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Contents

Research letters

Recent advances in the study of the neurobiological mechanisms behind the effects of physical activity on mood, resilience and emotional disorders

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
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Abstract

Physical activity (PA) significantly influences emotional wellbeing, from enhancing mood to counteracting emotional disorders such as depression and anxiety. This article offers an in-depth analysis of the neurobiological processes and theories underpinning the emotional benefits of PA which arise from exercise-induced physiological changes that simultaneously benefit brain function. We discuss the role of growth factors, neurotransmitters and biochemicals, as well as enhancements in mitochondrial biogenesis and antioxidant activity, and how they foster exercise performance and emotional health. Central to our discussion are theories related to depression: the "neurotrophic," "neurogenesis," "inflammation," "oxidative stress," and "monoamine" hypotheses. We also introduce the emergent "glutamate hypothesis" and discuss exercise-induced lactate release as a potential precursor for glutamate. Additionally, we explore the "endorphin" and "endocannabinoid" hypotheses, underscoring their implications in evoking feelings of euphoria, pain relief and diminished anxiety after exercise. In conclusion, PA exerts a diverse influence on brain health and emotional wellbeing. The dynamic interplay between PA and neurobiological processes signals a promising avenue for future research, with the potential to introduce innovative therapeutic strategies for emotional disorders.

Key words: neurotrophic hypothesis, neurogenesis hypothesis, glutamate hypothesis, monoamine hypothesis, endocannabinoid hypothesis

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Introduction

Numerous studies have demonstrated the positive influence of physical activity (PA) on emotional wellbeing,[1–4] including enhancing mood, vigor[5–7] and resilience,[8,9] and its therapeutic effects on depression[10–12] and anxiety.[13,14] Despite the fact that neurobiological mechanisms underlying these benefits remain a dynamic area of research,[9,15–19] comprehensive reviews offering a synthesis of existing knowledge are notably absent. Here, we extend our recent work on the cognitive impact of PA[20] to provide an exhaustive overview of the neurobiological mechanisms of the affective benefits of PA.

From exercise physiology to brain health

We have demonstrated that bodily adaptations that enhance exercise performance also benefit brain function.[20] Specifically, PA triggers the release of biochemicals, including growth factors such as brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), vascular endothelial-derived growth factor (VEGF), lactate, interleukin 6 (IL-6), and neurotransmitters such as dopamine and serotonin (5-HT). Additionally, PA facilitates mitochondrial biogenesis and bolsters antioxidant enzyme activity. These adaptations facilitate lipid and carbohydrate metabolism, and enhance tissue and blood vessel growth and repair, leading to optimized energy production. Both dopamine and 5-HT play vital roles in motor control, and together with growth factors, mitochondrial biogenesis and antioxidant enzyme activity, help mitigate central fatigue. Collectively, these adaptations bolster endurance and exercise performance.

Interestingly, physiological adaptations also confer brain benefits (Fig. 1). Growth factors cross the blood–brain barrier (BBB) and function as nutrients for neurons and blood vessels, facilitating enhanced angiogenesis and adult hippocampal neurogenesis, and increasing cerebral blood volume, dendritic spines, brain volume, and long-term potentiation (LTP). Lactate crosses the BBB and acts as an energy source for neurons and a precursor for the predominant excitatory neurotransmitter, glutamate, thereby augmenting LTP. Interleukin 6 activates IL-1 receptor antagonist and IL-10, which initiate anti-inflammatory actions, including reduced production of IL-1β, IL-8 and tumor necrosis factor alpha (TNF-α). Enhanced mitochondrial biogenesis and antioxidant enzyme activity decrease reactive oxygen species (ROS) production and oxidative stress. While chronic inflammation and oxidative stress impair the BBB, causing neuroinflammation, microglia activation and neuronal damage, PA protects against these effects (see Chen and Nakagawa[20] for details).

The multifaceted role of physical activity in boosting resilience and alleviating depression

Remarkably, physiological adaptations to PA play a significant role in promoting emotional wellbeing. The so-called "neurotrophic hypothesis"[21,22] and "neurogenesis hypothesis"[23,24] have established the crucial role of growth factors and adult hippocampal neurogenesis in resilience and depression. Essentially, both neurogenesis and growth factors diminish under chronic stress and depression, yet are restored or rejuvenated by antidepressants and PA. Moreover, growth factors are essential for adult neurogenesis, which mediates pattern separation, thus contributing to cognitive flexibility and resilience.[25,26] Growth factors also enhance the growth of dendritic spines, the primary locations for synaptic inputs to neurons. As such, PA increases dendritic spine density in the hippocampus and the prefrontal cortex (PFC).[27,28] In tandem, the "inflammation hypothesis"[29–31] and the "oxidative stress hypothesis"[32,33] have garnered considerable interest. Chronic stress triggers inflammation and oxidative stress that contribute to depressive and anxiety disorders. Antidepressant treatment and PA, however, reduce these effects. A potential mediator of PA-enhanced mitochondrial biogenesis is the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α),[34] which further regulates the kynurenine pathway, another mechanism through which PA reduces depression.[35–37]

Regarding neurotransmission, the "monoamine hypothesis," which proposes that antidepressants act on serotonin, noradrenaline and dopamine, has been a dominant theory for over half a century.[38] It posits that depression involves the depletion of these monoamines, and that agents increasing them alleviate depression. However, it does not explain the latency in depressive symptom relief,[39] prompting the emergence of the "glutamate hypothesis."[40] The alterations in glutamate and its metabolites identified in various brain regions of depressed patients, along with the rapid antidepressant effects of ketamine (a glutamate N-methyl-D-aspartate (NMDA) receptor antagonist), provide compelling evidence for this hypothesis. Notably, ketamine induces transient activation of glutamate neurotransmission in the PFC via gamma-aminobutyric acid (GABA) interneuron disinhibition, followed by a sustained increase in PFC synaptic connectivity.[41,42]

Physical activity not only boosts the release of dopamine and serotonin in the brain, but also increases the levels of lactate crossing the BBB, which serves as a glutamate precursor.[20,43] As per our previous work, voluntary wheel running increased prefrontal dopamine in rats,[15] which

