotherapy and chemotherapy, thus increasing their efficacy (42). It inhibits DNA repair and causes DNA damage by promoting ROS production (43). Hyperbaric oxygen therapy is applied by administration of 100% oxygen at a higher pressure than 1 atmosphere. In cancer treatment, HBOT aims to fight against the cancer-promoting effects of tumor hypoxia by increasing blood oxygen levels (20). Hyperbaric oxygen is known to work synergistically with radiation therapy and some chemotherapeutic agents (43). In addition, hyperbaric oxygen adds to the positive effect of ketogenic diets on the mean survival time of mice with systemic metastatic cancer. However, hyperbaric oxygen is not efficient on its own (44). Hyperbaric oxygen can also promote ROS production (45). Both hyperthermia and hyperbaric oxygen can synergistically work with prooxidant chemotherapy regimens. A ketogenic diet can compensate for this prooxidant status via its antioxidant effects on healthy cells, and it can also promote ROS production in cancer cells (46, 47).

Metabolism Antagonists

To target and prevent glycolysis and glutaminolysis in cancer cells, there are some molecules, including 2-deoxy-D-glucose (2-DG), which is a non-metabolizable glucose analog, and 6-diazo-5-oxo-L-norleucine (DON). These metabolism antagonists can be combined with a kind of ketogenic diet regimen, thus utilizing the synergistic effect and strengthening the standard therapy (48, 49).

Aerobic glycolysis is preferred by cancer cells due to its potential advantages. In order to sustain glycolysis, cancer cells can increase their glucose uptake 20-30 times compared to normal cells. This increased uptake requires the overexpression of GLUT (50). 2-DG radioisotope analogs are used to detect transformed, malignant cells by exploiting this glucose uptake characteristic (8). 2-DG itself competes with glucose and competitively inhibits its uptake. After entering the cell, 2-DG is phosphorylated by hexokinase II to form 2-deoxy-D-glucose-6-phosphate. However, it cannot be metabolized further and gets accumulated in the cell, where it allosterically inhibits hexokinase activity. Cell growth inhibition, arrest in the cell cycle, and eventually cellular death are seen (51). There are ongoing studies *in vivo* and *in vitro* investigating the potential use of 2-DG and its derivative, aiming to have more drug-like properties for use in combined anticancer therapies (52-54).

Even though DON has been studied for more than 60 years, it was abandoned in clinical trials because of its nausea and vomiting side effects. However, it is important to note that, in that period, acceptable side effect criteria were stricter. Most of the current chemotherapeutic agents cause similar side effects in patients. As a potent glutamine antagonist, it can be reclaimed as a DON prodrug with improved therapeutic index and side effects (55, 56). Sirpiglenastat is one of the DON prodrugs with a better therapeutic index, and it is being evaluated in phase I/IIa clinical trials (57).

Glioblastoma multiforme cells are dependent on glutamine as well as glucose. In addition, glucose deprivation, which can be achieved by a ketogenic diet, directs cells to use glutamine even more. Glutamine metabolism is significant in rapidly proliferating tissues to produce biomolecules (58). Therefore, the use of DON in the treatment of GBM has been considered. An *in vivo* study has used DON with a calorie-KD-R to target both glutaminolysis and glycolysis in GBM mouse models. KD-R-positive DON reduced cell proliferation. Synergistically, KD-R facilitated the delivery of DON to brain tissue. Furthermore, since cancers are heterogeneous, detection of glutamine dependence is sensible (59). As DON can cause significant side effects, better tolerability can be achieved with improved delivery methods, thus lowering the therapeutic doses (56).

When metabolic therapies are compared with chemotherapeutics in terms of drug delivery systems, derivatives, and similarity of side effects, metabolism antagonists are more appealing.

Press-pulse Strategy

Exploitable abnormal cancer cell metabolism offers a variety of therapeutic interventions, including a ketogenic diet, hyperbaric oxygen therapy, glucose, and glutamine antagonists. The use of these interventions in a systemic way is described as a "press-pulse strategy". Press disturbance describes the chronic stress induced by a calorie-restricted, isocaloric ketogenic diet. Pulse disturbance describes the acute stress caused by glucose-glutamine antagonists and hyperbaric oxygen therapy (35). Some clinicians also include HT in the pulse disturbance category (42, 43). Due to the abnormal metabolism mentioned in the review, these stress factors can boost the efficacy of standard therapies for particular types of cancer, such as chemoradiotherapy (Figure 1). These cost-effective, non-toxic, and encouraging therapies could be effective additions to standard therapy in the future of oncology.

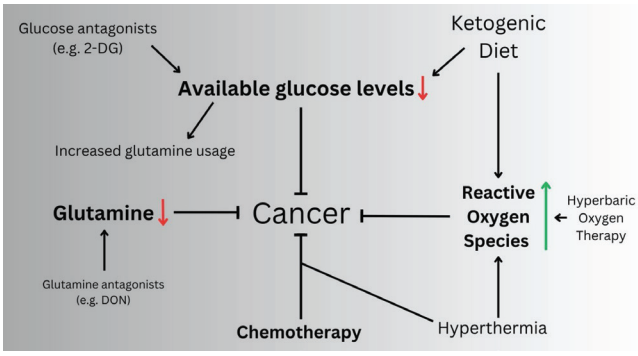

Figure 1: Schematic explanation of therapies exploiting abnormal cancer metabolism. Green arrows indicate an increase and red arrows indicate a decrease.

2-DG: 2-deoxy-D-glucose, DON: 6-diazo-5-oxo-L-norleucine

CONCLUSION

Cancer has been interpreted as a genetic disease for years. Studies, as well as treatment modalities, are conducted in line with this paradigm. This paradigm has made the field of oncology successful to some extent; however, for some cancer types, satisfying results have not been achieved. The abnormal metabolism of cancer cells, which was described by Warburg et al. (11) in the 1920s, has gained popularity in recent years to take oncology one step further. This metabolic perspective and its promising treatment options exploit the lack of metabolic flexibility in cancer cells. Therapeutic approaches centered on this aspect of tumor cells are mostly non-toxic and have anticancer properties. Furthermore, they enhance the efficacy of current therapies, thus making them more tolerable for patients due to their reducing effects on the minimum effective dose of chemotherapeutics. As lower doses of chemotherapeutics are given, chemotherapeutic resistance development decelerates.

One of the main targets of metabolic treatments is the glucose and fermentation dependence of cancer cells. The ketogenic diet, which is characterized by low carbohydrate and high lipid intake, exploits this status by reducing available glucose and increasing non-fermentable ketone bodies. Ketone bodies cannot be metabolized entirely by some cancer cells; however, normal cells can utilize them. Ketone bodies can also promote ROS production in cancer cells. However, there are common side effects of ketogenic diets, including constipation, asthenia, and hypoglycemia (34). HBOT and HT are other stressors for cancer cells that are known to work synergistically with a ketogenic diet. Also, there are studies investigating the use of metabolism antagonists such as 2-DG and DON. They compete with glucose and glutamine and prevent their metabolism (48, 49). These significant side effects are documented, so prescription and use require considerable attention. The press-pulse strategy describes the systematic combination of these metabolic stressors. Similar to how some chemotherapeutics may not be effective in some cases, the ketogenic diet and other metabolic therapies may not show anticancer properties unilaterally as well. Therefore, personalized medicine in cancer treatments is likely to play a major role not only in current therapies but also in metabolic therapies. As a result, the metabolic perspective of cancer is a rising topic in oncology. The emerging literature and evidence suggest that the use of metabolic treatment strategies, both separately and in combination with current therapies, will be beneficial.

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REVIEW

PCSK9 siRNA INHIBITOR INCLISIRAN AS A TREATMENT OPTION IN HYPERCHOLESTEROLEMIA: A BRIEF REVIEW

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ABSTRACT

Dyslipidemia and hypercholesterolemia are global health issues that require urgent and efficient treatments due to their major impact on cardiovascular disease. The incidence of these illnesses is impacted by population and time differences, with familial hypercholesterolemia and lifestyle changes exacerbating these disorders. Inclisiran, a recently licensed RNA interference therapy, specifically a proprotein convertase subtilisin/kexin type 9 siRNA inhibitor, appears to be a revolutionary treatment method. However, questions about its long-term safety, impact on lipid metabolism, and cost-effectiveness remain unanswered. Evidence from the ORION clinical trials shows that inclisiran is effective at significantly lowering low-density lipoprotein cholesterol, non-high-density lipoprotein cholesterol, and apolipoprotein B levels. It also demonstrates a low frequency of adverse events and a potential improvement in patient quality of life. Remarkably, inclisiran's low-density lipoprotein cholesterol reduction outperforms statins alone and is comparable to the efficacy of other proprotein convertase subtilisin/ kexin type 9 inhibitors such as evolocumab and alirocumab. It has the potential to revolutionize the coronary preventative medicine market by providing an economically viable long-term cardiovascular risk reduction option. Limited long-term safety data, cost-effectiveness concerns, and clinical experience with the medicine are all barriers to wider acceptance. Despite these obstacles, inclisiran appears to hold promise as an effective, safe, and potentially cost-effective treatment for hypercholesterolemia and dyslipidemia, particularly in high-risk and statin-intolerant patients. However, the precise association between low-density lipoprotein cholesterol lowering and improved cardiovascular outcomes remains unclear, prompting additional investigations. Future research should seek to overcome these knowledge gaps, comprehend inclisiran's broader impact on lipid metabolism, and investigate its usefulness in specific patient populations.

Keywords: Dyslipidemia, hypercholesterolemia, inclisiran, siRNA

INTRODUCTION

Dyslipidemia, a common lipid disorder, is defined by elevated levels of cholesterol and/or triglycerides in the blood, which increase the risk of cardiovascular disease (CVD) (1). Hypercholesterolemia, a type of dyslipidemia, is characterized by high levels of total cholesterol, low-density lipoprotein (LDL) cholesterol, or both (2). The interplay of dyslipidemia, hypercholesterolemia, and CVD is complex and important, necessitating a detailed understanding of their interconnections.

Cardiovascular diseases are the leading cause of mortality across the globe, responsible for 17.9 million deaths, or 31% of all worldwide deaths, in 2017. Over 80% of these fatalities occur in low- and middle-income countries, with more than half of all CVD-related deaths happening before the age of 70 years (3). A variety of factors, including lifestyle changes such as increased dietary fat intake, physical inactivity, and obesity, have contributed to the rising prevalence of dyslipidemia and hypercholesterolemia in recent years (4, 5). Both conditions are strongly correlated with high CVD risk and are considered significant modifiable risk factors for cardiovascular events (6).

It is important to note that the prevalence of hypercholesterolemia and dyslipidemia can vary significantly depending on the population studied and the time. For example, in the United States and China, recent studies have estimated the prevalence of hypercholesterolemia and dyslipidemia among adults aged 18-64 to be 11.4% and 35.5%, respectively (7, 8). However, direct comparisons between these results may be misleading due to differences in the definitions of hypercholesterolemia

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used in each study. Other research findings show an increase in the raw occurrence of initial hypercholesterolemia or mixed dyslipidemia in the United Kingdom from 13.5% in 2009 to 23.5% by 2019 (9), a prevalence of dyslipidemia of 78% among metropolitan overweight adults in South Delhi, India (10), and a prevalence of 75.9% among young adults in Karachi, Pakistan (11).

Hypercholesterolemia is the primary cause of atherosclerotic CVD, while dyslipidemia is also linked to an increased risk of cardiovascular events, death, and increased healthcare resource consumption and expenditures (12). An integral component of these conditions is elevated LDL-cholesterol (LDL-C) levels. As a key modifiable risk factor, LDL is central to the pathogenesis of CVDs and forms a bridge between different types of dyslipidemia, including hypercholesterolemia (7). This highlights how both genetic and lifestyle factors significantly contribute to the development of dyslipidemia and its subtypes, including hypercholesterolemia (13). Of the genetic influences, familial hypercholesterolemia is the most common cause of hypercholesterolemia, affecting 1 in 250 individuals worldwide (14). This autosomal dominant disorder results from mutations in the LDL receptor gene, highlighting the critical role of genetics in this condition (14).

Early diagnosis and intervention are essential in mitigating the risk of CVD associated with dyslipidemia, including hypercholesterolemia (15). Current treatment strategies consist of lifestyle modifications, such as dietary changes and increased physical activity, as well as pharmacological therapies like statins and proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors (16). Inclisiran, a recently approved PCSK9 inhibitor, has emerged as a promising treatment option for patients at high risk for CVD events. It functions by targeting the messenger ribonucleic acid (RNA) of the *PCSK9* gene, thereby reducing the expression of the PCSK9 protein, and ultimately decreasing cholesterol levels (17) (Figure 1).

Despite the potential benefits of inclisiran, there are still many unanswered questions surrounding its use in hypercholesterolemia and dyslipidemia. For example, it is unclear how long inclisiran can remain effective in reducing cholesterol levels or how it may affect other aspects of lipid metabolism. Additionally, there is limited data on the safety profile of inclisiran in this patient population. These gaps in knowledge highlight the need for further research into the efficacy and safety of inclisiran as a treatment option for hypercholesterolemia and dyslipidemia.

This narrative review aims to examine the current evidence on inclisiran for dyslipidemia treatment, with a focus on hypercholesterolemia. The review will analyze existing clinical studies, including the conceptual design of the ORION program, to assess the efficacy and safety profile of inclisiran in this patient population. Additionally, this review will identify knowledge gaps surrounding inclisiran's use and provide recommendations for future research.

RESULTS

Clinical Trials

Inclisiran is a double-stranded, modified RNA that binds to the carbohydrate molecule N-acetylgalactosamine (GalNAc), which is expressed by hepatocytes (18). Inclisiran cleaves matrix RNA and decreases PCSK9 protein synthesis after entering hepatocytes, increasing the absorption of circulating LDL by hepatocyte receptors, and reducing LDL levels in circulation (18). The administration schedule of inclisiran is twice a year, which may contribute to the patient compliance and the efficacy of the treatment.