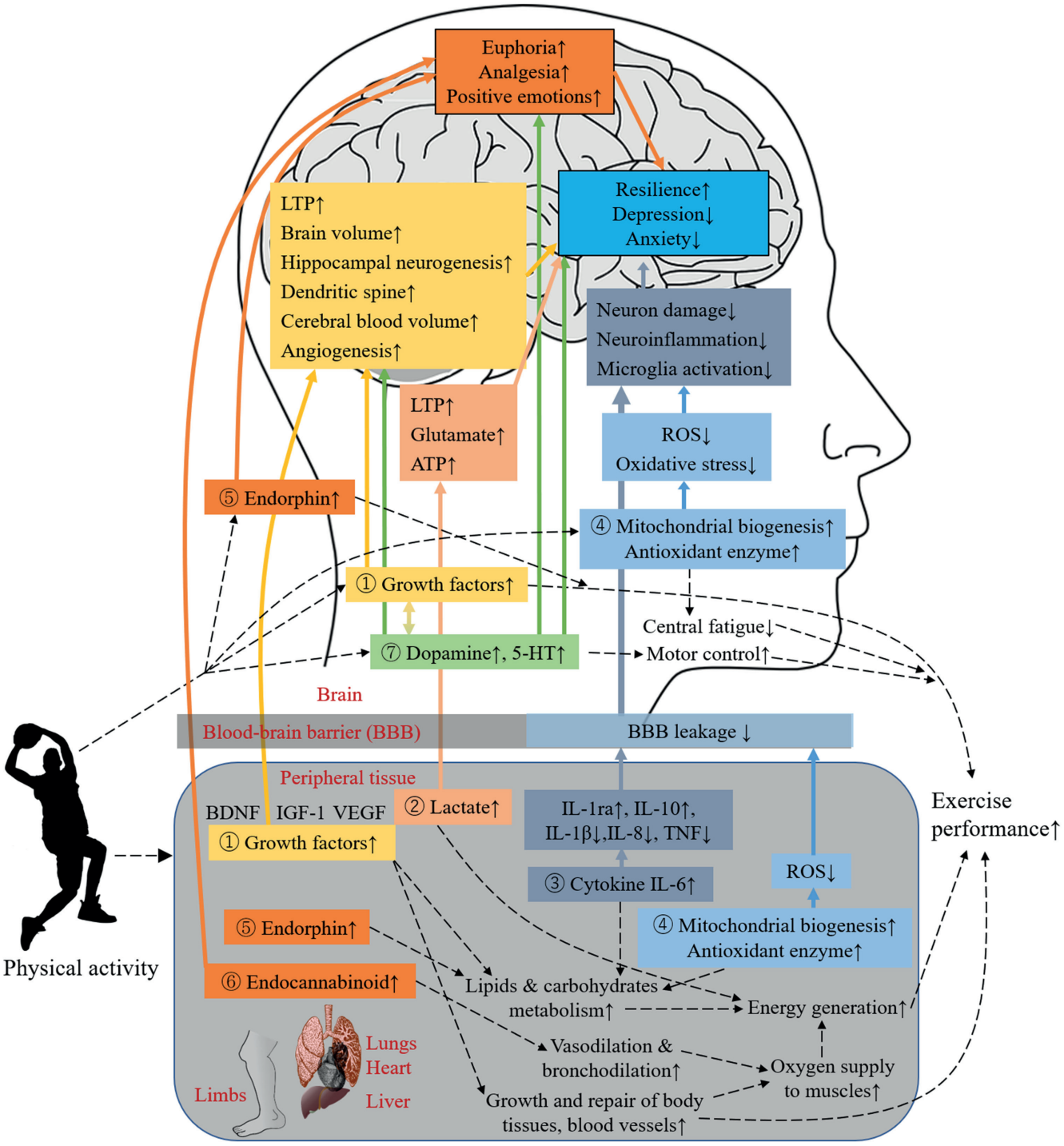

Fig. 1. A schematic illustration of exercise physiology and the neurobiological mechanisms through which physical activity augments emotional well-being. During physical activity, the body's physiological adaptations that are primarily aimed at improving exercise performance (represented by dashed black arrows) serve a dual purpose, as they also confer significant benefits to the brain, consequently enhancing emotional wellbeing (depicted with solid colored lines with each pathway distinguished by a unique color). Adapted from Chen and Nakagawa[20]

ATP – adenosine triphosphate; BBB – brain–blood barrier; BDNF – brain-derived neurotrophic factor; IGF-1 – insulin-like growth factor-1; IL-6 – interleukin 6; LTP – long-term potentiation; ROS – reactive oxygen species; 5-HT – serotonin; TNF-α – tumor necrosis factor alpha; VEGF – vascular endothelial-derived growth factor.

aligns with the functional role of prefrontal dopamine in working memory, with PA linked to heightened cognitive flexibility[6,44,45] and resilience.[15,46] Physical activity also raises dopamine levels in the midbrain and striatum,[47,48] which encode reward prediction errors and fuel action vigor.[49–51] Using similar and relevant mechanisms,[52–54] PA helps reduce anxiety, a condition commonly co-occurring with depression.[55–58]

Physical activity, endorphins, endocannabinoids, and positive emotions

Alongside the aforementioned mechanisms, the body also releases endorphins (a term derived from "endogenous" and "morphine") and endocannabinoids (an endogenous cannabinoid) during PA. Endorphins facilitate lipid and carbohydrate metabolism, while endocannabinoids promote vasodilation and bronchodilation, enhancing oxygen supply to muscles. These biochemicals augment exercise performance and deliver affective benefits, as captured in the "endorphin hypothesis"[59,60] and the "endocannabinoid hypothesis."[61]

The "endorphin hypothesis" was the initial theory to elucidate the phenomenon of "runner's high" or feelings of euphoria, pleasantness and analgesia after PA. Research has illustrated a strong correlation between changes in β-endorphin plasma concentrations after PA and shifts in feelings of pleasantness (Rho = 0.738, $p < 0.001$).[62] This hypothesis, however, has faced criticism since β-endorphin cannot cross the BBB to activate the μ-opioid receptors responsible for euphoria.[63] Despite this, subsequent research implies that PA may directly augment opioid binding in prefrontal/orbitofrontal cortices and the anterior cingulate cortex, thereby eliciting euphoria.[64]

The "endocannabinoid hypothesis" is an alternative theory that has garnered increasing attention, as peripheral endocannabinoids cross the BBB, activate cannabinoid receptors, and evoke feelings of euphoria and reduced anxiety.[65] The PA-induced changes in anandamide, a particular endocannabinoid, correlate with shifts in positive emotions ($r = 0.96$, $p < 0.0001$).[66] Moreover, genetic ablation of cannabinoid receptors on GABAergic neurons eliminates PA-induced anxiolysis, while pharmacological inhibition of central and peripheral cannabinoid receptors blocks analgesia in mice.[67]

Conclusions

The complex interactions between PA and the neurobiological mechanisms of affective benefits offer a rich, multifaceted field of study. Our comprehensive overview provides a robust framework for further research, one that may hold the key to developing novel therapeutic approaches for the treatment of emotional disorders and the promotion of emotional wellbeing.

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Lecanemab (Leqembi) is not the right drug for patients with Alzheimer's disease

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Abstract

On July 6, 2023, the U.S. Food and Drug Administration (FDA) approved lecanemab (Leqembi) for the treatment of Alzheimer's dementia (AD) patients. In 2 clinical trials, lecanemab reduced amyloid in the brain and slowed cognitive decline. Here, I review in detail the clinical trial by van Dyck et al. (2023) entitled "Lecanemab in early Alzheimer's disease", published in *The New England Journal of Medicine* on January 5, 2023. In this 18-month trial, lecanemab did not slow cognitive decline in women. This is especially significant because women have a twofold increased risk of AD compared to men, that is, there are 2 times more women than men living with AD. Lecanemab did not slow cognitive decline in *APOE4* carriers; rather, it enhanced the decline in study participants with 2 *APOE4* genes. This is bad news for AD patients, 60–75% of whom carry at least 1 *APOE4* gene. These negative results regarding lecanemab's therapeutic value make me wonder if the approval of lecanemab was the worst decision of the FDA up till now, after the approval of aducanumab on June 7, 2021.

Key words: immunotherapy, clinical trial, Alzheimer, lecanemab, *APOE4*

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Introduction

Lecanemab (branded Leqembi by Eisai and Biogen) is a monoclonal antibody targeting oligomeric Aβ peptides, which prevents amyloid formation.[1] In 2 clinical trials, lecanemab reduced amyloid deposits in the brain and slowed cognitive decline in early Alzheimer's dementia (AD) patients, as reported in 3 papers.[2–4] On July 6, 2023, The U.S. Food and Drug Administration (FDA) approved lecanemab for the treatment of AD patients,[5] following the Advisory Committee's endorsement by a 6-0 vote on June 9, 2023. In this commentary, I review the clinical trial study by van Dyck et al.[4] "Lecanemab in early Alzheimer's disease", published in *The New England Journal of Medicine* on January 5, 2023 (online November 28, 2022), by a way of telling two stories called "Apples and Oranges" and "*APOE4*". Further, I opine on the unusual and less than transparent fashion, to say the least, on how the results were disclosed, presented, interpreted, and discussed in van Dyck et al.'s paper.

Objectives

The purpose of the study was to review, re-analyze and re-interpret the clinical trial results of lecanemab that can be found in the paper and supplementary appendixes published by van Dyck et al.[4] Additionally, I aim to question the lecanemab's estimated 27% clinical benefit in slowing AD progression, when a better estimate is 9.3%, and to question the treatment of men and women as one 'statistical' population, when their responses to lecanemab were too different to happen by chance.

Apples and oranges

Table 1, which is adapted from Figure S1B (van Dyck et al., Supplementary Appendix),[4] shows mean changes of CDR-SB (Clinical Dementia Rating–Sum of Boxes) scores from baseline to 18 months and percent slowing of cognitive decline for men and women with and without lecanemab. Now, let us define (Equation 1):

$$x - y = z \quad (1)$$

Table 1. CDR-SB score change with and without lecanemab

APOE4	Number of participants (placebo, lecanemab)	Adjusted mean change	Percent slowing of decline
–/–	275, 267	–0.75	41
–/+	468, 456	–0.50	30
+/+	132, 136	0.28	–22
Female	446, 441	–0.20	12
Male	411, 416	–0.73	43

The content of the table is adapted from van Dyck et al. (Figure S1B, Supplementary Appendix).[4] Adjusted mean change (from baseline to 18 month) is the Clinical Dementia Rating–Sum of Boxes (CDR-SB) score difference of the lecanemab minus the placebo-treated group, negative difference indicating benefit of lecanemab treatment. The CDR-SB measures cognition and function on an 18-point scale, higher scores indicating worse performance.[6] Percent slowing of decline (of cognition and function) is calculated as score difference divided by placebo score.

where x indicates the lecanemab score, y stands for the placebo score and z represents the difference.

Let us define (Equation 2)

$$z/y = \% \text{ slowing} \quad (2)$$

where score difference divided by placebo score (multiplied by 100) gives percent slowing of cognitive decline with lecanemab compared to placebo.

From the data in Table 1, we can now calculate (*calculemus*, as Leibniz would say) x- and y-scores for men and women, as shown below

MEN 47.7%	**WOMEN** 52.3%
x – y = –0.73	x – y = –0.20
0.73/y = 0.43	0.20/y = 0.12
x = 0.97	x = 1.47
y = 1.70	y = 1.67

And from these values, we can calculate men (47.7%) plus women (52.3%) combined weight adjusted x- and y, as follows

MEN+WOMEN

x = 0.477 × 0.97 + 0.523 × 1.47 = 1.23

y = 0.477 × 1.70 + 0.523 × 1.67 = 1.68

The combined score difference 1.23 – 1.68 = –0.45 is the difference between the lecanemab and placebo groups reported by van Dyck et al.[4] It can also be derived from weigh adjusted score differences:

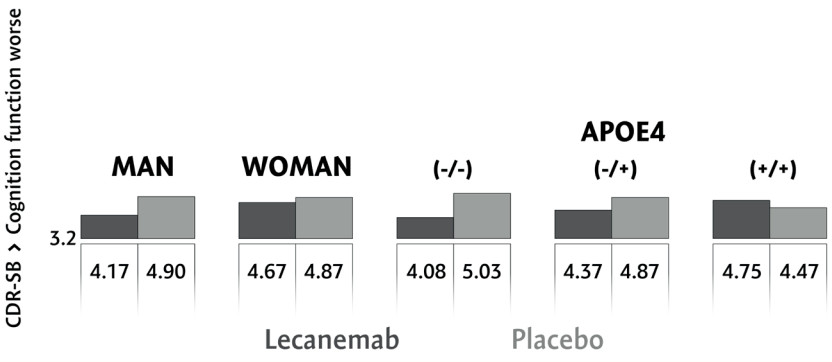

Fig. 1. Lecanemab does not work for women and enhances cognitive decline of *APOE4* (+/+) carriers.

Numbers show CDR-SB score at the end of 18-month trial. Dark grey (lecanemab) and light gray (placebo) bars show score increase from baseline (3.2). The –0.73 difference for men indicates 14.9% (0.73/4.90) less disease progression and cognitive decline with lecanemab compared to placebo, and the –0.20 difference for women indicates 4.1% (0.20/4.87) less decline. Score differences for the *APOE4* carriers indicate 18.9% (–/–), 10.3% (–/+) and –6.3% (+/+) less cognitive decline

0.477 × (–0.73) + 0.523 × (–0.20) = –0.455.

And finally, z/y = 0.45/1.68 = 26.8% slowing.

Accordingly, for men plus women, the score change was 1.23 with lecanemab and 1.68 without lecanemab, with a difference of –0.45. Note how these values are described in the paper: "The adjusted least-squares mean change from baseline at 18 months was 1.21 with lecanemab and 1.66 with placebo (difference, –0.45; 95% confidence interval [CI], –0.67 to –0.23; p < 0.001)."[4]

Importantly, although not explicitly stated, 1.21 and 1.66 are not measured values of the study population with and without lecanemab but are calculated (the way I did above) as a weight-adjusted change from the data for men and women. Isn't this like comparing apples and oranges? Clearly, a –0.73 difference for men and a –0.20 for women (Table 1) are too different to originate (statistically) from the same population. Therefore, I suggest that 1.21, 1.66 and –0.45 do not represent any population, do not characterize anybody, have no meaning, and are useless values.

Now, let's look at Fig. 2 in the van Dyck et al.'s paper,[4] which shows data on the aggregate of brain amyloid positron emission tomography (PET) and several other cognitive measures as an adjusted mean change from baseline in the study populations with and without lecanemab. Similar to the CDR-SB score changes discussed above, those cognitive measures apply to nobody or no group in particular, and therefore are useless and irrelevant in the real world.

Further, although not stated in the paper, commentaries and popular media have interpreted the –0.45 difference as a 27% (0.45/1.66) less cognitive decline in the lecanemab group compared to the placebo group. This is a very trivial miscalculation. The correct value is 9.3% (0.45/4.86), which pays attention to the 3.2 baseline, as observed in Kurkinen et al.[7] The 9.3% less cognitive decline is unlikely to make any difference for people living with early AD.[8]

APOE4

The *APOE4* is the strongest and most common inherited genetic risk factor for AD. Since 1993, it has been known that *APOE4* enhances the development and progression of AD. This conclusion can be deduced from the observation that *APOE4* lowers the age of onset and increases the risk of AD.[9–11] One *APOE4* gene (12% of the population) increases the AD risk by twofold and 2 genes (2%) increase the AD risk by 12-fold compared to *APOE3* carriers (68%).

From the data in Table 1, we can calculate x and y values for the *APOE4* carriers (not shown), and then on top of baseline (3.2), the scores at the trial end (the scores actually measured), as shown in Fig. 1. Note that lecanemab increases CDR-SB scores (making cognition and function worse) in the (–/+) and (+/+) carriers compared to (–/–) carriers, and even enhances cognitive decline of the (+/+) carriers compared to placebo. This has implications for the treatment of AD patients, 60–75% of whom carry at least one *APOE4* gene.[10] This is also bad news for the prevention therapy of individuals at high risk of developing AD associated with 1 (12% of the population) or 2 copies of the *APOE4* gene (2%).

Strangely enough, the *APOE4* gene appears to decrease CDR-SB scores (slowing cognitive decline) in a dose-dependent fashion in the order of (+/+) > (–/+) > (–/–). It has not escaped my attention that between the (+/+) and (–/–) carriers, the score difference is 4.47 – 5.03 (1.27 – 1.83 as increase from baseline) = –0.56, or 11% (0.56/5.03) or 31% (0.56/1.83) slowing of cognitive decline. The difference –0.56 and 31% are better than –0.45 and 27%, the most frequently mentioned clinically meaningful benefit of lecanemab in the treatment of patients with AD.

Wovon man nicht sprechen kann...

The van Dyck et al.'s paper[4] has major problems in how the results are presented, interpreted and discussed. The most conspicuous feature is the lack of reporting the negative results in women and *APOE4* carriers, the majority of the study participants (Fig. 1).

Lecanemab did not work for women (52.3% of the study population), and the CDR-SB score difference between the lecanemab and placebo groups was –0.20 (compared to –0.73 for men). This is especially significant, because women have a 2 times higher AD risk, that is, there are 2 times more women than men living with AD. These data can be found only in Figure S1B in the Supplementary Appendix.[4] Why were these data not disclosed, not even discussed, in the paper? Indeed, the words "man" and "woman" or "male" and "female" were not used, not even once, in the paper.

Lecanemab enhanced cognitive decline in study participants (15.5%) carrying 2 *APOE4* genes, and the CDR-SB score difference between the lecanemab and placebo groups was 0.28. Remarkably, *APOE4* was found to slow cognitive decline in a dose-dependent fashion, in the order of (+/+) > (–/+) > (–/–), so much that the score difference between the (+/+) and (–/–) carriers was –0.56 or a 31% less decline. These values report better outcomes than –0.45 or 27%, the most frequently mentioned clinically meaningful benefit for AD patients.

Why these results were not disclosed in the abstract, results or discussion of the paper, but were hidden in Figure S1B and passed over in silence? Is it because the data would not stand the light of day? Why these data were not brought up and discussed in public, rather than discussed among "insiders" at Clinical Trials on Alzheimer's Disease (CTAD) conference (San Francisco and online, November 29–December 2, 2022)?[12] It seems ironic that nobody was willing to look at the data in Figure S1B and see the forest for the trees, especially since Fig. S1B also displays data in graphs called, of all things, forest plots.

This strange practice of select reporting of the data and bordering on obstruction of science has resulted in news and commentaries that have only misinformed the public about the lecanemab study and lecanemab's clinical benefit, without any hint of the lack of benefit. Only recently, these and other issues noticed in the van Dyck et al.'s paper[4] were raised in the 4 "Letter to Editor" letters published in *The New England Journal of Medicine* on April 27, 2023.[13] One of the letters was from Valenzuela and Pascual-Leone, who write: "We are concerned about the possible lack of therapeutic efficacy among women in the trial by van Dyck and colleagues."

Discussion

Aducanumab is a monoclonal antibody against a conformational epitope found on Aβ peptides.[14] On June 7, 2021, the FDA approved aducanumab (branded Aduhelm by Biogen) for the treatment of AD. This controversial decision, which was against the FDA's Advisory Committee's 10-0 vote not to approve aducanumab, resulted in a congressional investigation concerning inappropriate contact between the FDA and Biogen during the approval process.[15,16] As of today, Aduhelm has not been approved anywhere else in the world, except the UAE.