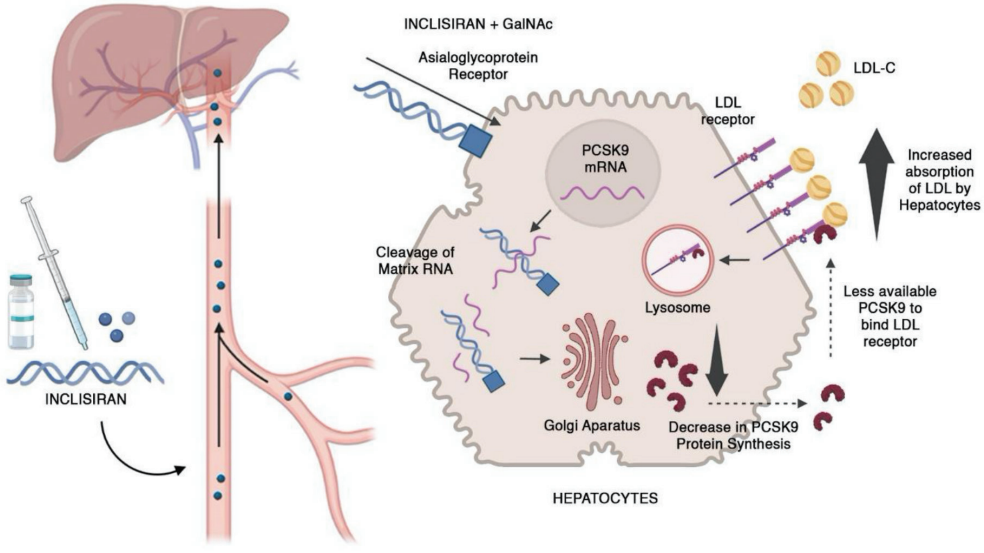

Figure 1: Simplified overview of mechanism of action.

LDL-C: Low-density lipoprotein cholesterol, PCSK9: Proprotein convertase subtilisin/kexin type 9, GalNAc: N-acetylgalactosamine

The ORION clinical initiative consists of a pair of stage 3 investigations, ORION-1, and ORION-2, in addition to an open-label extension examination, ORION-3. The fundamental goal of the ORION initiative was to assess the long-term effectiveness of inclisiran in patients with hypercholesterolemia or dyslipidemia and the well-being of patients. ORION-1 and ORION-2 were randomized, double-anonymous, placebo-controlled experiments that enrolled 2,519 participants. Subjects were administered either 300 mg of inclisiran or a placebo every six months for a maximum of 18 months, with the primary outcome being the LDL-C percentage change from baseline at 18 months (19, 20).

The ORION-1 trial, a randomized, double-anonymous, and placebo-controlled multicenter phase 2 study, aimed to investigate the impact of one or two doses of inclisiran on LDL-C levels (21). The trial enrolled patients who had increased LDL-C despite undergoing maximally tolerated treatment with statins. The primary objective of ORION-1 was to measure the time it took to return to within 20% of baseline for LDL-C levels and time-averaged LDL-C reductions over a year. The results of the study indicated that inclisiran effectively reduces LDL-C levels and lowers the incidence of cardiovascular problems with no clinically significant adverse effects (22).

The ORION-3 trial, an open-label extension study, evaluated the long-term efficacy and safety of inclisiran with four years of follow-up. This trial included patients who had completed the ORION-1 study and received either 300 mg of inclisiran or placebo every six months for an additional 18 months. The results showed that inclisiran significantly reduced LDL-C levels by an average of 53% compared to placebo at 24 months, with these effects sustained for up to 36 months (23).

Inclisiran was also tested in the phase 3 ORION-9 study, which assessed its effectiveness and safety in individuals with homozygous familial hypercholesterolemia. The results showed that inclisiran significantly reduced LDL-C by an average of 52% at day 270 compared to placebo. In addition, inclisiran demonstrated reductions in non-high-density lipoprotein cholesterol (HDL-C) and apolipoprotein B (ApoB) levels, with a safety profile similar to that of a placebo. These effects were sustained for up to 12 months (24).

The trials ORION-10 and ORION-11 evaluated the effectiveness and safety of inclisiran in reducing LDL-C levels in individuals with atherosclerotic CVD risk factors. A total of 3,177 patients were randomly assigned to inclisiran (n=1590) or placebo (n=1587). Patients received two subcutaneous doses of either inclisiran 300 mg or placebo at baseline and day 90, with the primary outcome being the percentage change in LDL-C from baseline to day 270. The studies ORION-10 and ORION-11 revealed that inclisiran led to a mean reduction of 52% and 50% in LDL-C levels at day 270 in comparison to the placebo (23, 25). Additionally, inclisiran demonstrated 44% and 47% reductions in non-HDL-C and ApoB levels, respectively, at day 510 (25) (Table 1).

Table 1: Percentage change in LDL-C subgroups.

Trial	Population	Arm	n	Timepoint (days)	Percentage change in LDL-C			
					Change (%)		Difference from placebo (%)	
					Mean	(95% CI)	Mean	(95% CI)
ORION-9 (24)	Overall (HeFH)	Inclisiran	242	510	-39.7	(-43.7, -35.7)	-47.9	(-53.5, -42.3)
		Placebo	240		8.2	(4.3, 12.2)	-	-
ORION-10 (25)	Overall (ASCVD)	Inclisiran	781	510	-51.3	NR	-52.3	(-55.7, -48.8)
		Placebo	780		1	NR	-	-
	Statin at BL	Inclisiran	701		NR		-57.3	(-60.7, -54.0)
		Placebo	692				-	-
	No statin at BL	Inclisiran	80				-54.8	(-62.0, -47.6)
		Placebo	88				-	-
ORION-11 (25)	Overall (ASCVD or RE)	Inclisiran	810	510	-45.8	NR	-49.9	(-53.1, -46.6)
		Placebo	807		4	NR	-	-
	Statin at BL	Inclisiran	766		NR		-53.3	(-56.5, -50.1)
		Placebo	766				-	-
	No statin at BL	Inclisiran	44				-41.6	(-51.1, -32.1)
		Placebo	41				-	-
	ASCVD	Inclisiran	712				-53.3	(-56.6, -50.1)
		Placebo	702				-	-
	ASCVD-risk equivalent	Inclisiran	98				-47.2	(-56.1, -38.3)
		Placebo	105				-	-
ORION-1 (26)	Overall	Inclisiran	59	180	-52.6	(-57.1, -48.1)	NR	NR
		Placebo	61		1.8	(-2.6, 6.3)	-	-

ASCVD: Atherosclerotic cardiovascular disease, BL: Baseline, HeFH: Heterozygous familial hypercholesterolemia, LDL-C: Low-density lipoprotein cholesterol, NR: Not reported, RE: Risk-equivalent, CI: Confidence interval

In terms of safety, reactions at the injection site have been identified as the most seen adverse effects. These were usually mild in severity and did not necessitate medical treatment. They included symptoms such as a slight self-limiting rash, hyperpigmentation, musculoskeletal pain, headaches, back pain, and acute nasopharyngitis or hiccups (26-30). Despite these reactions, no serious side effects from Inclisiran have been observed.

Notably, switching from PCSK9 monoclonal antibodies (mAb) to inclisiran did not affect the drug's efficacy. This finding implies that prior exposure to or treatment with a PCSK9 mAb does not influence inclisiran's effectiveness (31). However, more study is needed to confirm these findings and acquire a better understanding of the long-term effects of this therapeutic strategy.

Additionally, quality of life improvements have been observed in patients receiving inclisiran. Patients who received inclisiran reported improved physical functioning, role functioning, social functioning, and mental health compared to those receiving a placebo (32, 33). These improvements were maintained over 18 months of treatment with inclisiran. These results suggest that inclisiran can improve the quality of life in patients with hypercholesterolemia or dyslipidemia.

Finally, the use of inclisiran is not limited to the adult population. Inclisiran appears to be a promising strategy for controlling hyperlipidemia in younger patients, particularly those with familial hypercholesterolemia, according to the ORION-13 and ORION-16 trials. The investigation of the drug's efficacy, safety, and tolerability in children and adolescents aged 12 to 18 years provides promise for therapeutic choices other than the statins and ezetimibe that have usually been administered for this age range (34, 35).

In conclusion, the findings of these studies suggest that inclisiran has the potential to be an effective, well-tolerated treatment for hypercholesterolemia and dyslipidemia, enhancing patients' quality of life over a wide age range. However, more study is needed to confirm these findings and understand the long-term therapeutic consequences of this developing therapy technique.

Cost-effectiveness

Cost-effectiveness is a crucial aspect of evaluating new treatment options like inclisiran, a novel PCSK9 small interfering RNA (siRNA) inhibitor. Comparing the cost-effectiveness of inclisiran to other treatments for hypercholesterolemia and dyslipidemia is essential to understanding its potential impact on clinical practice. The cost-effectiveness analysis evaluates both the costs and health benefits of various therapies, offering an unbiased evaluation of their merits (32).

The cost-effectiveness of inclisiran in treating atherosclerotic cardiovascular patients with elevated LDL-C despite statin therapy has been studied. With an incremental cost-effectiveness ratio (ICER) of $51,686, inclisiran was assessed to be cost-effective for the US health system at a price just above $50,000 per quality-adjusted life year (QALY) (36). Another study looked at the clinical and economic feasibility of increasing the frequency of use of ezetimibe, alirocumab, evolocumab, and inclisiran in combination with statins in adult patients at very high cardiovascular risk, including those who have not met lipid targets on statin therapy. In comparison to current practice, the study found that increasing the frequency of PCSK9 inhibitor prescriptions, including inclisiran, was cost-effective (37).

According to the ICER Final Report 2021, the evidence was evaluated as sufficient to demonstrate a net health advantage for inclisiran over conventional treatment alone. If priced at parity with current PCSK9 inhibitor prices, the committee determined that inclisiran would provide low-to-intermediate long-term value for money. The $3,600-6,000 annual net price benchmark range for inclisiran is advised by ICER. In addition, inclisiran is cost-effective in terms of QALY gained, with an ICER of $1,686 per QALY gained (38).

Table 2: Advantages and disadvantages of inclisiran.

Advantages	Disadvantages
Reduction in LDL-C levels by approximately 50% compared to placebo (23, 25)	Unknown long-term benefits and safety profile due to its recent approval (31)
Favorable administration regimen (0-90-180 days), which should lead to better compliance (49)	Adverse effects could persist for six months due to its long-acting nature (35, 45)
Reduced plasma PCSK9 levels by approximately 80%, altering lipoprotein profile favorably (23, 29)	Injection site reactions, which were more frequent in the inclisiran group (26-30)
Long-acting duration, remaining effective for up to six months (25)	Uncertainty about whether LDL-C level reduction improves cardiovascular outcomes (45)
Well-tolerated in clinical trials with mostly mild side effects (50)	It's only approved for adults 18 years or older, excluding pediatric patients or those under 18 years of age (49)
Proven efficacy in LDL-C level reduction in familial hypercholesterolemia, patients with elevated cardiovascular risk, statin intolerance, or hyperlipoproteinemia(a) (51)	It is an injectable therapy requiring frequent injections (44)
Rapid liver uptake, a short plasma half-life, and long-lasting effects on PCSK9 inhibition and LDL-C lowering due to Inclisiran's GalNAc attachment (18)	Possible adverse effects that might be noticed in the future after several years of treatment

LDL-C: Low-density lipoprotein cholesterol, PCSK9: Proprotein convertase subtilisin/kexin type 9, GalNAc: N-acetylgalactosamine

In summary, inclisiran appears to be a cost-effective and valuable treatment option for hypercholesterolemia and dyslipidemia. The cost-effectiveness analyses suggest that inclisiran may be an economically viable way to reduce long-term cardiovascular risk in patients with these conditions. Moreover, the clinical effectiveness of inclisiran in reducing LDL-C and major cardiovascular events supports its potential role in managing high-risk patients, including those who are statin-intolerant.

Comparative Analysis

Inclisiran, a novel PCSK9 siRNA inhibitor, was granted authorization for subcutaneous injection and is recommended to be administered every 3 to 6 months (19). This less frequent dose schedule, as compared to statins, adds to the drug's encouraging results in the treatment of hypercholesterolemia and dyslipidemia. In addition to this benefit, inclisiran is associated with more significant decreases in LDL-C levels, with an average 55% reduction in LDL levels after six months of treatment, compared to a 40% reduction with statins alone (22, 39). When combined with statins, inclisiran has demonstrated even greater reductions in LDL-C levels. Combination therapy with atorvastatin and inclisiran reduced LDL-C levels by up to 65%, whereas atorvastatin monotherapy resulted in a reduction of up to 40% (40-42). This shows that combination medication, rather than monotherapy, may be more effective in lowering both LDL-C and non-HDL-C levels.

In terms of potential side effects, the literature has associated statins with a variety of adverse events, including toxicity to the liver, statin-related myopathy, rhabdomyolysis, the development of new diabetes, cataracts, and hemorrhagic stroke (43). On the other hand, none of the research has associated inclisiran with liver toxicity or clinically evident liver injury. Furthermore, alanine transaminase elevations have been reported in less than 1% of patients after inclisiran medication, and they were mild-to-moderate, temporary, and without associated symptoms or jaundice (24). Despite these preliminary findings, additional detailed and direct comparison studies will be required to have a more comprehensive understanding of the adverse effects of inclisiran and statins.

The ORION-3 research evaluated inclisiran's long-term efficacy and safety, finding that it is as effective as other PCSK9 inhibitors in terms of LDL-C lowering and has a comparable safety profile. In the ORION-10 study, inclisiran was found to be equal to evolocumab in terms of LDL-C reduction at day 180, while it was superior to alirocumab at day 270 in the ORION-11 trial. In terms of safety, there were no notable differences between inclisiran and either evolocumab or alirocumab (23, 25). The most prevalent adverse events reported with inclisiran were injection site reactions, along with other PCSK9 inhibitors (25).

Inclisiran has also been used as a public policy to treat patients with coronary heart disease or at a highly elevated risk, representing an innovative way to introduce a drug to the market (44, 45). However, barriers to its widespread use should be analyzed, such as accessibility, cost, and patient acceptance. Moreover, potential candidates for inclisiran therapy include patients who are statin-intolerant or those who require additional cholesterol reduction beyond what can be achieved with statin monotherapy.

Strengths and Limitations of Inclisiran

Inclisiran, an RNA interference therapy, offers several advantages as a treatment option for hypercholesterolemia and dyslipidemia. As a highly targeted therapy, it reduces the expression of specific genes, potentially causing fewer side effects than other treatments (46-48). Inclisiran has proven effective in reducing LDL-C levels, the primary treatment goal for these conditions, with some patients experiencing up to a 50% reduction in LDL-C levels (23, 25). Notably, inclisiran has been associated with significant decreases in LDL-C and PCSK9 levels in both diabetic and non-diabetic patients, implying its potential as a novel therapeutic option for controlling dyslipidemia regardless of diabetes status (19). The long duration of action of inclisiran is another advantage; a single dose can remain effective for up to six months, significantly longer than statins or PCSK9 inhibitors (25). This makes it an appealing option for patients requiring long-term LDL-C level maintenance without frequent injections or other treatments (49).

In clinical trials, inclisiran has demonstrated good tolerability. The most common side effects were mild, quickly resolving injection site reactions (50). This aspect makes it an attractive option for patients concerned about potential side effects from other treatments.

Potential candidates for inclisiran therapy include patients with familial hypercholesterolemia, those with elevated cardiovascular risk, statin intolerance, or hyperlipoproteinemia(a) (51). Inclisiran's GalNAc attachment results in rapid liver uptake, a short plasma half-life, and long-lasting effects on PCSK9 inhibition and LDL-C lowering (18).

However, there are limitations to inclisiran as a treatment option for hypercholesterolemia and dyslipidemia. It is only approved for use in adults aged 18 years or older, excluding those under 18 years of age (49). Although effective in reducing LDL-C levels, it remains unclear whether this reduction translates to improved cardiovascular outcomes, such as reduced heart attack or stroke risk. Long-term benefits are uncertain, and further research is needed to determine if inclisiran can improve patient outcomes over time (44).

The safety profile of inclisiran is relatively unknown due to its recent approval and limited use in clinical practice. There may be potential side effects or adverse events not yet identified. While it has a long duration of action, inclisiran is still an injectable therapy requiring frequent injections to maintain efficacy over time, which may be inconvenient for some patients preferring oral medications or other less invasive treatment options (44, 45) (Table 2).

In conclusion, inclisiran is a promising treatment option for specific patient populations, such as those with familial

hypercholesterolemia, elevated cardiovascular risk, statin intolerance, or hyperlipoproteinemia(a). However, more research is needed to evaluate its long-term safety, efficacy, and cost-effectiveness before it can be widely used in coronary prevention.

CONCLUSION

In summary, the ORION clinical trials have convincingly demonstrated that inclisiran, a siRNA molecule, is an effective and safe treatment for hypercholesterolemia and dyslipidemia. This novel therapeutic agent effectively targets the *PCSK9* gene, leading to significant reductions in LDL-C levels, non-HDL-C levels, and ApoB levels. Additionally, patients receiving inclisiran have reported quality of life improvements, reinforcing its potential value in clinical practice.

Comparative analyses have highlighted inclisiran's superior efficacy and longer duration of action compared to statins, and the combination of the two may offer even greater LDL-C reductions. In terms of efficacy and safety, inclisiran has shown comparable results to other PCSK9 inhibitors. Moreover, the cost-effectiveness analysis suggests that inclisiran could provide value for money, especially considering its potential to reduce long-term cardiovascular risk.

Nonetheless, there are still limitations to consider. Inclisiran is approved for use only in adults aged 18 years or older, and while it effectively reduces LDL-C levels, it is not yet certain whether this will translate into improved long-term cardiovascular outcomes. Furthermore, its safety profile, though seemingly promising, is not fully known due to its recent approval and limited usage. Lastly, as an injectable therapy, some patients may find the administration less convenient than an oral medication.

In conclusion, the evidence presented in this review supports further research into the use of inclisiran as a treatment option for hypercholesterolemia and dyslipidemia. Future studies should focus on long-term safety, efficacy, cost-effectiveness, and potential for reducing other cardiovascular risk factors, considering the insights and experience of the authors to contribute to a comprehensive understanding of inclisiran's potential.

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ORIGINAL ARTICLE

EFFECTS OF NICKEL CHLORIDE ON CELL MORPHOLOGY AND MIGRATION IN NON-SMALL CELL LUNG CANCER CELL LINES

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ABSTRACT

Aims: The discovery of the anti-cancer properties of the metal-based compound cisplatin and its effective use in cancer treatment has opened the door to the investigation of the anti-cancer properties of many other metals and metal-based compounds. Studies have shown that nickel chloride ($NiCl_2$) could suppress cell migration and metastasis in some types of cancer and could even be a promising anti-cancer agent in oral cancers, although the activity of $NiCl_2$ on the cell morphology and cell migration in non-small cell lung cancer cell lines (A549) is unknown. Thus, we aimed to investigate the role of $NiCl_2$ on cell morphology and migration in non-small cell lung cancer cell lines.

Methods: The present study investigates the effect of $NiCl_2$ on cell morphology and cell migration in A549 cell lines using a Giemsa staining technique, with an *in vitro* scratch analysis performed to determine the effect of $NiCl_2$ on cell migration.

Results: No significant change was observed in cell morphology in the group treated with 200 µM $NiCl_2$ compared to the control group, while the cellular morphology was changed in the cell lines treated with 600 µM $NiCl_2$. The cells lost cell-to-cell contacts, the cytoplasm shrank, and their morphology diverged from that of their ancestors, taking on a spindle-shaped and more unhealthy appearance. In addition, it was observed that cell confluency was decreased by half. It was found that $NiCl_2$ treatment at a dose at which cell morphology changed (600 µM) significantly reduced cell migration after 12 hours, and the effect was sustained at 24 and 48 hours, with cell migration significantly suppressed.

Conclusion: Our results suggested that treatment of non-small cell lung cancer cell lines with $NiCl_2$ changed cell morphology in a dose-dependent manner and suppressed the migration of cancer cells.

Keywords: Neoplasms, therapeutics, lung neoplasms, nickel

INTRODUCTION

Nickel (Ni), the 28th element in the periodic table, is a hard and ductile transition metal that is silvery-white in color (1). It has several oxidative forms (from -1 to +4), and the +2 oxidative form (Ni2+) is the most common form in the environment and biological systems (2). In general, Ni exposure most frequently occurs through the oral route in water and nutrients (3). Ni exposure can also occur through skin contact and inhalation (4). Ni, defined as immunotoxic and cancerogenic depending on the dose and duration of exposure, can cause various health problems such as contact dermatitis, cardiovascular disease, asthma, pulmonary fibrosis, and respiratory tract irritation (5). Soluble and insoluble Ni were identified as human carcinogens by the International Agency for Research on Cancer based on these toxic effects (6).

Nickel chloride ($NiCl_2$), a Ni compound, has been demonstrated to be a very weak carcinogen, producing no tumor after intramuscular injection in rats, and the administration of $NiCl_2$ alone did not cause skin tumors in mice, although studies have reported carcinogenic effects of elementary Ni and Ni salts (7). $NiCl_2$ has been defined as a non-genotoxic carcinogen as it does not directly cause changes in DNA, although the mechanisms underlying $NiCl_2$-induced cancer development have yet to be elucidated (8).

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There have been many recent studies reporting that Ni chloride suppresses cellular proliferation and induces cell death in some cancer types, and these characteristics could indicate anticancer properties (9, 10). Moreover, the treatment of $NiCl_2$ in some cancer types has been shown to suppress metastasis, and because of these effects, it has been suggested that $NiCl_2$ may be a new promising anticancer agent for the treatment of oral cancer. Although studies have demonstrated the efficacy of $NiCl_2$ against cell migration and cellular morphology in some cancer cell lines, there is no evidence of the effects of $NiCl_2$ on non-small cell lung cancer cell lines (9).

This study investigates the effects of $NiCl_2$ on cellular morphology and cell migration in non-small cell lung cancer cell lines.

MATERIAL AND METHODS

Cell Culture

The human non-small cell lung cancer cell lines (A549) used in the present study were purchased from the commercial American-type cell culture collection (ATCC, CCL-185). The cells were cultured at 37 °C in a humidified incubator with 5% CO_2 in a DMEM/F12 (Sigma, Cat. No: D6421) medium containing 10% fetal bovine serum (Fetal Bovine Serum, Biological Industries, Cat. No: 01-121-1A), 2 mM L-Glutamine (Biological Industries, Cat. No: 03-020-1B) and 100 µg/mL penicillin/streptomycin (Biological Industries, Cat. No: 03-031-1B).

Giemsa Staining

Giemsa staining was used to determine the effect of $NiCl_2$ on the cell morphology in A549 cell lines. The cells were seeded in 96-well culture plates, with each well containing 5,000 cells. The cells were treated with $NiCl_2$ for 24 hours at doses of 200 µM and 600 µM, as determined in previous preliminary studies (10-12). The mediums were discarded at the end of the specified period, and the cells were washed once with phosphate buffered saline (PBS). The cells were then incubated with PBS/methanol at a rate of 1:1 for two minutes. The content of the wells was discarded, and the cells were incubated with fresh methanol for 10 minutes, then the methanol was discarded and the cells were incubated for two minutes after adding Giemsa staining to the wells. The Giemsa stain was removed, and the wells were washed with distilled water for two minutes. The morphology of cells was examined under a light microscope.

Cell Migration Analysis

An *in vitro* scratch analysis was used to evaluate the effects of $NiCl_2$ on cell migration in A549 cell lines. The A549 cells were seeded in 96-well culture plates, each well containing $2x10^4$ cells. After 24 hours, a 200 µL pipet was used to scratch the well in the middle from one end to the other to create an artificial wound. The cells were treated with 600 µM $NiCl_2$. The area between the two ends of the wound was calculated at 0, 6, 12, 24, and 48 hours to evaluate the degree of cell migration. Migration areas were calculated using ImageJ 1.53 software.

Statistical Analysis

All data were processed using GraphPad Prism 8.0 statistical software. Differences between control groups and nickel chloride treated groups were analyzed using Student's t-test. p values less than 0.05 (*), 0.01 (**), and 0.001 (***) were considered statistically significant.

RESULTS

Treatment with Nickel Chloride Affects A549 Cell Morphology in a Dose-dependent Manner

Giemsa staining was used to determine the effect of $NiCl_2$ on the cellular morphology of A549 cell lines for which the A549 cell lines were subjected to $NiCl_2$ at doses of 200 µM and 600 µM for 24 hours. At the end of this period, the changes in cellular morphology were observed by examining the $NiCl_2$-treated groups in comparison with the group not treated with $NiCl_2$ under a light microscope. The microscopic examination revealed no significant changes in cellular morphology in the group treated with a dose of 200 µM compared to the control group, while the cellular morphology changed in the cell lines treated with 600 µM $NiCl_2$ with a loss of cell-to-cell contact, a decrease in the cytoplasm, and a morphological divergence from their ancestors in gaining a spindle-shaped and unhealthier appearance. Furthermore, although an equal number of cells was seeded, cell confluency in the cells treated with 200 µM $NiCl_2$ was similar to that of the control group, while cell confluency in the group of cells treated with 600 µM $NiCl_2$ was decreased almost by half (Figure 1).

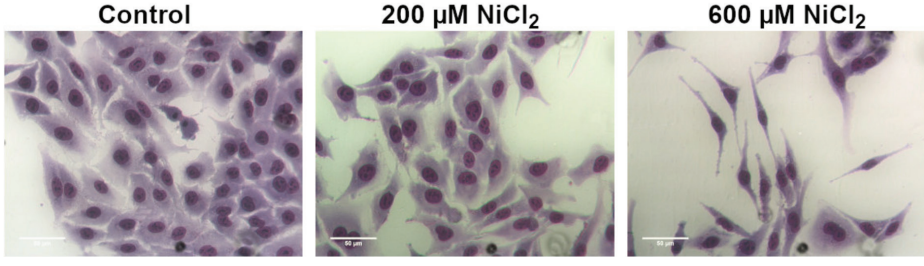

Figure 1: Effect of nickel chloride on cell morphology. Giemsa staining technique was used for determination of cellular morphological changes. All samples were examined under the same magnification level under a brightfield microscope.

$NiCl_2$: Nickel chloride

Thus, our study demonstrated that $NiCl_2$ administration changed the cellular morphology of A549 cell lines in a dose-dependent manner.

$NiCl_2$ Treatment Suppresses Cell Migration in A549 Cell Lines

For the application of $NiCl_2$ at a dose affecting cellular morphology, an *in vitro* scratch analysis was carried out to determine the changes in the migration abilities of A549 cell lines. Following the treatment of cells with 600 µM $NiCl_2$, the areas of migration were evaluated at 6, 12, 24, and 48 hours to determine migration status. The migration area in the control group was found to be significantly reduced after 12 hours when compared to the $NiCl_2$-treated group ($p<0.05$). Similarly, the cell migration was sustained at 24 and 48 hours in the control group, but was suppressed in the $NiCl_2$-treated group, and the migration area was significantly larger ($p<0.001$) (Figure 2).

The findings of the present study reveal that $NiCl_2$ treatment suppresses cell migration in A549 cell lines.

DISCUSSION

The use of metal-based compounds is of great importance in medicine. Many metals and metal-based compounds such as antimony (Sb), gold (Au), iron (Fe), silver (Ag), and platinum (Pt) are used effectively in the treatment of cancer protozoal, arthritic, malarial, and microbial diseases. Also, compounds containing a wide spectrum of metals from transition elements to main elements have been extensively studied to identify any anti-tumor activity (13). Following the years of success of the metal-based cisplatin and other platinum-based drugs on cancer treatment, great advances have been made in the use of both essential and non-essential metals and their complexes (14).

One such metal, Ni is found in vast amounts in nature and has been identified as a human carcinogen by the International Agency for Research on Cancer (6). However, no DNA damage attributable to the $NiCl_2$ Ni compound has been demonstrated, leading it to be regarded as a non-genotoxic Ni compound with weak carcinogenic effects among the other Ni compounds (8).