In AD trials, several monoclonal antibodies against Aβ peptides, oligomers, fibrils, and amyloids have reduced brain amyloid deposits as detected using PET imaging, but they have not slowed cognitive decline. On the contrary, their use has consistently resulted in significant health problems caused by adverse events due to amyloid-related imaging abnormalities (ARIA), as seen on magnetic resonance imaging (MRI) pictures. They have been associated with brain edema and brain hemorrhages that can be fatal.[17–23] For example, in the lecanemab trial,[4] the most common adverse events were infusion-related reactions (26.4% with lecanemab and 7.4% with placebo), ARIA-H with microhemorrhages, macrohemorrhages, or superficial siderosis (17.3% with lecanemab and 9.0% with placebo), and ARIA-E with brain edema (12.6% with lecanemab and 1.7% with placebo).

Brain amyloid removal using monoclonal antibodies that bind to soluble oligomeric forms of Aβ peptide, but not to deposited β-sheet fibrillar forms of Aβ amyloid plaques, immediately raises 2 questions. How does the amyloid get removed from the brain as measured with amyloid PET imaging and what does the amyloid PET measure? The Aβ amyloid is found in the cortex (a 1.55–3-mm thick tissue made of neuron cell bodies) and around microvasculature associated with cerebral amyloid angiopathy (CAA), which is present in 90% of AD patients.[24] These areas of amyloid deposition are very different than what can be observed with PET imaging. Indeed, as argued by Høilund-Carlsen et al., the specificity and sensitivity of PET in measuring Aβ amyloid in the brain have not been proven.[25]

The idea to use anti-Aβ antibodies in AD immunotherapy is based on the amyloid hypothesis,[26,27] which proposes that Aβ peptide amyloid formations in the brain cause AD. Thus, the hypothesis predicts that the removal of brain amyloid provides a treatment for AD patients, and preventing brain amyloid formation inhibits the development of AD.[28] Ever since its formulation in 1991–1992, the amyloid hypothesis has almost singularly misguided AD research, drug development and clinical trials. The amyloid hypothesis has been tested in hundreds of clinical trials and shown to be wrong. While a hypothesis can never be proven right, it can be proven wrong, in theory or by experiment.[29]

Limitations

This study is a review and analysis of the clinical trial results published by van Dyck et al.[4] and has the same limitation as the original study. That is, the 1734 study participants (47.7% men, 52.3% women) were treated as one statistical population, which turned out not to be true. For example, the CDR-SB score change in men was 0.97 with lecanemab and 1.70 without, a difference of −0.73, and for women, it was 1.47 with lecanemab and 1.67 without, a difference of −0.20. The −0.50 difference between men and women in the lecanemab group is more than the −0.45 weight-adjusted mean difference of the men+women population with and without lecanemab. As I have suggested in this study, −0.45 does not characterize anybody, because men with a −0.73 difference and women with a −0.20 difference are too different.

Conclusions

In the USA, 6.7 million people are living with AD, and there are 50 million AD patients worldwide.[30] For the treatment of AD, the FDA has approved aducanumab and lecanemab, 2 anti-Aβ antibodies, and I guess, next year comes donanemab, an antibody against the pyroglutamate form of Aβ found only in amyloid plaques.[31,32] These very controversial decisions by the FDA have not been supported by evidence-based science, and clearly do not "promote, protect, and ensure the full enjoyment of human rights by persons with disabilities", as articulated in the United Nations Convention on the Rights of Persons with Disabilities (CRPD), May 3, 2008, that is in force in 186 nations.

Understanding and the treatment of disease go hand in hand. Despite decades of research efforts in academia and the drug industry and hundreds of clinical trials, we have no treatment or prevention for AD. Why is that? The short answer is that we do not understand AD, its origin and disease mechanisms.[33,34]

It is fair to say the amyloid hypothesis is the reason we have no disease-modifying treatment for AD.[35] Therefore, in the spirit of "prevention is the only cure", we need

to invest more into the research on preventive therapies as well as increase public awareness of the role healthy lifestyles can play in delaying the onset of AD.[36–38]

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In a blink of an eye: Graphical abstracts in *Advances in Clinical and Experimental Medicine*

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Abstract

This editorial discusses graphical abstracts (GAs) as a relatively new tool used to concisely summarize a scientific paper and promote it on social media to boost the visibility of research and the number of citations. This article attempts to define GA as clearly as possible and to explain the role of GAs as scientific communication tools in medical journals.

A clear definition of a GA is lacking. Several definitions from the literature are presented, which illustrates that the terms "visual abstract" and "graphical abstract" can be used interchangeably. The role of GAs can be described in 3 aspects: 1) time required for communication (GAs are meant to convey the key contents of a scientific paper in a time much shorter than required for reading the full text), 2) means of communication (social media), and 3) mechanism of communication (research results in many fields of medicine can be better conveyed through visual or at least more visual means rather than plain text).

A review of the existing literature concerning the effectiveness of GAs presents studies regarding the use of GAs in promoting scientific papers on Twitter – visual abstracts attracted significantly more engagement than plain English ones, especially from medical professionals. Visual abstract tweets were associated with a significantly higher number of impressions, retweets, and link clicks compared to text abstract tweets. Journals that have introduced GAs demonstrated significantly higher impact factor (IF) increases for the past 3 years than those of journals without GAs. The longer GAs have been utilized in a journal, the higher the IF the journal had.

The experience of the editors of *Advances in Clinical and Experimental Medicine* (ACEM) concerning GAs are discussed, divided by types of papers published in this journal (original papers, meta-analyses, reviews, research-in-progress articles, and editorials), illustrated with examples of well-prepared GAs, and supplemented with a brief description of the feedback from authors and readers amassed following the introduction of GAs in ACEM. Finally, the authors offer the readership of ACEM 8 practical tips on how to prepare a useful GA, and list 8 common mistakes and misconceptions regarding GAs – both in text form and summarized in tables.

The conclusion of the paper is that there is currently no universal standard for GAs, which can lead to inconsistencies in their formats and content; therefore, more detailed guidelines to standardize GAs for scientific research are warranted.

Key words: graphical abstract, visual abstract, social media, dissemination of science, visual communication

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Introduction

With thousands of scientific papers published every day, strong competition involves not only their scientific merit but also visibility. It can be easily imagined that an important paper is simply overlooked by both specialists in the field and the general public, because it becomes overlapped by other publications momentarily after its release. Therefore, both authors of papers and editors of scientific journals strive to enhance the visibility of published research – among peers and the general public. The present editorial concerns primarily the former target group. Such visibility encompasses both reading the paper online and downloading it from the journal's website, as well as posting information about it on social media (mainly Facebook, Twitter and LinkedIn).

Along with the rise of social media, it became apparent that "visibility" in this context should not be understood only metaphorically, since content with visual elements (illustrations, videos) is much more popular among the users of such media and attracts broader audiences than plain text only. Such rise has been scientifically observed already in 2011 when Eysenbach showed that tweets presenting or citing already published papers can predict highly cited articles within the first 3 days of article publication,[1] while subsequent research by Fox et al. in 2015–2016 did not confirm a direct relationship between promoting papers on social media and the number of views or citations.[2,3] Some of the more recent studies show that a positive correlation between exposure to social media and article citations indeed exists, as argued, e.g., by Özkent.[4] Studies analyzing the efficacy of scientific communication using social media and offering suggestions on how to optimize its usage have appeared (e.g., Habibi and Salim analyzed static compared to dynamic methods of delivery for scientific communications on Twitter and TikTok).[5] Research concerning the potential of social media as a means for competence development and professional communication among practicing physicians has also been performed, as evidenced in a study by Khan et al., who assessed the effects of social media usage among healthcare providers.[6] In some scientific medical journals, already in 2016, a dedicated position of social media editor was present among the editorial staff. Their responsibilities, as well as goals and barriers to their position, were studied by Lopez et al.[7] Qualitative analysis of scientific communication on social media has also commenced: Pandey et al. chose 6 popular Facebook pages presenting scientific materials and performed their content analysis.[8] The bibliometric approach has also been implemented in such research: Yeung et al. analyzed the presence of medical scientific literature on Twitter to obtain quantitative information on the dominant research topics, trending themes, key publications, scientific institutions, and prolific researchers.[9]

According to an anonymous editorial published in *Nature Chemistry*,[10] single-panel graphics presenting key issues described in the articles were employed as a regular feature in 1976 in the West German *Angewandte Chemie* chemistry journal, and then again in 1977, in the international edition of the same journal. In 1986, another chemistry journal adopted this idea (*Tetrahedron Letters*), but until recently, this trend concerned only single journals (e.g., *Chemical Communications* in 1994 and *Journal of the American Chemical Society* in 2002) among many, and only in chemistry – a field in which visual representation of findings is unavoidable.[10] For medical scientific periodicals, the adventure with visual/graphical abstracts began in 2016 when Andrew M. Ibrahim, chief editor of *Annals of Surgery*, decided to implement visual abstracts in this journal to improve the dissemination of publications, primarily on social media. Of note, Ibrahim and his editorial team produced the visual abstracts themselves after the papers were accepted for publication. As the practice of using such abstracts has been adopted by more and more medical journals, some editorial offices decided to prepare visual/graphical abstracts for papers that qualified for publication. However, others required that the author design them. As of now, there are no studies discerning how many journals stipulate that visual/graphical abstracts are mandatory and in how many journals they remain only an option for authors who wish to boost the circulation of their work.