While various studies have demonstrated the potential carcinogenic properties of $NiCl_2$ (15-17), many others have identified its potential use for the treatment of cancer (9). Studies of several cell lines for the evaluation of the anticancer activity of $NiCl_2$ have investigated concentrations that suppress cellular proliferation by 50% (IC50) (10). The IC50 has been reported to be 1.5 mM in osteosarcoma cell lines (U2OS), 2 mM in human keratinocytes (HaCat) (10), and 400 µM in hepatocellular carcinoma cell lines (HepG2) (12), and $NiCl_2$ has been demonstrated to suppress cellular proliferation and induce apoptosis in cancer cells (10). Our study evaluated cell confluency in A549 cell lines after $NiCl_2$ treatment at doses of 200 µM and 600 µM and recorded the changes in the cellular morphology. Accordingly, the cell confluency rates did not change with a 200 µM dose, while cell confluency decreased by 50% at a dose of 600 µM. In addition, Giemsa staining revealed no significant changes in cellular morphology in the cell lines treated with 200 µM $NiCl_2$, whereas, in the groups treated with 600 µM $NiCl_2$, the cell morphology has been disrupted, with a loss of cell-to-cell contact, a decrease in the cytoplasm, and a morphological divergence from their ancestors by gaining a spindle-shaped and unhealthier appearance.

The migration of cancer cells and their ability to metastasize to distant organs leads to failure in cancer treatment and makes the greatest contribution to cancer-related deaths (18). Indeed,

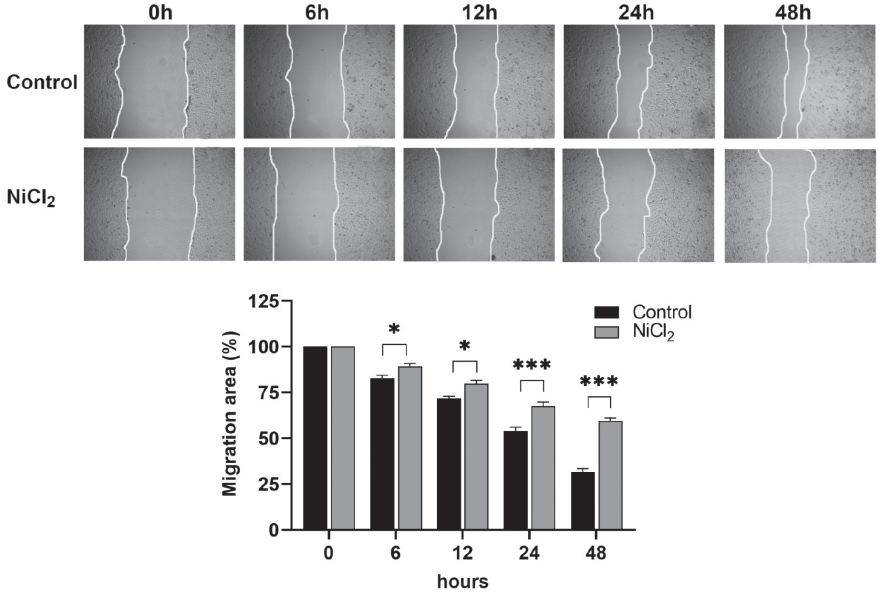

Figure 2: Effect on nickel chloride on cell migration. An *in vitro* scratch assay was used to define the migration status of A549 cells. Asterisk indicates *p<0.05, ***p<0.001

$NiCl_2$: Nickel chloride

approximately 90% of cancer-related deaths have been linked to metastasis, being a complex process involving the migration of cancer cells after separation from the primary local tumor and invasion of the surrounding tissues and colonization in distant organs (19). In the light of these data, gaining insight into the key molecular actors in the metastasis process and how to target these actors through therapeutic interventions is vital in the suppression of this process.

In a study conducted by Ota et al. (9), $NiCl_2$ was shown to decrease matrix metalloproteinase expressions significantly at messenger ribonucleic acid (mRNA) and protein levels, and to decrease the expression of angiogenic factors such as IL-8 and vascular endothelial growth factor (VEGF) at mRNA level. Moreover, the authors demonstrated that various genes involved in cancer metastasis were suppressed in mice fed with $NiCl_2$ (9). Based on these effects, $NiCl_2$ was determined to suppress cell migration and metastasis in oral squamous cell carcinoma, identifying the potential of $NiCl_2$ as a new and promising therapeutic anti-cancer agent (9). Similar to their study, our study demonstrated that $NiCl_2$ suppressed cell migration in A549 cell lines at a dose of 600 µM from 12 hours, and this effect was sustained and augmented at 24 and 48 hours after administration.

CONCLUSION

The findings of this study reveal that cell morphology was affected, and cellular migration was suppressed in non-small cell lung cancer cell lines treated with $NiCl_2$.

Ethics Committee Approval: *All assays were performed in in-vitro using commercially purchased cell lines. This study does not include any human or animal data.*

Informed Consent: *The study does not require patient consent.*

Conflict of Interest: *The authors declared no conflict of interest.*

Author Contributions: *Concept: E.G., Design: H.T.K, E.G., Data Collection or Processing: H.T.K, Ç.T., A.B., Analysis or Interpretation: H.T.K., E.G., Literature Search: H.T.K., Writing: H.T.K., Ç.T.K., A.B., E.G.*

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ORIGINAL ARTICLE

EDUCATION AFTER THE PANDEMIC: QUALITY IMPROVEMENT IS POSSIBLE WITH EASY AND RESOURCE-FRIENDLY VISUAL MODULES

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ABSTRACT

Aims: The pandemic has increased the importance of remote teaching resources in medical education and highlighted the importance of out-of-class learning in a hybrid setting. Achieving such a model seems to be challenging, especially for institutions with limited experience and resources. This study aims to demonstrate the educational benefit of such hybrid curricula by using simple modules, reproducible in various settings even with limited resources.

Methods: All fifth-year medical students at our institution (n=43) were included in this study. Their original random grouping to take internships at different times at our institution was utilized. The first group to undergo their psychiatry internship was registered as a control group (group 1, n=20) and the latter as an intervention group (group 2, n=23). First, an initial need assessment was administered to both groups before their internships to guide the design of the intervention. According to the needs identified, we came up with a plan consisting of clips and simple animations corresponding to three disorders in psychiatry. We integrated this module into the curriculum of the intervention group and checked its efficacy using the pre/post-survey method. We later compared the two groups for knowledge retention, self-evaluated sufficiency, and satisfaction with multiple-choice questions and a late-post survey. Statistical significance within the intervention group had been determined by dependent samples t-test whereas it was determined by independent samples t-test between the two groups, following normality analysis by the Shapiro-Wilk test.

Results: In the initial need assessment, Likert scores (1-5) of both groups showed agreement with low concentration [mean =4.3 (±0.85) and 4.1 (±0.85)] and stated disagreement about "patient variety," [mean =2.5 (±1.28) and 1.8 (±0.94)] and "management" [mean =3.0 (±1.15) and 2.9 (±1.01)]. After the visual display, Likert scores of the intervention group improved significantly in certain items reflecting self-sufficiency, and the post-survey had more correct answers (+21.5%, p=0.017). When the two groups were compared, the intervention group answered more questions correctly (+12.6%, p=0.058). They also stated benefits in memory, exam-preparedness, and sufficiency.

Conclusion: Simplistic, affordable, and easily prepared visual supplementation can offer an improvement in quality and increase student satisfaction with online teaching.

Keywords: Medical education, teaching methods, distance learning, quality improvement

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INTRODUCTION

The coronavirus disease-2019 pandemic has made it compulsory to transition into remote teaching techniques in a rapid trial and error method (1-3). Medical schools tried to incorporate different methods within their online curricula such as online simulations with simulated patients or supervised virtual consultations (4-7). In fields like psychiatry where student-patient interaction has become limited, the use of such add-ons has been reported to be fruitful in terms of both satisfaction and knowledge retention (8-10).

However, in countries such as Türkiye, most institutions could not transition fast enough and had to either postpone their lectures indefinitely or move on with two-dimensional slide-based online sessions (11). In those countries where there is underreporting and general dependence on face-to-face conventional teaching, it can be hard to estimate the real repercussions of this situation on students (11). There are limited reports around the world about the negative effects the students face in resource-limited settings such as the lack of "social presence," limited patient encounters, and decreased sense of clinical competency and motivation among the students (6, 12, 13). This can be worrisome as it can create negative prejudice towards innovative teaching methods and technology in medical education (11-13). Under the influence of such a negative connotation, graduates may not be equipped well with the requirements of modern medical practice (14).

Almost three years after the onset of the pandemic, face-to-face medical education ensues today, but remote teaching methods also continue to proliferate. It has therefore become important for institutions to translate the experience they gained during the pandemic into practice now to catch up with the state-of-the-art level of teaching. This can be especially hard for those institutions that could not incorporate such methods into their curricula (11-13). That is why we conducted this study on remote teaching to determine student demands, self-reported insufficiencies and respond with an intervention plan that is applicable and efficient. Specifically, we believe that the diversification of the education material by using simplistic visual supplementation can yield to improvements -large or small- in online medical education even in resource-limited settings. We would like to demonstrate that such innovations need not be heavily dependent on resources and experience but can still influence medical learning even after the pandemic.

MATERIAL AND METHODS

Procedure

We received formal approval from the Koç University Ethics Committee on 25 February 2021, before conducting this study (decision no: 2021.098.IRB3.059). Our study involved all the medical students in their fifth year -totaling 43- who were to begin their psychiatry rotations at different times. This approach to include most students -if not all- in one term has been demonstrated in the literature for similar interventions (7, 8, 10). Since fifth-year medical students are already randomly divided into two groups in our institution, we decided to study each group separately and designated the first group as a control group and the second group as an intervention group (Figure 1). Enrollment within the analysis was voluntary and formal consent was given by all student participants.

During the planning phase, we first devised an initial needs assessment. It targeted both the psychiatric curriculum completed by group 1 and the general state of online education.

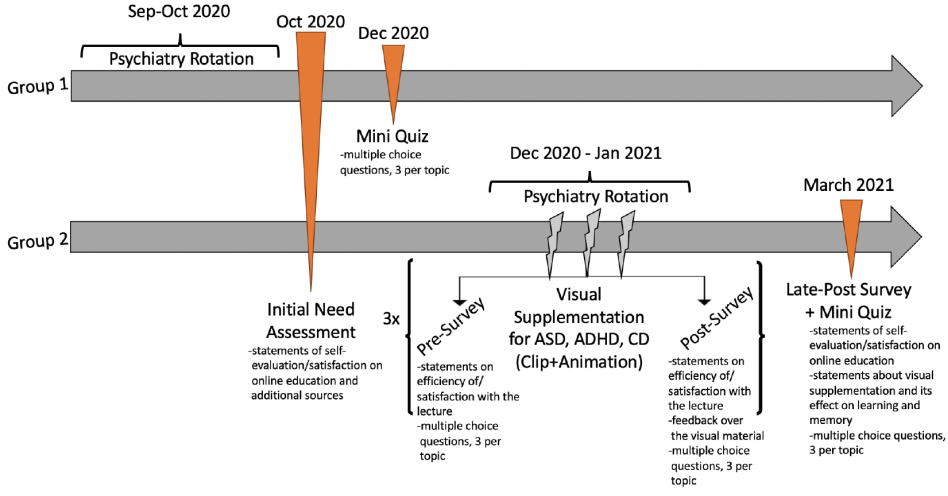

Figure 1: Our timeline of events depicting both the intervention and the measurements taken. Note that there is a separate pre-survey, visual material, and post-survey for each of the three topics selected. For each topic, the two surveys and the intervention in between took place on the same day that the corresponding lecture was scheduled. The statements found in the initial need assessment are the same with the ones in the late-post survey of the second group. The statements found in the pre-survey and the post-survey are also the same and different from the ones in the initial need assessment. The multiple-choice questions consist of three questions per topic with 5 choices. The questions used in the mini-quizzes are the same. The questions used in the pre-survey and the post-survey are also the same and different from the ones used in the mini-quizzes.

ASD: Autism spectrum disorder, ADHD: Attention deficit hyperactivity disorder, CD: Conduct disorder

The survey consisted of three domains: online education compared to prior face-to-face settings, self-evaluated sufficiency, and types of additional external resources. Each domain had a list of statements measured by the Likert 5 scale (15). The responses and the types of additional resources guided the design of the intervention (refer to Table 1 in the results section for the questions involved). Following that, the initial topics to target with our intervention were selected from the child and adolescent psychiatry curriculum. We prioritized three core topics: Attention deficit hyperactivity disorder (ADHD), conduct disorder (CD), and autism spectrum disorder (ASD). These topics were later dissected into their learning objectives as approach, diagnosis, and management.

Two months after completing their psychiatry rotation, the first group was administered a MiniQuiz to determine memory without the intervention. It included three multiple-choice questions (MCQs) for each topic (ADHD, CD, ASD) focused on formerly mentioned objectives; approach, diagnosis, and management. Questions were prepared by us using the most recent diagnostic criteria (16).

Before the beginning of the psychiatry rotation of group 2, intervention design was carried out based on the responses within the initial needs assessment and auxiliary material already being used. We narrowed down our options of easy and applicable materials: clips from TV shows/movies containing psychiatric elements, and software-generated custom animations. For the first visual element, a list of TV shows/movies/clips was put together (Supplement A) by the psychiatric faculty members. We aimed to select the most representative footage to display symptom patterns of CD, ASD, and ADHD. Our selection included Modern Family (2010) Season 1 Episode 18 for ADHD, Temple Grandin (2010) for ASD, and the 400 Blows (from

Table 1. The results of the initial need assessments of both groups and their change before and after our intervention in group 2.