Objectives

This editorial has the following aims: 1) to present a definition of a visual/graphical abstract as clearly as possible, 2) to explain the role of visual/graphical abstracts in scientific communication using the example of medical journals, 3) to summarize the most important rules for creating a clear visual/graphical abstract formulated by professional graphic designers, 4) to review existing literature concerning the effectiveness of visual/graphical abstracts, 5) to discuss the experiences of the editors of *Advances in Clinical and Experimental Medicine* (ACEM) concerning visual/graphical abstracts and present remarks based on these experiences, and 6) to offer the readership of ACEM practical tips on how to prepare a useful visual/graphical abstract.

What is a graphical abstract?

A clear definition of a graphical abstract (GA) is lacking; moreover, there seems to be a terminological ambiguity concerning 2 expressions: "graphical abstract" and "visual abstract". For example, Hoffberg et al. treated a visual abstract as a subset of GAs but neglected to explain the difference between them.[11] Ibrahim consequently refers to visual abstracts,[12] and the same term is used by Chapman et al.,[13] Klaassen et al.,[14] Aggarwal,[15] Stahl-Timmins et al.,[16] Lindquist and Ramirez-Zohfeld,[17] Chisari et al.,[18]

and Ramos and Concepcion.[19] However, in more recent literature, the type of abstract in question is more frequently called a GA, as seen in studies by Kim et al.,[20] Zong et al.,[21] Pferschy-Wenzig et al.,[22] Lee and Yoo,[23] and Bennett and Slattery.[24] However, no reason has been provided for this shift. Also, in a primer by Ibrahim et al. on the professional creation of such materials, they are called visual abstracts.[25] In other sources – both scientific and popular – these 2 terms are used alternately, and it can be safely assumed that the authors deem them interchangeable. For the sake of clarity, in this editorial, only the term "graphical abstract" will be consequently used. The reason for adopting this term in ACEM was to avoid confusion since some readers can understand a visual abstract as any abstract that uses visual means of conveying the message – i.e., a video abstract or a key figure would also be a visual abstract.

Graphical abstract is described as a visual representation of the key messages of a research paper,[23] a visual representation of the key findings typically found in the abstract portion of an article, enabling the reader to quickly grasp the key findings and take-home message,[12] a visual summary of the paper's main content,[20] a single, concise, pictorial and visual summary of the main findings of the paper,[26] or a visual distillate of the take-home message of an article into an image that is not too cluttered, somewhat eye-catching and relatively simple to interpret.[10] It is also stressed that such perception should be possible at a single glance,[22] which we understand as follows: the GA should be comprehensible in its entirety when viewed in full-screen mode. It is important to note a significant difference in the above definitions. While some authors point out that the GA is a summary of only the text abstract of a scientific paper, others underline that the GA presents the whole article in a single-panel illustration. From the experience of the editors of ACEM, it transpires that such differentiation is purely academic. If the plain English abstract is well prepared (i.e., it reflects the structure of the paper and concisely presents its main materials and methods, results, and conclusions), the GA based on both solely the text abstract and full text of the paper will contain all required information, while a GA based on an incomplete abstract will also be incomplete in terms of content.

Role of GAs in scientific communication

The role of GAs can be described in 3 aspects: 1) time required for communication, 2) means of communication and 3) mechanism of communication. Regarding the 1st factor, the basic function of a GA is similar to that of plain text one – it is meant to convey the key contents of a scientific paper in a time much shorter than required for reading the full text. The main difference when compared to text abstracts is that GAs are more varied regarding the degree to which they attract attention and can "make readers pick out one's article from a plethora of potentially interesting literature", as put by Pferschy-Wenzig et al.[22] Therefore, they not only help spare time but also direct the attention of the reader. Moreover, the "single glance" concept – when implemented expertly – may allow further shortening of the time required for comprehending the abstract. The function of GA and plain-text abstract is therefore virtually identical: They both do not substitute the whole paper but convey its main content at the same time enticing to read the whole article. The latter function is even more important regarding GA since its potential in "avertising" the paper is broader. A GA should truly summarize the paper, not only complement it.

Means of communication are – in this context – the channels through which GAs are disseminated, i.e., the journal's website and social media. Social media (particularly Twitter, Facebook and Instagram) favor visual elements of the message, with longer texts often offered only as a URL to access material for further reading. Twitter restricts the length of a single tweet to 260 characters, while Instagram offers the possibility of providing text only as a description of an image, which the user has to click to view the description. Social media accounts of scientific journals are perceived as a powerful means of disseminating knowledge and promoting individual papers to encourage a broader audience to read them. It should be noted that social media is used both by professional circles and larger audiences, so using GAs in such media does not necessarily mean that GAs must be understood as a means of disseminating the results of scientific research among the general public. A potential for the latter always exists, but popularizing science among lay people requires specifically dedicated design of GAs, often resembling infographics encountered, e.g., in public healthcare facilities.

The 3rd aspect is particularly visible in the branches of science in which the GAs have been implemented so far – and medicine is a particularly good example. In many fields of medicine, research results can be better conveyed through visual or at least more visual means rather than plain text – tables, charts, graphs, schemes, flowcharts, and similar tools. In many scientific medical papers, visuals constitute the core of the publication, with the text being more of a commentary. If the same message were to be only in text form, it could become virtually incomprehensible or at least confusing. Although complex information presented using these tools must be (to some extent) simplified to fit a GA, a GA as an idea is still in concert with the ways of depicting scientific investigation in modern journals.

Limitations of GAs

Based on the ACEM editors' experience with GAs, several limitations and challenges connected with such a form of science dissemination can be identified. The main

limitation results from the restricted size of a GA – even the most creative researcher would be unable to fit very complex content into a design that would be comprehensible when viewed in full-screen without enlarging respective sections of the picture. Therefore, it can be assumed that there are papers that can be summarized using a GA only to a certain extent, albeit the ACEM editors encountered not a single situation when a given paper was unsuitable to be accompanied by a GA at all. Some general terms may be represented only symbolically – and inventing unambiguous symbols without additional commentary may be challenging. In many medical papers, the results are expressed as results of multi-step statistical calculations, and the final outcomes cannot be concisely formulated in a single sentence or picture. This is the case especially in manuscripts of a very limited scope (e.g., relations of expression of certain gene polymorphisms with certain tumors), but examining multiple problems at the same time (e.g., 20 polymorphisms in 3 types of cancer). In such publications, the possibility to express the results and conclusions concisely is limited, even in plain-text abstracts, not to mention GAs.

The most significant challenge when preparing a GA is the danger of oversimplification, and – in consequence – misrepresentation of the article content. Some authors seem to assume that a visual depiction of some key terms used in the paper and connecting them with lines or arrows will provide an appropriate visual summary of the manuscript, while a GA designed that way may easily create an impression that the terms in question are in quite different relationships than in reality. Misrepresentation may occur also when oversimplification has been avoided. One of the dangers is an error of proportion: some sections of the paper (e.g., methods) can occupy disproportionately more space than other sections, equally important in the whole paper, which in turn may suggest that the content featured more prominently in the GA is the main idea of the whole article.

Effectiveness of GAs

Since 2016, the effectiveness of GAs as a means of disseminating knowledge has been studied using different study designs and in both single journals and groups of periodicals. The researchers mainly focused on the following issues:

1) whether – and if yes, to what extent – GAs boost the number of citations of each paper,

2) whether – and if yes, to what extent – GAs enhance the visibility of papers on social media (primarily Twitter), and

3) whether – and if yes, to what extent – GAs cause the full text of a given paper to be downloaded from the journal's website.

One of the first studies concerning the feasibility of introducing GAs in a scientific medical journal was helmed by their originator in the field of medicine, Andrew M. Ibrahim. A prospective case-control crossover study by Ibrahim et al. performed between July and December 2016 used 44 original research articles published that same year in *Annals of Surgery*.[27] The study measured only Twitter visibility and revealed that when the same articles were tweeted as a GA, they had a 7.7-fold increase in the number of impressions and an 8.4-fold increase in the number of retweets.