	Group 1 (control) (n=20)	Pre-intervention group 2 (n=23)	Significance between two groups (p-value)	Post-intervention group 2 (n=23)	Significance of change after intervention (p-value)
A. Online vs. prior face-to-face education [mean (SD)][a]					
1. "I believe face-to-face lectures are more efficient when compared to online lectures."	4.1 (0.97)	3.5 (1.27)	0.083	3.3 (0.93)	0.680
2. "It is easier to concentrate during face-to-face lectures than during online lectures."	4.3 (0.85)	4.1 (0.85)	0.534	3.4 (1.03)	0.043*
3. "I had to allot more time for self-study in the online system when compared to the prior face-to-face setting."	3.8 (1.12)	3.6 (1.08)	0.585	3.1(1.20)	0.185
4. "Face-to-face lectures were easier to follow when compared to online lectures."	4.8 (1.23)	3.8 (1.15)	0.542	3.9 (0.97)	0.888
5. "Cases in online lectures are as informative as cases we encounter in bedsides/rounds/clinic hours."	2.25 (1.28)	2.0 (1.00)	0.203	3.2 (0.89)	0.000*
B. Self-evaluated sufficiency during online education [mean (SD)]					
1. "I believe I have encountered enough number/variety of cases/ patients."	2.5 (1.28)	1.8 (0.94)	0.053	2.3 (0.78)	0.056
2. "It is comfortable for me to take history/converse with patients."	3.0 (1.17)	2.7 (1.10)	0.455	3.7 (0.82)	0.005*
3. "I can easily form differential diagnoses upon patient encounter."	3.5 (1.00)	3.2 (1.11)	0.400	3.6 (0.99)	0.186
4. "It is easy for me to find the best approach to patients."	3.2 (1.06)	3.0 (1.07)	0.632	3.8 (0.90)	0.014*
5. "I can easily formulate management options and understand the benefits/risks."	3.0 (1.15)	2.9 (1.01)	0.808	3.7 (0.93)	0.028*
C. Additional resources during online education [percentage (n)][b]	**Group 1 (n=20)**	**Group 2 (n=23)**			
Textbooks	6.7 (5)	14.9 (15)			
Amboss™ (Miamed Inc. Cologne, Germany)	26.7 (20)	21.8 (22)			
UpToDate™ (UpToDate Inc. Wellesley, MA, USA)	13.3 (10)	5.0 (5)			
Osmosis™ (Osmosis Inc. Baltimore, MD, USA)	10.7 (8)	14.9 (15)			
Youtube™ (Youtube Inc. San Bruno, CA, USA)	14.7 (11)	7.9 (8)			
Sketchy™ (Sketchy Medical Inc. Los Angeles, CA, USA)	5.3 (4)	4.0 (4)			
Lecturio™ (Lecturio Medical Magazine, Leipzig, Germany)	0 (0)	1.0 (1)			
Boards & Beyond™ (Boards and Beyond, CT, USA)	6.7 (5)	7.9 (8)			
Question Bank [i.e Uworld™ (UWorld Inc. Dallas, TX, USA)]	14.7 (11)	20.0 (20)			

SD: Standard deviation
[a]The mean Likert scores are given from 1 to 5. Scores above 3 indicate agreement. SDs are given in parentheses.
[b]The percentage of the given source in relation to total answers within that group are given. Note that one participant could have selected multiple options for this part of the survey.
*Statistical significance, p<0.05.

French Original "Les Quatre Cents Coups") (1959) for CD. In the end, three 5-minute clips were prepared to be displayed for group 2. For the second visual element, the online Toonly™ (Bryxen Inc. Dublin, OH, USA) software is selected to generate animations of exemplary patient-doctor interviews. Using the software, a script is played out using 2D characters to generate a roughly 5-minute animation (Supplement B). It included the first encounter with the patient, the initial history, and the first steps of management. The scripts were written by the research team, using academic case reviews and up-to-date DSM5 criteria (17, 18). The final products were shown to students via separate links on the days of corresponding lectures.

In order to check the response of group 2 students, we used the pre-and-post-survey method with multiple choice questions and Likert 5 statements (Figure 1). It had three multiple choice questions referring to each of the abovementioned sections of learning objectives, prepared by the psychiatry department. Their answers did not consist of anything exclusive to the online lectures already available. Upon completion of the psychiatry rotation by group 2 students, a late-post survey was given two months later (Figure 1). The late post-survey tried to measure any change in the needs of students by using the same layout of statements in the "initial needs assessment." The same Mini-Quiz applied to group 1 was then applied to group 2, post-intervention.

Statistical Analysis

All the participant data is assembled in a single SPSS™ (IBM Inc. Armonk NY, USA) sheet. We based our analysis on mean Likert scores given to each survey item and analyzed the distribution ($n<30$ in each group) using the Shapiro-Wilk test. The distribution of the measurements yielded results in parallel to normal distribution with $p>0.05$. Taking this into consideration, we used independent samples t-test to compare the answers given to the initial need assessment by the two groups or the statements scored separately by group 2 about each type of visual modality. We additionally used paired samples t-test to determine the significance of change within group 2. The change from the pre-survey to the post-survey and from the initial need assessment to the late-post survey are determined this way. The answers given to the MCQs are compared in terms of correctness by the chi-square test. We analyzed the power of our intervention within group 2 for the statements of the needs assessment with significant change in a post-hoc fashion with an online tool (19).

RESULTS

All 43 of the students volunteered to participate in the study with 20 students in group 1 and 23 in group 2. However, four students in group 2 failed to be present during either one or all the days of visual intervention and therefore were excluded from the pre-survey/post-survey analysis. Since the visual supplementation was later separately shared with them, they were included in later steps (late-post survey and mini-quiz). The study population consisted of 21 males and 22 females with a mean age of 22.7 years. In parallel to this study, all students completed and passed their psychiatry rotation on the expected dates. The initial need assessment conducted at the beginning of the study revealed similar trends (Table 1) in both groups. After the intervention, in part A, agreement by mean Likert scores of the first three statements decreased, unlike the last two statements. In part B, agreement with all statements climbed up.

Significantly positive changes were observed in statements about "concentration" (statement A2), "case informativeness" (statement A5), "history taking" (statement B), "approach" (statement B), and "management" (statement B) with post-hoc powers 65%, 98%, 90.3%, 72.5%, and 74.1%, respectively.

The visual intervention introduced changes to both students' perspectives and also their grasp of knowledge (Table 2, Sections A and B) as highlighted by the improvements in the post-survey results of group 2 students.

The number of correct answers given to multiple choice questions also rose (+20.5%, $p=0.017$) within the pre- and post-survey results of group 2 (Table 2, Section B). Two questions (Q1 of CD and Q1 of ASD) were answered correctly by everyone even in the pre-survey.

Regarding the comparison between the intervention group and the control group, even though there was a difference in the correct answers (group 2: 87% vs. group 1: 74.4%) given to MCQs in the mini-quizzes, it was not significant and therefore further analysis is not shown (+12.6%, $p=0.058$). The feedback reflecting at the visual material on the late-post survey returned mainly positive and comparable between the two modalities (Table 2, Sections C and D).

DISCUSSION

Our study highlighted certain needs within a hybrid medical education setting, unlike a certain number of reports that stated increased student satisfaction (9, 20). However, the term "online education" on its own is a broad concept, and applications change even from one medical school to another in the same region. In schools where the online content was limited to didactic recordings of lectures and low-quality synchronous meetings, reports signify the decrease in student motivation and the lack of dimensionality within the current medical education (4, 5). This might have yielded a negative connotation for such innovative methods in settings where experience with such tools remained limited (11-13).

As identified by our study, students agreed with the "efficiency" and "realism" of the prior educational setting and stated higher "concentration" before (Table 1). They also reported insecurities about "approaching patients," running "diagnostics," and "management." When we take a look at the list of complementary resources used, visual platforms like Osmosis™ (Osmosis Inc. Baltimore, MD, USA), Youtube™ (Youtube Inc. San Bruno, CA, USA), and Sketchy™ (Sketchy Medical Inc. Los Angeles, CA, USA) collectively seem to be highly preferred by both groups. The role of such materials

Table 2. The pre-, post- and late-post survey results, reflecting responses given before and after the visual supplementation (A, B) and separate feedback about the visual modalities (C, D).

	Pre-survey (n=19)	Post-survey (n=19)	Significance of change (p-value)[b]
A. Overall satisfaction with/perceived efficiency of the online lectures [mean (SD)][a]			
1. "I believe I understood this subject well."	4.2 (0.63)	4.3 (0.58)	0.578
2. "The materials used were sufficient."	3.6 (0.83)	4.3 (0.75)	0.023*
3. "There were memorable elements in this lecture."	3.2 (1.17)	3.8 (0.71)	0.048*
4. "Participating in this lecture was enjoyable."	3.3 (0.99)	4.3 (0.75)	0.001*
5. "It was easy to concentrate in this lecture."	3.2 (0.83)	4.1 (0.71)	0.000*
6. "I can establish the diagnosis of this condition."	4.1 (0.78)	4.1 (0.57)	0.804
7. "I can recognize the symptoms of this condition."	3.8 (0.83)	4.1 (0.62)	0.429
8. "I know what to inquire in a patient with this condition."	3.9 (0.85)	4.0 (0.67)	0.816
9. "I can follow the steps of management with this condition."	3.6 (0.84)	3.9 (0.71)	0.167
10. "I feel sufficient as a doctor on this condition."	3.4 (0.90)	3.9 (0.74)	0.058
B. Multiple choice questions			
Overall [correct %]	65.5	86.0	0.017*
ASD [correct %]c			
ASD subtotal	75.4	94.7	0.031*
Q1 (Approach)	100	100	-
Q2 (Diagnosis)	68.42	94.7	0.056
Q3 (Management)	57.89	89.5	0.055
ADHD [correct %]			
ADHD subtotal	47.4	73.7	0.017*
Q1 (Approach)	68.4	89.5	0.104
Q2 Diagnosis)	52.6	68.4	0.454
Q3 (Management)	21.1	63.2	0.007*
CD [correct %]			
CD subtotal	73.7	89.5	0.083
Q1 (Approach)	100	100	-
Q2 (Diagnosis)	73.7	100	0.021*
Q3 (Management)	47.4	68.4	0.331
C. Feedback statements [mean (SD)][a]	**Animations (n=19)**	**Clips/Movies (n=19)**	**Significance (p-value)[d]**
1. "It made me understand the subject better."	4.4 (0.61)	3.7 (0.89)	0.005*
2. "It made the lecture more enjoyable."	4.1 (0.99)	4.2 (0.76)	0.856
3. "I believe it is an efficient method of learning."	4.5 (0.51)	4.0 (0.75)	0.016*
4. "It made it easier to remember key concepts."	4.3 (0.75)	3.9 (0.99)	0.149
5."I feel more motivated after watching it."	3.0 (0.75)	4.4 (0.69)	0.001*
6. "I believe it will be beneficial to widen its use in other topics as well."	4.6 (0.60)	4.1 (0.94)	0.046*
D. Long-term feedback given by group 2 students [mean (SD)][c]	**Group 2 Late-post survey (n=23)**		
1. "Visual materials helped me remember concepts in the long-term."	4.3 (0.63)		
2. "I can recall lectures with visual materials better than the ones without."	4.3 (0.76)		
3. "Visual materials used were preparatory for real-life patients I encountered."	4.2 (0.90)		
4. "I felt more prepared for the exams with the visual materials."	4.1 (0.79)		
5. "I feel it is necessary to increase the use of visual materials within online education."	4.5 (0.67)		

SD: Standard deviation, ASD: Autism spectrum disorder, ADHD: Attention deficit hyperactivity disorder, CD: Conduct disorder
[a]The mean Likert scores are given from 1 to 5. Scores above 3 indicate agreement. SDs are given in parentheses.
*Statistical significance, p<0.05.

and popular culture in medical education is shown by multiple studies such as organizing movie nights, teleconferences with patients, and showing patient footage (8-10, 21, 22). We, therefore, shaped our intervention design accordingly and preferred to use animations and clips because it was easy to find, disseminate, and operate with positive reports in the literature (9, 23). The first improvement we observed was reported by group 2 students in their post-survey. They assigned higher scores to "sufficiency of the material," "memorability," "enjoyability," and "ease of concentration" (Table 1). Other statements such as "recognition of symptoms," "taking history," and "management" -although statistically insignificant- also were scored higher after the intervention (Tables 1 and 2). Being a more objective measure, the overall correct answers given to multiple choice questions also improved significantly (Table 1) after the intervention. Since there was no lecturing present in the supplementation, the visual input we provided could have helped the students to use the information they have previously learned more efficiently. Therefore, beyond being simply a tool for enjoyment and motivation, our study displays that visual materials can have a role in turning theory into practice. When students were asked to compare the two modalities we used in our intervention, statistically significant differences (Table 2) in responses were given. Students deemed the animations to be more helpful in "understanding" the subject and "efficiency."This can be due to the fact that the animations contained an ideal encounter and therefore had more teaching value than the clips. For the clips, the students significantly gave higher scores to the statements about "motivation" and "enjoyability."

In terms of the answers given to multiple choice questions in the mini-quizzes to assess long-term memory, it may seem hard to conclude since certain questions are answered correctly by all and statistical significance was limited. However, the effect of visual content on memory has also been analyzed before, especially for movies (23). Therefore, even though the benefit of the information retained is unclear, our study displays that simple visual supplementation can provide students with further motivation to look back on previous topics when preparing for exams and when they encounter psychiatric patients in other rotations.

We had several limitations within this study. The first one was about the number of students who were included in this study due to the small annual capacity of our institution. This likely has affected the statistical significance of our results and weakened the testing of our hypothesis. This scarcity of responders has also affected our capability to perform a healthy power analysis. Even though some of our results in the significant statements have relatively high power, these values are calculated with post-hoc calculation which is known to yield relatively unreliable results (19). We are aware that our findings would become more convincing if supported by the addition of the upcoming fifth-year medical students each year. Furthermore, we would like to overcome this limitation by extending the supplementation to other rotations as well to include more students. Other institutions can also benefit from the content we have created (Supplements A, B) and replicate the study. This way, we can perform sampling out of a larger student body and have a healthier outcome about the population of medical students. It can also boost the generalizability of our results and persuade other regions to take part in similar initiatives. Another issue we have faced was regarding the quality of the MCQs we prepared. The discrimination between the two groups and within the second group was challenging since some questions were either too easy or too well-known by the students. This has obscured the effect on memory and knowledge retention. Better quality assessment tools with a larger design team can overcome this problem.

CONCLUSION

We present a form of visual supplementation as a simple, cheap, readily available, and efficient method of diversifying the content of a hybrid medical education setting. As demonstrated here, disproportionate to the small scale of the intervention, a modest but positive outcome in multiple domains of teaching can be achieved with such a module. Institutions and teaching faculty can therefore be encouraged to engage in similar initiatives which make use of the resources at hand regardless of how limited. This way, even if a fully online educational setting is abandoned after the pandemic, the new and innovative methods introduced by the hybrid classrooms can be utilized to maintain a high standard of teaching in medical schools. We believe this can alter the negative connotation behind "online education" in regions with less experience and access by rendering it a complementary tool to support traditional learning in medicine.

Ethics Committee Approval: *This study was approved by the Committee on Human Research of Koç University (decision no: 2021.098.IRB3.059).*

Informed Consent: *Informed consent was obtained from all of the subjects.*

Conflict of Interest: *The authors declared no conflict of interest.*

Author Contributions: *Concept: Z.B.K., S.C.S., Ş.K., A.Ö.M., A.E.Y., Design: Z.B.K., T.M., Data Collection or Processing: Z.B.K., S.C.S., F.Y., O.E., T.M., Analysis or Interpretation: Z.B.K., S.C.S., T.M., Literature Search: Z.B.K., S.C.S., F.Y., O.E., Ş.K., A.Ö.M., Writing: Z.B.K., S.C.S., F.Y., O.E., A.Ö.M., A.E.Y.*

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Supplement A
The list of resources found for the three conditions: ASD, ADHD, CD
A) Autism spectrum disorder
Temple Grandin-HBO Films-2010
Forrest Gump-Paramount Pictures-1994
The Good Doctor-Disney (series)-2017
Atypical-Netflix (series)-2017
The Boy Who Could Fly-20th Century Fox-1986
Miracle Run-Lifetime Television-2004
Snow Cake-IFC Films-2006
A Boy Called Po-New Coast Productions-2016
A Mile In His Shoes-NGN Productions-2011
Adam-Fox Searchlight-2009
Bad Hurt-Screen Media-2015
David's Mother-CBS-1994
Extremely Loud and Incredibly Close-Warner Bros.-2011
Fly Away-New Video-2011
Jack of the Red Hearts-ARC Entertainment-2015
Mozart and the Whale-Millennium-2005
My Name Is Khan-Fox Searchlight-2010
The Imitation Game-Weinstein-2014
B) Attention deficit hyperactivity disorder
Modern Family-20th Century (series)-2009-2020
Take Your Pills Netflix (documentary)-2018
Wunderlich's World-Zodiac Pictures-2016
Finding Nemo-Pixar-2003
Mrs. Doubtfire-Blue Wolf Productions-1993
Juno-Fox Searchlight-2007
C) Conduct disorder
400 Blows-Les Films du Carrosse-1959
We Need to Talk About Kevin-BBC Films-2011
Donnie Darko-Pandora Cinema-2001
Sucker Punch-Warner Bros. Pictures-2011
Breakfast Club-A&M Films-1985
Euphoria-HBO (series)-2019-Present
A Clockwork Orange-Warner Bros. Pictures-1971

ASD: Autism spectrum disorder, ADHD: Attention deficit hyperactivity disorder, CD: Conduct disorder

Supplement B
The links to the animation and clip footages
Animation Footage-ADHD: https://www.youtube.com/watch?v=YHcnI1ZVxSI&t=4s
Animation Footage-CD: https://www.youtube.com/watch?v=G8gEpkfWWUY&t=6s
Animation Footage-ASD: https://www.youtube.com/watch?v=GmrqP8UItYo&t=3s
Clip Footage-ADHD: https://www.youtube.com/watch?v=_3HFFolyQWk&t=52s
Clip Footage-CD: https://www.youtube.com/watch?v=xoWCjJl-Pag&t=6s
Clip Footage-ASD: https://www.youtube.com/watch?v=ARC3Us1JaTk&t=49s

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ORIGINAL ARTICLE

THE MENTAL STATUS AND SMOKING BEHAVIORS OF MEDICAL STUDENTS DURING THE COVID-19 PANDEMIC: A CROSS-SECTIONAL STUDY

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ABSTRACT

Aims: This study aimed to assess the frequency of smoking and the mental states of students who were studying in a medical faculty during the coronavirus disease-2019 pandemic and to identify associated factors.

Methods: The cross-sectional study was conducted with 432 medical students between June and August 2021. A demographic information collection form, the General Health Questionnaire-12, and the Fagerström Nicotine Addiction test were used as data collection tools in the study.

Results: The mean General Health Questionnaire-12 score of students was determined as 3.36±3.54. 63.4% of the students had a General Health Questionnaire-12 score above the cut-off score (≥2), which was determined as a risk for mental problems. In terms of general health questionnaire scores in multivariate logistic regression analysis, it was determined that those aged 21 and under [odds ratio (OR) =1.70, 95% confidence interval (CI) =1.121-2.594), women (OR =2.22, CI =1.455-3.379), those who reported that they did not eat regularly (OR =1.60, CI =1.039-2.451), and smokers (OR =2.34, CI =1.311-4.204) were at risk, and living at home with family or a group of friends was a protective factor. It was determined that addiction levels in smokers increased with age (22 years and older) (OR =3.303, 95% CI =1.244-8.765) and with drinking alcohol (OR =8.702, CI =1.024-73.975).

Conclusion: It was found that the COVID-19 pandemic significantly contributed to the increase in mental problems in medical students, and there was a slight increase in smoking behaviors.

Keywords: COVID-19 pandemic, medical students, mental health, smoking

INTRODUCTION

The already difficult medical education has been negatively affected due to the coronavirus disease-2019 (COVID-19) pandemic. During the pandemic period, medical education was greatly interrupted, clinical training was reduced, and only some pieces of training could be conducted remotely (1). With the closure of schools, students' daily lives have changed to a great extent, their motivation to study has decreased, and their anxiety and depression levels have increased (2, 3). According to a systematic review, medical students went through periods of moderate and high stress during the pandemic (4).

According to current literature, stress and anxiety are known emotional triggers for smoking (3). People are known to start smoking more during difficult situations like epidemics as a coping or defense mechanism (3). Most smokers attribute this behavior to the feeling of relaxation they get while smoking (3). However, since COVID-19 in particular is an acute respiratory disease, tobacco use in individuals has been associated with experiencing the disease more severely, hospitalization in intensive care, and death (5, 6). Smoking increases the risk for the prognosis of COVID-19 by a factor of 14.28 (5). There are a limited number of studies investigating whether smoking behaviors have changed during the COVID-19 pandemic (6).

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The overall effect of the COVID-19 pandemic on tobacco addiction in the general population is reported to be uncertain at the macro level (7). It is estimated that the COVID-19 pandemic led to changes in lifestyle, disruptions in education, quarantines, and isolation that resulted in mental illnesses and nicotine addiction in young people (8). However, there is not enough empirical evidence to support these findings. Smoking addiction is a serious health problem in our country, as it is in many developed and developing countries. Smoking is an important psychosocial problem in terms of its causes and is one of the most harmful behaviors for human health. Therefore this study aimed to evaluate the mental status of medical students, who are health professionals of the future, during the COVID-19 pandemic to examine their smoking behaviors and to determine the affecting factors.

MATERIAL AND METHODS

Ethics Committee Approval

Approval for the research was obtained from the Ministry of Health and Trakya University Ethics Committee (decision no: 13/16, date: 14.06.2021) for the study.

Study Design

The study was designed as a cross-sectional study. The study was conducted through face-to-face interviews with students studying at a medical school of a state university between June and August 2021. The population of the study consisted of 1693 students in the medical school. Using the sample size calculation program G*Power v3.1.9.7 (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany), the minimum (min) sample size was calculated as 364 people in the calculation made by taking the pattern effect as 0.25, α =0.05, power =0.90. Considering possible data loss, the study was conducted with 432 students. In the study, stratified sampling was conducted, and students were selected from each class using a simple random sampling method. However, since the 6th-year medical students (interns) were on duty in pandemic clinics, a sufficient number of students could not be reached from this class, and only 30 students from this class participated in the study. The study included students who were enrolled in Trakya University School of Medicine and volunteered to participate.

Data Collection Tools

The study data were collected using the Personal Information Form, the 12-item General Health Questionnaire (GHQ-12) (9), and the Fagerström Nicotine Addiction test (10).

Personal Information Form: The form consists of 21 questions developed by the researchers in line with the literature. Students were asked about their age, gender, chronic diseases, economic level, place of stay, smoking status, alcohol consumption, exercise status, sleep patterns, diets, whether they were vaccinated against COVID-19, whether they or their families were diagnosed with COVID-19, and whether there were people who died due to COVID-19 in their families.

General Health Questionnaire-12 (GHQ-12): The General Health Questionnaire (GHQ), developed by Goldberg in the 1970s, is a test that can be filled out by individuals and is used to identify mental health problems, especially in primary care. GHQ-12 consists of 12 questions in which the respondents indicate their agreement on a four-point scale (0= Not at all; 3= More than usual) (9). The validity and reliability study of the scale in Türkiye was conducted by Kılıç (11). The responses of the scale, which has a sensitivity of 0.74 and a specificity of 0.84, can be scored as Likert type (0-1-2-3) items or as recommended in the GHQ manual (0-0-1-1). In our study, the answers selected as "a" and "b" options of the questions were scored as "1" points. The GHQ-12 score ranges from 0-12. In this study, those with a GHQ-12 score ≥2 were considered at risk in terms of mental problems.

Fagerström Nicotine Addiction Test: Karl O. Fagerström designed the test, which consists of six questions, to assess the degree of physical addiction to smoking. The questions are all closed-ended (10). The test score increases according to the degree of smoking addiction. A mild nicotine addiction is one with a test score of less than 5, a moderate addiction is one with a score of 5 or 6, and a severe addiction is one with a score of 7 or more. Uysal et al. (12) conducted a validity and reliability study of the Turkish version of the test, and its reliability was found to be moderate (α =0.56).

Statistical Analysis

SPSS 21.0 package program was used for the data analysis. While evaluating the data, its compliance with normal distribution was investigated with the Kolmogorov-Smirnov test, and it was observed that the obtained data conformed to the normal distribution ($p>0.05$). In the analysis of the findings, the chi-square test was used for discrete variables, while the t-test and one-way analysis of variance were used for continuous variables. Linear regression and multivariate logistic regression analysis were used to determine the extent of independent variables affecting the GHQ-12 total score. The level of statistical significance was set as $p<0.05$.

RESULTS

The mean age of the students participating in the study was 21.5±2.2 years, 56% were female, 54.7% had an income equal to their expenses, 31.3% lived alone, and 14.1% had a chronic disease. It was determined that 18.8% of the students had COVID-19 infection, 16% lost a relative due to COVID-19, and 90.3% thought that they had sufficient information about COVID-19.

According to the information from the students, 72.3% of them never or rarely exercised during the COVID-19 pandemic period, approximately half (44.7%) could not eat regularly, and 38% stated that their daily sleep duration changed (Table 1).

The mean GHQ-12 score of the medical school students was determined as 3.36±3.54 (min: 0, maximum: 12), and the GHQ-12 score of 63.4% of the students was found to be above the

Table 1: GHQ score averages of medical school students according to some demographic characteristics.

Characteristics	Value (Percentage)	GHQ-12 Mean ± SD	p-value
Gender			
Female	242 (56.0)	4.12±3.65	**0.002**
Male	190 (44.0)	3.06±3.31	
Age			
21 years and under	229 (53.0)	3.85±3.51	0.239
22 years and older	203 (47.0)	3.44±3.57	
Mother's education			
Primary school	89 (20.6)	3.76±3.56	
Middle school	37 (8.6)	3.86±3.89	
High school	129 (29.9)	4.06±3.60	0.279
College or higher	144 (33.3)	3.27±3.40	
Father's education			
Primary school	45 (10.4)	3.28±3.20	
Middle school	30 (6.9)	5.03±3.79	
High school	128 (29.6)	4.21±3.74	**0.009**
College or higher	224 (51.9)	3.24±3.55	
Income			
Income < expenses	71 (16.4)	4.52±3.82	
Income = expenses	236 (54.7)	3.69±3.49	**0.028**
Income > expenses	125 (28.9)	3.12±3.40	
Accommodation			
Family members	98 (22.6)	4.08±3.80	
Dormitory/student housing	130 (30.1)	3.19±3.48	**0.018**
Friends	69 (16.0)	4.62±3.90	
Alone	135 (31.3)	3.31±4.49	
Chronic disease			
Yes	61 (14.1)	4.19±4.29	0.204
No	371 (85.9)	3.57±3.40	
Physical activity			
Sometimes, never	107 (24.8)	3.98±3.61	**0.002**
Always, regularly	325 (75.2)	2.83±3.21	
Regular diet			
Yes	239 (55.3)	3.04±3.17	**0.000**
No	193 (44.7)	4.43±3.82	
Sleep habits			
Changed	164 (37.9)	4.25±3.90	**0.007**
No change	268 (62.1)	3.30±3.86	
COVID-19 infection			
Yes	81 (18.8)	4.40±4.15	**0.036**
No	351 (81.2)	3.49±3.37	
COVID-19 knowledge level			
Sufficient	390 (90.3)	3.58±3.50	0.167
Insufficient	42 (9.7)	4.38±3.87	
Smoking status			
Yes	90 (20.8)	5.21±3.30	**0.001**
No	342 (79.2)	3.25±3.99	
Alcohol consumption*			
Never-former drinker	256 (59.2)	2.86±3.05	**0.001**
Current	172 (39.8)	4.17±3.76	
Smoking status during the COVID-19 pandemic**			
Increased	46 (10.6)	7.0444±3.78	
Decreased	24 (5.6)	5.0833±3.86	**0.001**
No change	29 (6.7)	2.2759±2.21	

Significant values are marked as bold. *Percentages do not add up to 100% because of the missing data in the survey responses. **Percentages are calculated based on 432 survey respondents

GHQ-12: General Health Questionnaire-12, COVID-19: Coronavirus disease-2019, SD: Standard deviation

cut-off score (≥2), which indicates a risk for mental problems. The mean GHQ-12 scores of female students, those with low economic status, those who do not regularly exercise, and have regular eating habits, those who have changed their sleep duration, those who drink alcohol, and those who have had COVID-19 infection were found to be higher (p<0.05) (Table 1).