In 2019, Chapman et al. performed a randomized controlled trial of the impact of papers published in *BJS* and presented on the journal's Twitter account. Forty-one articles were randomized into plain English abstract (14 papers), visual abstract (14) and standard tweet (13) groups. Most engagements were from healthcare professionals (95%). Visual abstracts attracted significantly more engagements than plain English ones.[13] A year later, Hoffberg et al. obtained similar results in a prospective, randomized crossover trial concerning papers on suicide prevention published in a medical journal indexed in PubMed by researchers from the Rocky Mountain Mental Illness Research, Education and Clinical Center (part of the MIRECC network; Aurora, USA). They compared Twitter posts with a visual abstract to those with a simple screen grab of the PubMed abstract (50 journal publications in total). Visual abstract tweets were associated with a significantly higher number of impressions, retweets and link clicks compared to text abstract tweets.[11] In a similar vein and in the same year (2020), Chisari et al. performed a two-arm randomized controlled trial with crossover. Manuscripts from the *Journal of Arthroplasty* were allocated to one of 2 arms and disseminated via the journal's Twitter account as either a text-based tweet or a GA. Their conclusion was that when GAs are used to communicate research through social media outlets such as Twitter, the overall research engagement significantly increases compared to plain-text tweets.[18]

In the last 2 years, studies on the influence of GAs on the number of citations of given papers started to appear. In 2022, Kim et al. stated that there has been no study on the influence of GAs on the impact factor (IF) of journals and the citation index or social media exposure of individual articles.[20] Therefore, they investigated the presence of GAs, total citations and social media exposure of full-length original articles in the top 10 journals in the field of gastroenterology and hepatology over 3 years (2019–2021). Their research had a pronouncedly broader scope than most of the cited studies, as 4205 articles from 10 journals were evaluated over 3 years. Journals that adopted GAs demonstrated significantly higher IF increases over the course of 3 years than journals without GAs. The longer GAs have been utilized in a journal, the higher the IF the journal had. Moreover, individual articles with GAs had significantly higher Web of Science citation counts (median 14 compared to 12), more social media exposure (median 23 compared to 5) and more Altmetric.com tweet counts (median 15 compared to 7) than those without GAs.[20]

Examples of GAs in medical papers: the ACEM experience

Graphical abstracts were implemented in ACEM beginning with the January 2023 issue, but since most of the papers published in this journal are disseminated online in advance of the print release, the first articles containing GAs were published in September 2022. Until June 30, 2023, approx. 100 papers with GAs appeared in ACEM. Of note, the editorial office of this journal requires that a GA accompany all types of papers – including editorials, research letters and research-in-progress works. The ACEM editors' experiences from these 10 months (September 2022–June 2023) can be summarized as follows.

Graphical abstracts in original papers presenting clinical research

Preparing a GA for a paper about clinical research (including randomized controlled trials and other clinical trials) is especially challenging since, in this type of research, the material (i.e., enrolled participants) and the methods employed are equally important as the results, and the conclusion in this regard can lead to a serious misinterpretation. Although – as has been stated above – a GA is never a substitute for the text, it cannot be misleading. Therefore, we hypothesize that the type of paper for which GAs utilize the 3-panel concept is submitted most frequently. Moreover, this design can be realized in different forms – e.g., Kisiel et al. divided their GA into background, materials and methods, and results sections – although not titled, they are clearly distinguishable, and the whole GA is very clear (Fig. 1).[28] A similar design was used by Wnuk et al.[29] Przybylski et al. employed a more bulleted text in their GA, so titles of the panels proved necessary.[30] Among GAs for clinical research published in ACEM, there is also an example of a GA containing primarily text but optimally communicative – the work by Gao et al.[31] This last GA shows that if graphical elements (e.g., drawings) within a GA are to be purely ornamental in function, it is sometimes better to abandon them altogether. Gao et al. also employed a 3-panel design with the title serving as the background section.

However, there is another option of GA design for clinical research, encountered especially in research letters covering clinical research and published in ACEM. In their GA for a study encompassing a cohort that qualified for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccination, Stępień et al. concentrated on the composition of the study population and the outcomes, deeming them the most important (which shows the importance of information selection; Fig. 2).[32] A more radical approach was adopted by Paszkiewicz-Kozik et al., who took the risk of incorporating 2 tables and 2 figures (charts) into the GA, but the simple tables had been prepared specifically for this purpose and the charts are comprehensible in this context.[33] Together with a clear composition in 2 columns and the title of the paper serving as the background section, this GA can be called a model example of such an abstract for articles describing clinical research.

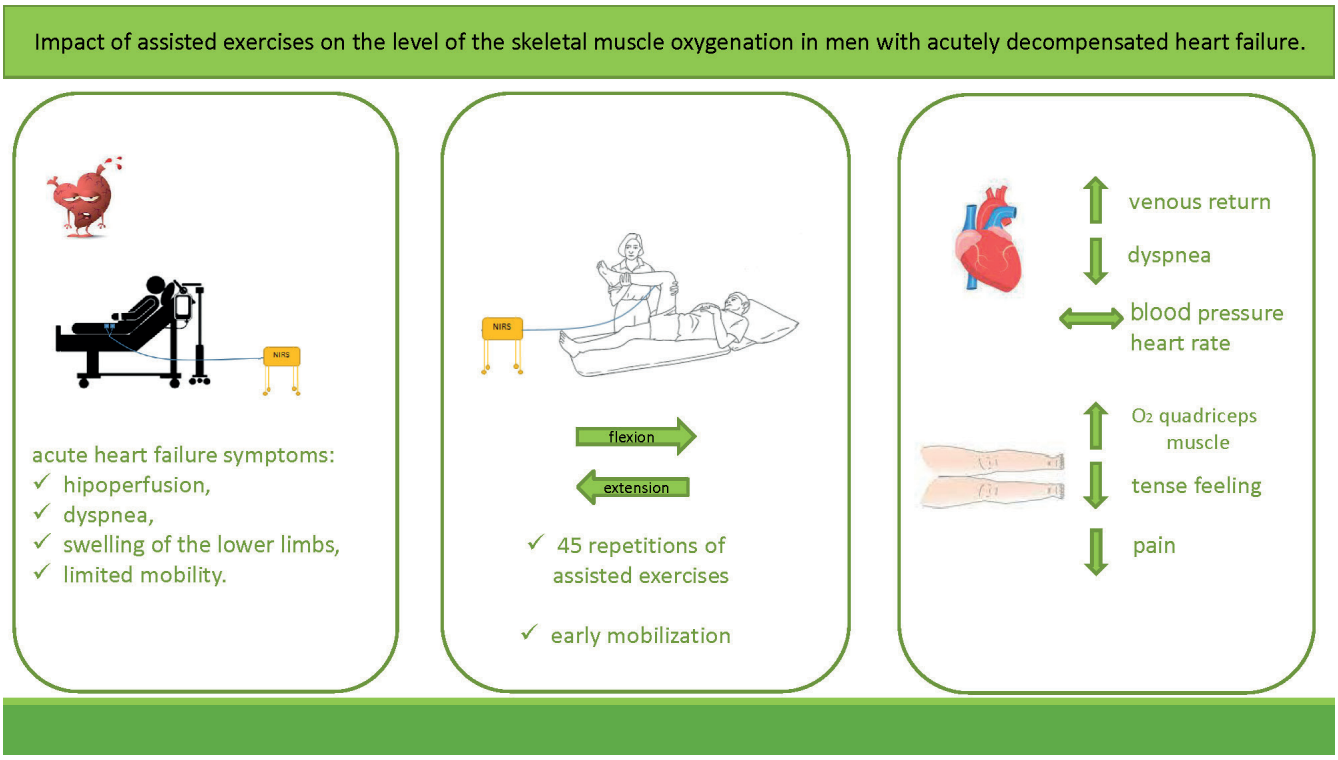

Fig. 1. Graphical abstract for an original paper by Kisiel et al.[28]