In addition, it was determined that 14.8% of the students consumed alcohol 1-2 times a week, 26.6% smoked, and 39.8% of the smokers had moderate and high addiction levels. When asked if their smoking habits had changed during the pandemic, 10.4% stated that their smoking behavior increased during this period. In addition, it was found that the GHQ-12 mean scores of the students who smoked at an increasing rate during the pandemic were higher (p<0.05) (Table 1).

According to the multivariate logistic regression analysis, it was determined that those aged 21 and under (odds ratio (OR) =1.70, 95% CI =1.121-2.594), women OR=2.217, confidence interval (CI) =1.455-3.379], those who reported that they did not eat regularly (OR =1.596, CI =1.039-2.451), and smokers (OR =2.348, CI =1.311-4.204) were at risk in terms of their general health status. It was observed that students living at home with their families or friends had a better level of protective health. The highest risk was found to be in the smoking group, with an elevated risk of 2.35 times. This result revealed the suitability of the multivariate binary logistic regression model that was created to estimate the variables that affect students' overall health status. The multivariate binary logistic regression model explains 11.6% of the variance (Nagelkerke R^2 =0.116) (Table 2).

When the smoking status of the students was compared with demographic data, it was determined that those who lived with their friends at home, those who did not eat regularly, those whose sleep patterns changed during the pandemic period, and those who drank alcohol had higher smoking rates (p<0.05) (Table 3).

According to the multivariate logistic regression analysis, it was determined that the smoking addiction levels of the students increased by being in the older age group (22 years and older) (OR =3.303, 95% CI =1.244-8.765) and by drinking alcohol (OR =8.702, CI =1.024-0.975). It was determined that the highest risk was 8.70 times higher in the alcohol group. This result revealed the suitability of the multivariate binary logistic regression model created to estimate the variables affecting the smoking addiction level of the students and explained 27% of the variance (Nagelkerke R^2 =0.270) (Table 4).

DISCUSSION

The results demonstrate that 63.4% of the medical students scored above the cut-off score; that is, their mental health was poor according to the GHQ-12 scale. In some overseas studies conducted on medical students using the same scale, this rate was 62% in Sri Lanka, 70% in India, and 77% in the USA (13-15). When these studies focusing on the mental health of students are compared with the pre-pandemic studies, it is seen that these problems have increased during the pandemic period (16). For example, in pre-pandemic studies, this rate was found to be 21% lower in New Zealand, 47.4% lower in Australia, and 54.4%

Table 2: Multivariate regression analysis for GHQ-12 level of medical students.

Variables	B	SE	OR	(95% CI)	p-value
Age					
22 years and older (reference)	1				
21 years and under	0.534	0.214	1.707	(1.121-2.594)	**0.013**
Gender					
Male (reference)	1				
Female	0.796	215	2.217	(1.455-3.379)	**0.001**
Regular diet					
Yes (reference)	1				
No	0.467	219	1.596	(1.039-2.451)	**0.033**
Smoking status					
No (reference)	1				
Yes	0.853	297	2.348	(1.311-4.204)	**0.004**
Stay					
Alone (reference)	1				
Family or friends	-0.763	324	0.466	(0.247-0.880)	**0.019**
COVID-19 infection					
No (reference)	1				
Yes	0.090	0.266	1.095	(0.650-1.843)	0.734

Significant values are marked as bold.*Parameters for logistic regression model: Age, gender, nutritional status, living arrangement, smoking and COVID-19 infection status. Model x^2 =9.134, Hosmer-Lemeshow test: p=0.243, Nagelkerke R^2: 0.116
OR: Odds ratio, CI: Confidence interval, SE: Standard error, COVID-19: Coronavirus disease-2019, GHQ: General Health Questionnaire-12

lower in Iran (16-18). The results obtained from this study are similar to those of other studies, and high GHQ-12 mean scores showed that the effect of COVID-19 on the mental health of medical students was substantial and that their mental health was negatively affected.

In a systemic review, it was reported that medical students went through periods of moderate and extreme stress during the pandemic, and therefore, students should be seen as a "vulnerable population" (4). Hence, urgent preventive measures such as screening and education programs should be implemented because university students are accepted as a high-risk group in terms of depression and anxiety symptoms (4, 19, 20). Due to the epidemic's nationwide spread, strict isolation policies and the shutdown of educational facilities, students' mental health has been negatively impacted (20). Especially with the disruption of daily routines, while the motivation to study decreases, the pressure on independent learning increases, and uncertainty about the future can be experienced (20). Additionally, this anxiety that worsens at unusual and unexpected times may be an important risk factor for unhealthy behaviors like smoking. In a study conducted on university students, it

Table 3: Smoking status of medical school students according to some demographic characteristics.

Characteristics	Smoking status				p-value
	Yes		No		
Gender	**Value**	**%**	**Value**	**%**	
Female	43	17.8	199	82.2	**0.049**
Male	47	24.7	143	75.3	
Age					
21 years and under	42	18.3	187	81.7	0.108
22 years and older	48	23.7	155	76.4	
Accommodation					
Family members	16	16.3	82	83.7	**0.001**
Dormitory/student housing	16	12.3	114	87.7	
Friends	27	39.1	42	60.9	
Alone	31	23.0	104	77.0	
Regular diet					
Yes	29	12.1	210	87.9	**0.001**
No	61	31.6	132	68.4	
Sleep habits					
Changed	43	26.2	121	73.8	**0.022**
No change	47	17.5	222	82.5	
COVID-19 infection					
Yes	20	24.7	61	75.3	0.211
No	70	19.9	281	80.1	
Alcohol consumption					
Yes	79	30.9	177	69.1	**0.001**
No	11	6.4	161	93.6	

Significant values are marked as bold.
COVID-19: Coronavirus disease-2019

Table 4: Multivariate regression analysis for FTND (Fagerström test for Nicotine Dependence) level of students.

Variables	B	SE	OR	(95% CI)	p-value
Age					
21 years and under	1				
22 years and older	1.195	0.498	3.303	(1.244)-(8.765)	**0.016**
Alcohol consumption					
No	1				
Yes	2.164	1.092	8.702	(1.024)-(73.975)	**0.048**

Significant values are marked as bold.
*Parameters for logistic regression model: Age, alcohol consumption status. Model x^2 =18.7641, Hosmer-Lemeshow test: p=0.625, Nagelkerke R^2: 0.270
OR: Odds ratio, CI: Confidence interval, SE: Standard error

was emphasized that nicotine addiction was associated with higher levels of anxiety (3).

In our study, it was determined that there was an increase in students' smoking behavior during the pandemic period, that one out of every four students smoked, and that the mental state of the students who smoked was worse. Although it is known that the COVID-19 infection will be experienced more severely in smokers, the increase in smoking behavior during the pandemic period reflects that COVID-19 negatively affects the mental health of students. One of the ways to cope with negative emotions can be in the form of abusing all kinds of stimuli, including cigarettes and alcohol (13). The results are in line with those of other research in which it was shown that there was an increase in the number of smokers during the pandemic (8, 21, 22).

According to studies, smoking more cigarettes may be linked to more mental distress (21). Additionally, quarantining causes a 9.1% increase in smoking, which is most frequently linked to a decline in quality of life, less sleep, increased anxiety, and depressive symptoms (21). This may suggest that increased stress brought on by the coronavirus pandemic in general causes individuals to use stimulants more frequently.

In this study, it was determined that one out of every five students smoked. In a study conducted by Sönmez et al. (23); among medical students, 19.5% of the students were found to be smokers. The smoking rate was 18.26% among first-year students and 21.27% among sixth-year students (23). In a study conducted on 1208 students at Uludağ University School of Medicine, the smoking rate was 17.3%, while in a study conducted on 230 students at Düzce University School of Medicine, it was 31.3% (24, 25). In a study conducted at Gazi University School of Medicine in 2004, the smoking rate among first-year students was determined to be 17.6% (26). In this study, although there was no significant difference by age, the smoking rate was higher in the group aged 22 and above. Although the observed rates vary, the common result in all of these studies is that smoking behavior among medical students is significant, and it tends to increase in higher academic years.

Smoking behavior is more common in males in general (27). In student studies, it is emphasized that male students demonstrate a higher prevalence of nicotine addiction than women, both during the COVID-19 outbreak and in general (3, 27). Smoking among women has increased as much as among men in developed countries since 2000 (28). In this study, it can be said that there is no significant difference between the smoking behaviors of male and female students.

On the other hand, female students had a higher risk of mental health problems than males (19, 29). According to the literature, mental health issues are more common in women (30). Women experience emotions more intensely than men do, due to physiologic differences such as genetic sensitivity, hormones, and cortisol levels (31). Women may therefore feel more depressed and anxious since they are more sensitive to stress and pain (31). There are also hypotheses indicating that this increased frailty in women is caused by physiological reactivity and hormones (32, 33). In this regard, our results are consistent with the literature.

In the present study, it was determined that students who live alone, who are under 21 years of age, who do not eat regularly, who do not exercise, and who are infected with COVID-19 have poor mental status. According to estimations made by the American Psychiatric Association, over 50% of people will experience the detrimental effects of the situation caused by severe acute respiratory syndrome (SARS) coronavirus-2 on their mental health (34). It is also expected that these problems will occur at a higher rate, especially in those who have had COVID-19. Socialization is an important factor in the protection of mental health. In the study of Tahara et al. (19) in Japan, it was reported that less communication with friends was a risk factor for negative mental health in students. Therefore, it can be said that staying with family or friends is a protective factor for mental health.

In the current study, the fact that students in the younger age group had more mental problems is in line with other studies. In the study of Li et al. (35) the stress levels of nurses with less professional experience were higher. As individuals' vocational training increases with age, their levels of negative affect are likely to decrease due to the development of their clinical performance (36). Improving the professional knowledge of healthcare professionals on issues such as disasters or epidemics during training periods prepares them for such events and may contribute to their becoming less affected (36). In this process, there is a need to organize training programs by evaluating new technological opportunities.

According to literature, individual's diets may change because of high levels of stress brought on by natural disasters, especially there may be a decrease in healthy eating behaviors (37). Considering that changes in appetite and behavioral changes are common among the physical symptoms of mental health problems, the results are consistent with the literature (38). The relationship between stress and nutrition can affect each other bilaterally (39). On one hand, while the organism increases the level of stress hormones when it cannot get enough nutrients for itself, on the other hand, the increase in stress leads individuals to consume unhealthy food, including instant food, snacks, and food with intense calories (39, 40). In fact, nutrition and lifestyle changes have occurred with the increase in time spent idle and the decrease in physical activity due to online lessons during the pandemic period (8). In particular, curfews to reduce the risk of disease transmission have led to a restriction of physical activities and an increase in stress (37). Contrary to the studies reporting that physical activity decreased during the COVID-19 pandemic period, there are also studies reporting an increase (8, 41). In some studies, it has been stated that some people try to cope with mental problems related to the pandemic by relying on negative health behaviors such as smoking or alcohol consumption, contrary to health-enhancing behaviors such as

physical activity (5). In the present study, it is seen that the higher smoking behavior in students with poor nutrition and sleep problems is consistent with the literature.

In this study, while smoking behavior was found to be higher in students who drink alcohol, live with friends, and are 22 years of age or older, it was determined that those who live alone have more mental problems than those who live with their family or friends, but their smoking addiction is lower. Smoking and alcohol use can also be signs of troubled environmental conditions. In this study, the rate of alcohol consumption among students is consistent with the literature. In a study conducted by Yengil et al. (42); among medical students, the alcohol consumption rate was found to be 43.8%. In Western countries, the lifetime alcohol consumption rate among university students varies between 88% and 96% (42). In Türkiye, the rate of alcohol consumption among university students is lower compared to Western countries (42). The environment can accommodate both advantages and disadvantages for health. Research shows that a circle of friends who smoke and a sense of curiosity are important reasons for starting to smoke (3, 28). Students who lived alone were thought to have a lower tendency to develop bad habits because they were more sensitive to mental problems and more focused on academic achievement. The use of all other substances is more common in smokers than non-smokers (43). The fight against smoking is the most critical step in protecting students from harmful habits. It would be useful to question the reasons with different independent variables in new studies.

Those with low incomes have a higher risk of mental health problems than those with high incomes (Table 1). Low socioeconomic status is associated with high anxiety. Loss of income and education in the pandemic and curfews have been important sources of stress (44). Moreover, the negative economic effects of the pandemic on thousands of people are also emphasized (3). As a matter of fact, low income can put participants in a vicious circle. For example, when needs are not met due to a loss of income, anxiety begins to occur, nutrition and sleep patterns change depending on anxiety, and it may trigger an increase in negative behaviors such as nicotine consumption. Therefore, it can be said that the status of income is a factor affecting mental health and smoking behavior.

CONCLUSION

In this study, it was determined that more than half of the medical students experienced mental problems during the COVID-19 pandemic, and there was an increase in their smoking behavior. Mental health problems in medical students, who are future physicians, are too critical to ignore. Studies on the SARS epidemic, for instance, have demonstrated that psychological impacts are not always temporary and can result in severe, ongoing mental health issues (45, 46). Additionally, students who experience hardships are more likely to use tobacco and other drugs. Educational measures and training programs should be implemented to protect young people against smoking and prevent the development of addiction. Universities should promote the accessible psychological support sources, guidelines, and psychological counseling services, starting with the identification of students at risk. It will be important to determine the dimensions and determinants of the problem with more comprehensive research.