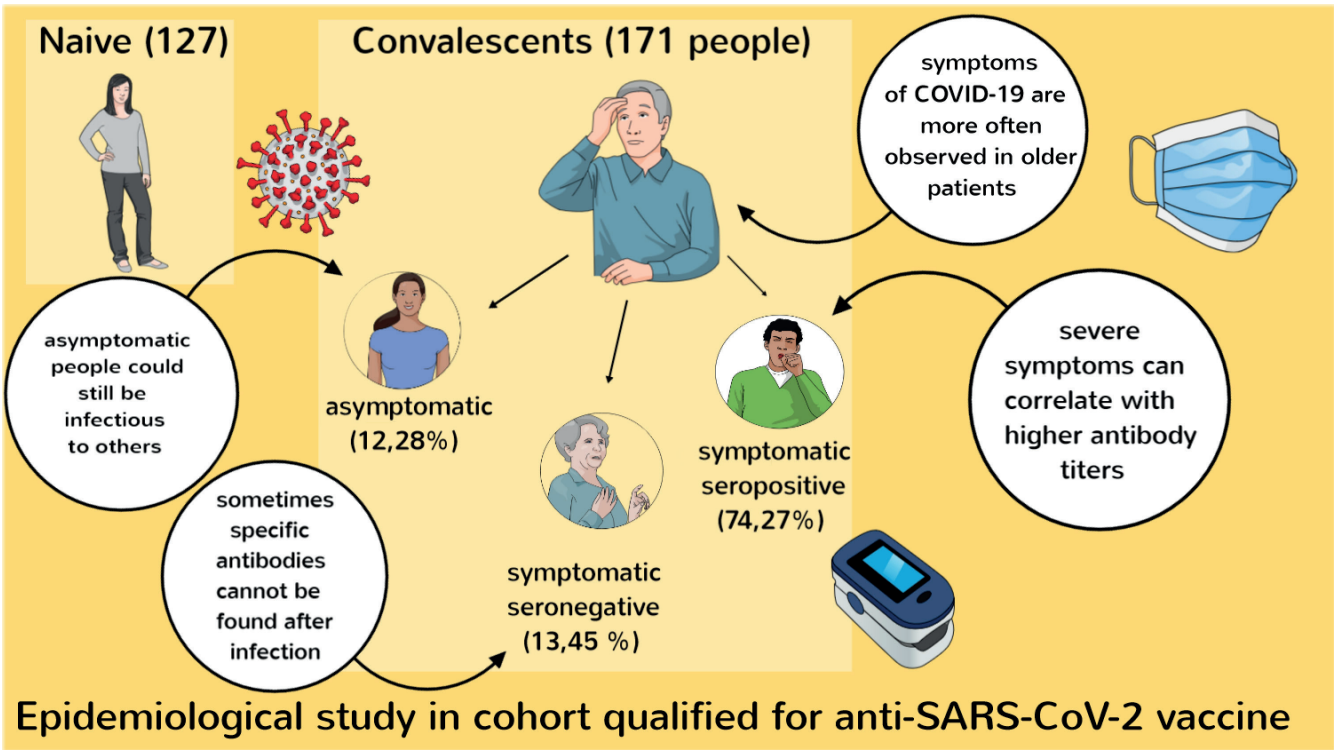

Fig. 2. Graphical abstract for a research letter by Stępień et al.[32]

Graphical abstracts in other original papers

This is certainly the most diverse pool of GAs – not only because original papers not describing clinical research constitute roughly 60% of all articles published in ACEM, but also because almost all types of content organization within a GA can be found in them. A circular way of reading the GA was employed by Chen et al.[34] and Wang et al.[35] – such design proves sound in compositions aimed to familiarize the reader only with the general thematic field and the idea of the presented research, because of the scarce use of text. The circularity enables viewing the GA at a single glance. A less detailed abstract can also be more vertical, like in Wang et al.,[36] combining schematic drawings and single terms. Similarly to GAs for original papers presenting clinical research, a dominant type of design is a 3-section one, with the sections with or without titles. However, implementations of this general concept can vary, with 2 discernible patterns.

1) The sections are arranged vertically, whereas the content of each section is read horizontally – with the dominance of graphical material (Grotowska et al.[37]) or text (Ventruba et al.[38]). The latter GA is an example of a well-thought use of text in such abstract – the title serves as the background section, and the 2 whole sentences stating the conclusions are not an excess because they provide the key message which could not be expressed with any illustrations (or at least not that clearly), and at the same moment do not dominate the whole composition because the main part of the text is represented by minimalistic boxes and clearly marked relationships between them. The former GA is a good example of a non-intrusive application of a slightly tongue-in-cheek approach – drawings of experimental animals are not meant to be fully serious. A similar horizontal design was used by Lubiński et al., with titles of the subsections placed in the middle panel (Fig. 3).[39] In this GA, the illustrations evoke desired associations with the described problem, while the text clarifies the necessary details. Deliberate use of 2 contrasts, but not overly bright colors (light yellow and deep red) should also be noted. Kosowski et al. reduced the number of subsections in the middle panel to 2, which allowed for more extensive use of schematic illustrations that help visually discern multiple points in each subsection.[40]

2) The sections are arranged vertically, with the contents of each section also read vertically. In the GA for a paper by Li et al., the upper panel (title) and lower panel (conclusions) are text-only, while the middle panel (the largest one) employs 3 separate flowcharts in 3 subsections (background – group & design – key results).[41] Using different, contrasting colors prevents confusing the subsections and directs attention to the "key results" subsection. In the GA by Tian et al., text and pictograms are balanced, and therefore, the color palette could be narrower (shades of blue with only the title highlighted).[42]

Other authors offered less sophisticated but no less communicative designs with a simple vertical orientation and dominance of text (Özgökçe et al.[43]) or a balance

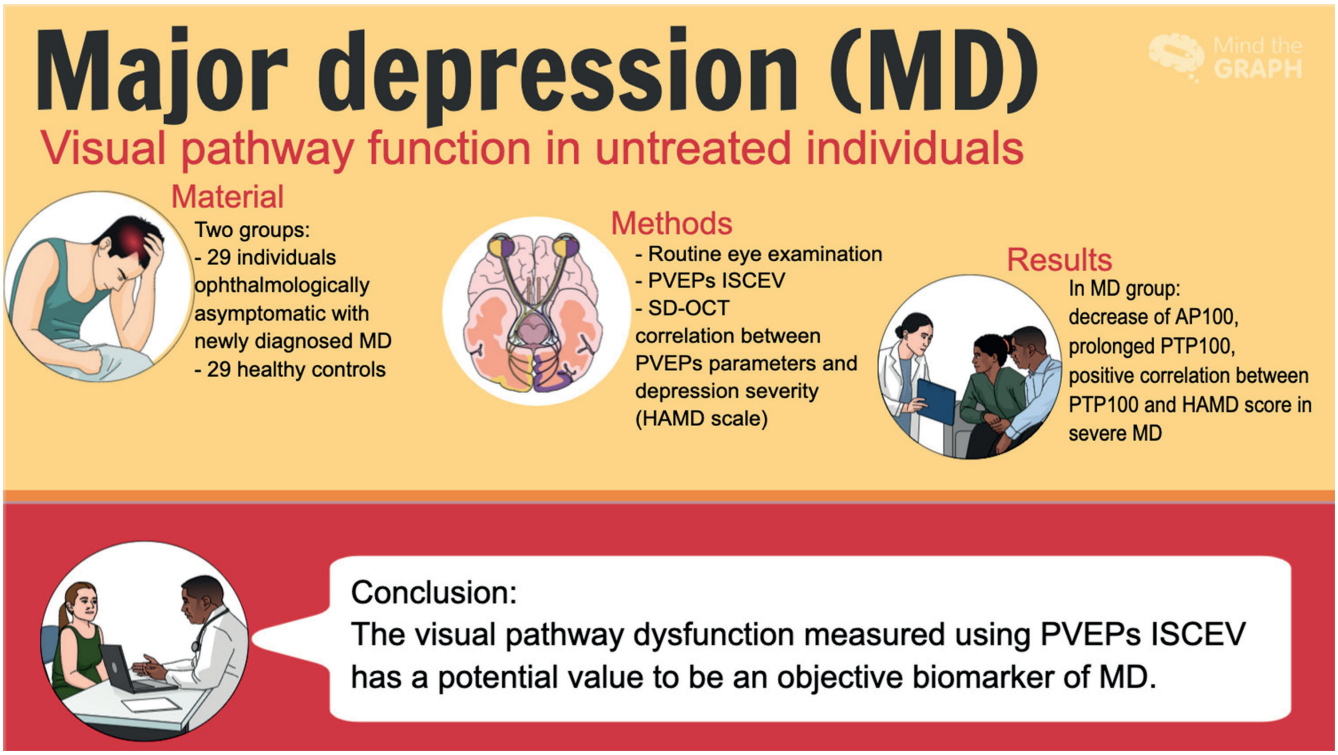

Fig. 3. Graphical abstract for a research letter by Lubiński et al.[39]