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CASE REPORT

AUTOIMMUNE HEMOLYTIC ANEMIA ASSOCIATED WITH COVID-19 IN AN INFANT: A CASE REPORT

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ABSTRACT

Autoimmune complications such as autoimmune hemolytic anemia (AIHA), an uncommon entity in pediatric patients, have been associated with infectious diseases. Knowledge of the pathophysiological mechanisms that contribute to the immune dysregulation caused by severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) infection is highly important, and clarifying the hypotheses about the molecular mimicry linked to these complications is essential to overcome potentially life-threatening hematologic manifestations in the underestimated pediatric population during the pandemic. A 10-month-old male with respiratory and gastrointestinal symptoms due to a SARS-CoV-2 infection documented by a molecular biology test developed severe anemia symptoms with an autoimmune hemolytic etiology. Management with corticosteroids improved his clinical condition and hematologic parameters. AIHA is a pathology with a broad presentation spectrum. Many illnesses are associated with triggers of AIHA; however, only a few related to Coronavirus disease-2019 (COVID-19) in infants have been described. This case report reminds us to consider the AIHA condition as a possible complication of COVID-19 in children under five years old.

Keywords: Anemia, autoimmunity, molecular mimicry, SARS-CoV-2

INTRODUCTION

Coronavirus disease-2019 (COVID-19) was first described in 2019 in Wuhan, China, and is associated with severe acute respiratory syndrome (SARS); it was declared a pandemic in March 2020 by the World Health Organization (WHO) (1).

According to the Korean Center for Control and Prevention of Diseases, 6.3% of COVID-19 cases occur in children under 19 years old, although the pediatric population is not likely to develop severe forms of the disease (2). Emerging evidence indicates that, despite initial statements, young children have a great risk of contracting SARS coronavirus-2 (SARS-CoV-2) infection. However, the epidemiology of this illness in the pediatric population, especially in children under five years old, is not clear (3).

Studies have highlighted the connection between viral infection and autoimmunity (4). Autoimmune disease after infection has been reported in adults. Autoimmune hemolytic anemia (AIHA) is one such disease in which antibodies cause hemolysis (5). AIHA can be idiopathic or related to a microorganism infection, such as the Epstein-Barr virus or *Mycoplasma pneumoniae* (6).

Other causes have also been identified, such as drug-dependent and independent antibodies. Drug-dependent antibodies involve hapten-mediated antibodies, which recognize a mixed epitope composed of erythrocyte parts and drug non-covalently bound to red blood cells, which includes penicillin and ceftriaxone. The second group induces AIHA via adsorption and immune dysregulation such as methyldopa and fludarabine (7, 8). Two children who developed AIHA after vaccination were described. The patients were a 20-month-

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old girl and a 21-month-old boy. The girl experienced two hemolytic episodes; one after oral polio vaccination, and the other after mumps, rubella, and measles vaccinations received simultaneously. The boy experienced hemolysis after revaccination against diphtheria-pertussis-tetanus, hepatitis B, *Haemophilus influenzae*, and polio simultaneously (9).

Until now, the majority of AIHA cases secondary to SARS-CoV-2 infection have been reported in adults, with only a few in pediatric patients (5). The following case report concerns an infant who developed AIHA after COVID-19.

CASE REPORT

A 10-month-old male from a rural area of the Caribbean coast of Colombia presented with a clinical picture of a one-month evolution consistent with diminished physical activity and regression of psychomotor skills. The caregiver noticed mucocutaneous pallor. Previously, the patient presented with a dry cough and liquid depositions over an approximate four-day period that coexisted with the beginning of the deterioration of the infant's general well-being. On physical examination, anthropometric measurements were evaluated as 8 kg for weight and 77 cm for height, with z-scores of -1.48 and +1.22, respectively. The z-score for weight-for-length was -3.39, according to child growth standards by the WHO (10). Considering these measurements, the patient was at risk of poor nutrition.

The child also presented with reactive cervical adenopathies, an active respiratory process, and an ejective systolic murmur of grade II/VI secondary to severe anemia. Upon evaluation at the local primary care center, fever was not documented, though several paraclinical studies were performed in which several abnormalities were noted: hemoglobin 4.6 g/dL, mean corpuscular volume (MCV) 113 µm^3, 14% reticulocytes, and lactic dehydrogenase (LDH) positivity. A blood transfusion was performed with packed red blood cells leukoreduced to 10 mL/kg without adverse reactions post-transfusion and with an increment of hemoglobin to 7.2 g/dL, decreasing after 24 hours to 6.6 g/dL. Considering the family history of unspecified anemia in his mother and maternal grandmother, qualitative antiglobulin, anti-IgG, and anti-C3d tests were requested, which showed a positive result. The patient was referred to "Fundación Hospital Infantil Napoleón Franco Pareja" where, due to respiratory and gastrointestinal symptoms, reverse transcription-polymerase chain reaction for SARS-CoV-2 was performed. Extension paraclinical studies were performed (Table 1) to support diagnostic imaging, such as a chest X-ray, which showed evidence of a reticular interstitial pattern without other relevant clinical findings. A thoracic computed tomography scan revealed vascular engorgement and thickening of the perilobular septa (Figures 1a, b). Doppler ultrasound did not indicate anomalies.

A qualitative polyspecific human antiglobulin anti-IgG and anti-C3d direct tests were negative, with an MCV of 106 µm^3, a rise in reticulocytes (17.2%), low hyperbilirubinemia (1.99 mg/dL), high indirect bilirubin (1.46 mg/dL), and elevated glucose-6-phosphate dehydrogenase (16.8 µg/mL). A new transfusion of packed red blood cells was indicated, and COVID-19 was confirmed by molecular testing. Post-transfusion paraclinical results showed qualitative anti-IgG and anti-C3d positivity, elevated LDH (853 U/L), and falsely elevated MCV due to agglutination and reticulocytosis. The patient was treated with nutritional support, 1 mg/kg of prednisone orally per day, and transfusion was indicated to manage the patient's hemoglobin stabilization after three days of treatment (Figure 1c). He was discharged with multidisciplinary outpatient follow-up and oral corticoid medication. Three months later, the patient's weight increased to 9 kg with a z-score of -0.97 and his height to 78 cm with a z-score of +0.16. However, the risk of poor nutrition persisted, as his z-score for weight-for-length was -1.9, according to WHO standards (10). Anthropometric parameters were still

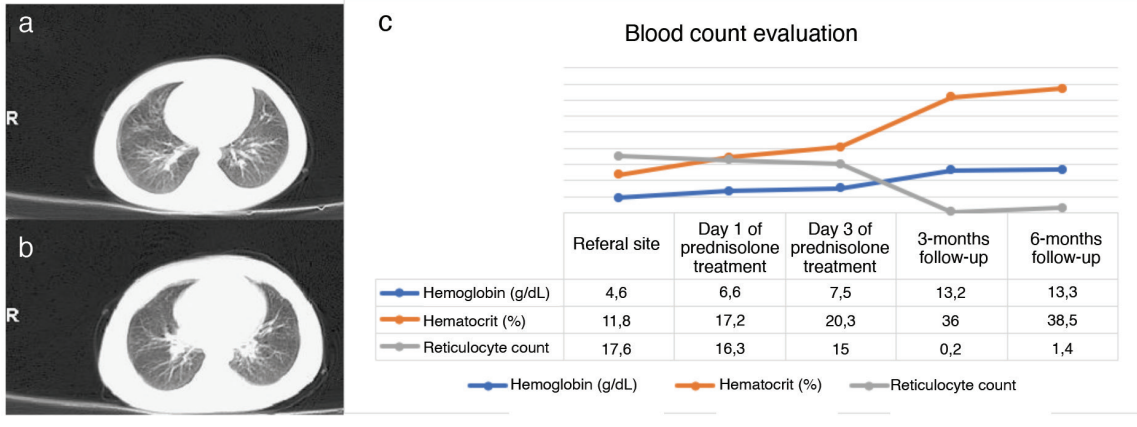

Figure 1: CT scan and evolution in blood count with corticoid therapy. The coronal cut of the CT scan, the parenchymal window, denotes a tendency toward an increase in interlobular septa (a), and vascular engorgement (b). All these findings are described as indeterminate in SARS-CoV-2 infection. The figure shows the evolution in blood count results for hemoglobin, hematocrit, and reticulocyte count before and after corticoid therapy. Hemoglobin and hematocrit levels increased 72 hours after oral corticosteroid therapy. Three and six months after hospitalization, the blood count parameters were normalized with remission of symptoms (c).

SARS-CoV-2: Severe acute respiratory syndrome-coronavirus-2, CT: Computed tomography

recovering six months later, and multidisciplinary follow-up is being continued.

DISCUSSION

In this clinical case, AIHA overlapped with an active SARS-CoV-2 infection without any platelet or coagulation alteration. The qualitative polyspecific anti-human globulin test was negative at admission, which might be a false-negative due to technical difficulties such as cellular concentration, inadequate centrifugation, sample concentration, incubation temperature, or host reasons such as rheumatoid factor, mediated hemolysis for IgA or IgM (6). With this negative result, it was impossible to make a proper diagnosis. The antiglobulin test was repeated, improving the technique, with qualitative anti-IgG and anti-C3d positivity. The initially altered blood count (hemoglobin, hematocrit, and reticulocytes) parameters stabilized between 48 and 72 hours after the use of oral corticoids (Figure 1c). MCV was also normalized.

Other infectious and noninfectious etiologies of AIHA were discarded, as were medications. In this case, the epidemiological nexus was SARS-CoV-2 infection, a cause not considered in pediatric patients and even less in infants.

Hematological complications of COVID-19 are few and primarily related to idiopathic thrombocytopenia purpura and Evans syndrome. The mechanism that mediates the autoimmune response to COVID-19 remains unclear. The possible abnormal expression on the endothelial surface of host protein epitopes has been proposed as a cause (4). Another hypothesis is

Table 1: Blood analysis of the patient.

Paraclinical test for infectious etiology	
Epstein-Barr, VCA IgM antibody	2.7 (negative)
Epstein-Barr, EBNA IgM	0.1 (negative)
Mycoplasma pneumoniae	IgG (-) IgM (-)
Parvovirus B19	IgG (-) IgM (-)
HIV-1 and HIV-2	Non-reactive
Cytomegalovirus (CMV)	IgG (-) IgM (-)
SARS-CoV-2 RT-PCR	Positive
SARS-CoV-2 antibodies	IgG positive IgM negative
VDRL	Non-reactive
Immunoglobulins	
IgA	96 mg/dL (10-100)
IgG	1669 mg/dL (330-1160)
IgM	104 mg/dL (40-170)
Others	
Ferritin	641 ng/mL (7-140)
D-dimer	2.46 mg/mL (<0.25 mg/dL)
Bilirubin	Total bilirubin 1.99 mg/dL Indirect bilirubin: 1.46 mg/dL Direct bilirubin: 0.53 mg/dL
Coagulation time	Prothrombin time test: 12 seconds (control 13) Partial thromboplastin time: 23.7 seconds (control 27.4)
Qualitative antiglobulin test	Negative (admission) Positive (control at 48 hours)
Troponin I	0.004 ng/mL (Negative)
Lactic dehydrogenase (LDH)	853 U/L (170-580)
Erythrocyte sedimentation rate (ESR)	23 mm/h (0-10)
Ureic nitrogen (BUN)	8.1 mg/dL (5-18)
Creatinine	0.55 mg/dL (control 0.46)
Blood smear	Erythroid line: Moderate hypochromia and polychromatophilia, macrocytes +, microcytes ++. Leukocyte line: Without alterations. Platelet line: Without alterations.
Hemoglobin electrophoresis	Hemoglobin: type A: 97.8% type A2: 2.2%
Glucose-6-phosphate dehydrogenase	16.8 ug/mL (3.4-8.8)
Antinuclear antibodies (ANA)	Negative

RT-PCR: Reverse transcription-polymerase chain reaction, SARS-CoV-2: Severe acute respiratory syndrome-coronavirus-2, VCA: Viral capsid antigen, EBNA: Epstein-Barr nuclear antigen, HIV: Human immunodeficiency virus, BUN: blood urea nitrogen

regarding molecular mimicry between ankyrin-1 (ANK-1) and the spike protein, which is the most supported cause of AIHA to date. ANK-1 is a protein that participates in the differentiation and formation of the skeleton of red blood cell membranes. Defects in this protein are associated with hemolytic anemia, such as hereditary spherocytosis; this protein 100% shares an immunogenic-antigenic epitope with the spike protein of SARS-CoV-2 (11).

Immune alteration due to SARS-CoV-2 in severe cases can produce multisystem inflammatory syndrome in children (MIS-C). Moreover, complications such as hemolytic anemia may occur even in children under five years old (3).

Although it has been documented in adults, few pediatric cases have been published before. One study about this condition in pediatric patients included seven patients, four of whom also had B-lymphoid malignancies previously discovered or already diagnosed during hemolytic syndrome (12). Cases such as this one have been reported, but not in children under five years old.

Autoimmune hemolytic anemia is a rare condition in childhood, and the incidence is underestimated. In 2011, a French study reported an annual incidence of 1 to 3 cases per 100,000 people and approximately 0.2 cases per 1,000,000 people under 20 years of age (13). In another study, eight children with systemic complications for COVID-19 were reported, providing evidence of the similarities between the pathology and autoimmune diseases (4, 14). The immune response is mediated by two pathways: warm antibodies and cold antibodies. The most common pathway in pediatric patients is the disease via warm antibodies. Because viral infections have been associated with immune disruption, this entity is deadly, and it is essential for pediatricians to be aware of it; strict multidisciplinary follow-up is required (15).

The parents of this patient were informed about his clinical situation and showed receptivity to the current medical treatment. The patient was discharged with 1 mg/kg/d of prednisone by mouth and poor adherence to management. However, at the 3- and 6-month follow-ups, his anthropometric and neurological state had improved, and normalization of cellular counts and inflammatory markers occurred.

This was a case of an infant without comorbidities who was previously healthy. Anthropometric measurements of the patient one month prior to the disease were in the normal range according to WHO standards (10). The findings suggest an association between COVID-19 and AIHA, making it necessary to explore and consider AIHA as a possible hematologic and immune complication.

It is necessary to clarify the pathophysiological pathways implicated in the dysregulation of the immune system during SARS-CoV-2 infection, such as the molecular mimicry hypothesis. More research on this topic is needed. To date, all AIHA patients with COVID-19 have shown great recovery.

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2023 REFEREE INDEX

2023 AUTHOR INDEX

2023 SUBJECT INDEX

NOTES

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