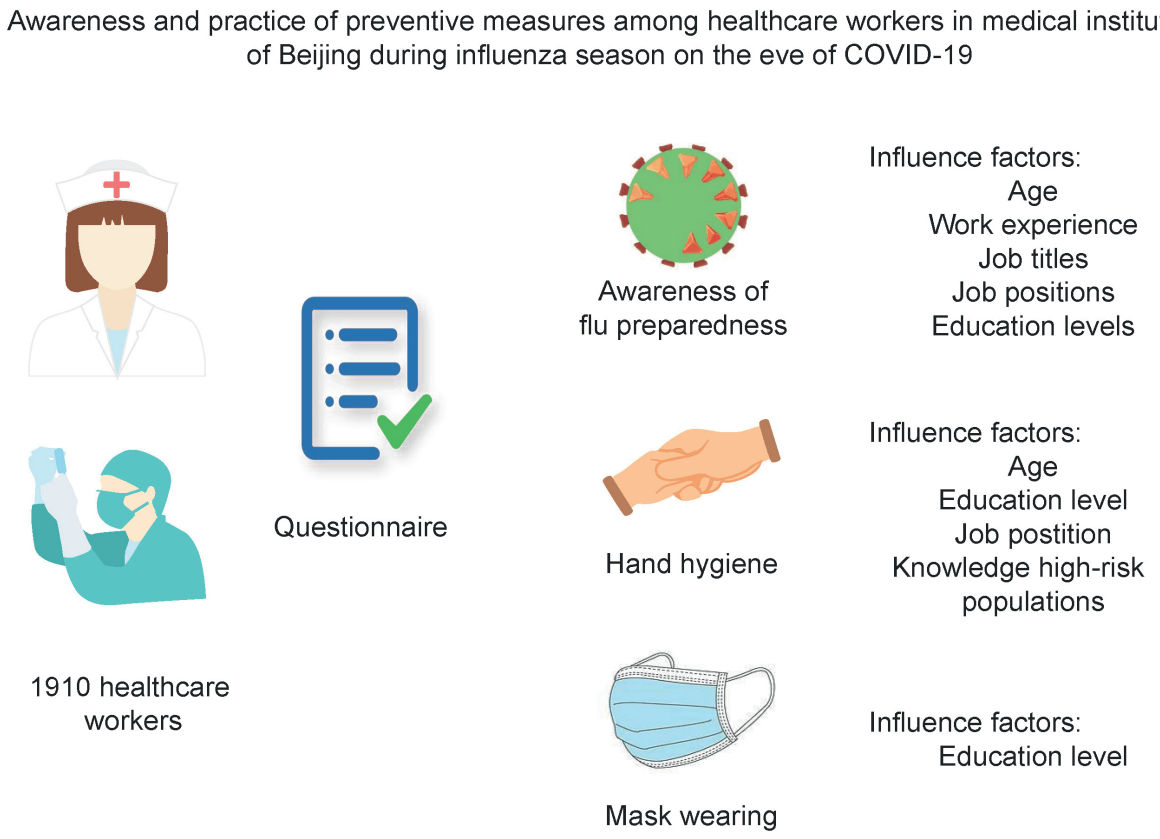

Fig. 4. Graphical abstract for a research letter by Xie et al.[45]

between text and graphical material, and with deliberate use of white space (Demirtas et al.[44]). Xie et al. proved that text (only single words or short expressions) combined with pictograms can allow for a cohesive, but relatively detailed understanding (Fig. 4).[45] Okur et al. used a horizontal flowchart with text only, but employed 2 well-considered

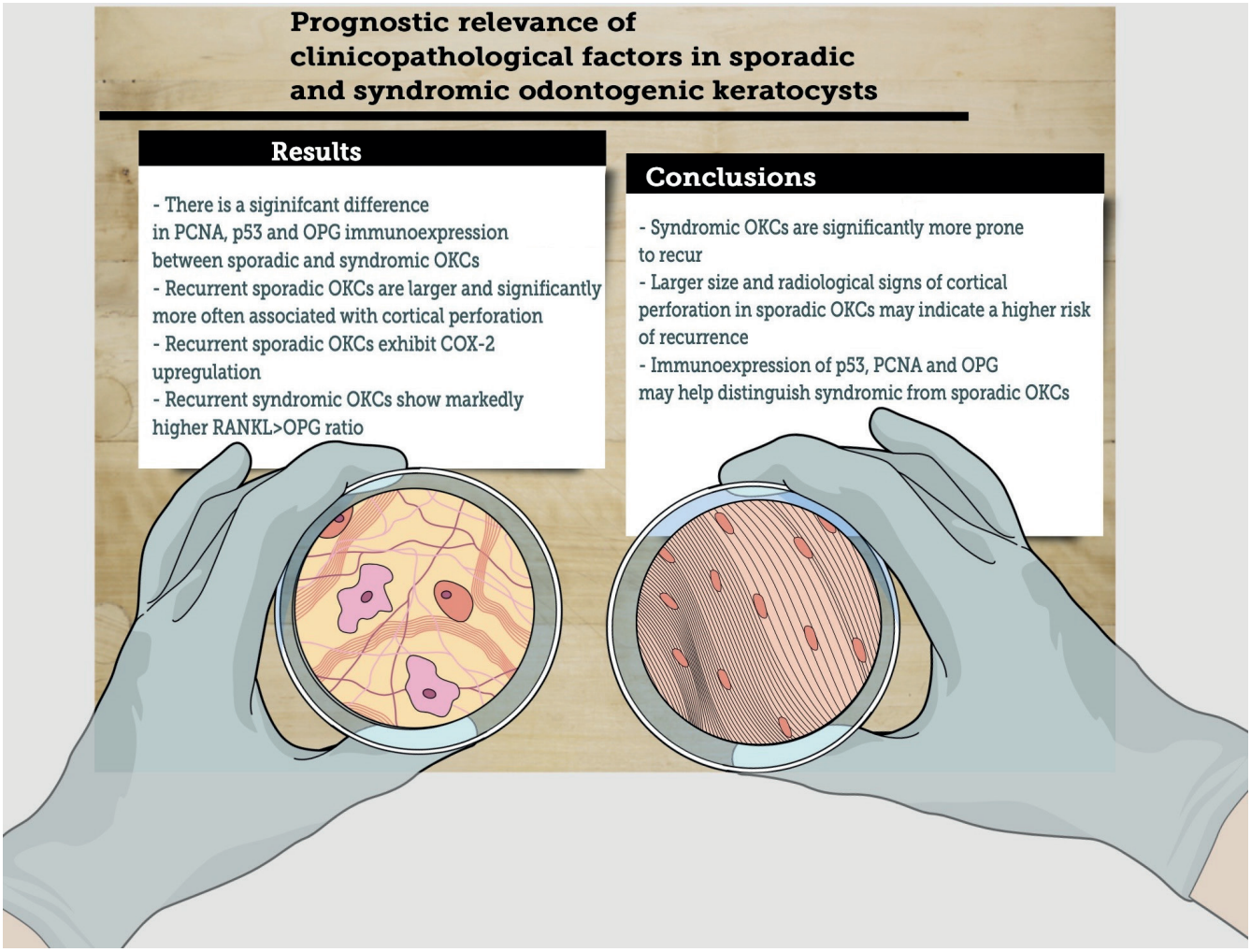

Fig. 5. Graphical abstract for an original paper by Kisielowski et al.[48]

visual tools: 1) boxes stating the nature of the information in green, while boxes with the information itself were blue, 2) a single pictogram on the left side of the GA that attracts attention and provides a clear suggestion who the members of the study population were.[46] Ferlengez et al. sparsely used text in horizontal material and the methods section and combined it with black-and-white schematic illustrations while keeping the conclusions in the text form – such design is aesthetically consequent and prevents distraction during reading.[47]

When the aesthetics are in question, 3 more GAs for original papers deserve particular attention. The work by Kisielowski et al. uses professional artwork, which, together with the title, gives a specialist in the field a general idea of the study design and methods, while results and conclusions are provided in short sentences and do not dominate the picture. Thoughtful choice of typeface is also worth noting (Fig. 5).[48] Edebal and Doğan were clearly inspired by infographics as a form of disseminating scientific knowledge among the lay public – the level of complication of their GA still allows for full-screen reading on desktop computers and laptops and the amount of lucidly conveyed content is truly massive (Fig. 6).[49] Finally, Wojtera et al. abandoned illustrations altogether in favor of a geometrical composition consisting of hexagons in different shades of blue as box-clusters.[50]

Graphical abstracts in meta-analyses

The highly abstract nature of a meta-analysis – a paper in which the material is other scientific papers – causes many authors to think that it is very hard or nearly impossible to prepare a proper GA for an article of such type. However, several research teams who published their meta-analyses in ACEM show several creative ways to deal with this challenge. Although re-using one of the figures from the paper is prohibited, the editors can make an exception if the meta-analysis flowchart is combined with a new graphic or other graphics, preferably with some additional text comments. Such exceptions are granted because – contrary to figures in other types of papers – a flowchart can provide comprehensible information about the materials and methods employed in the meta-analysis, and this information cannot be successfully conveyed by other

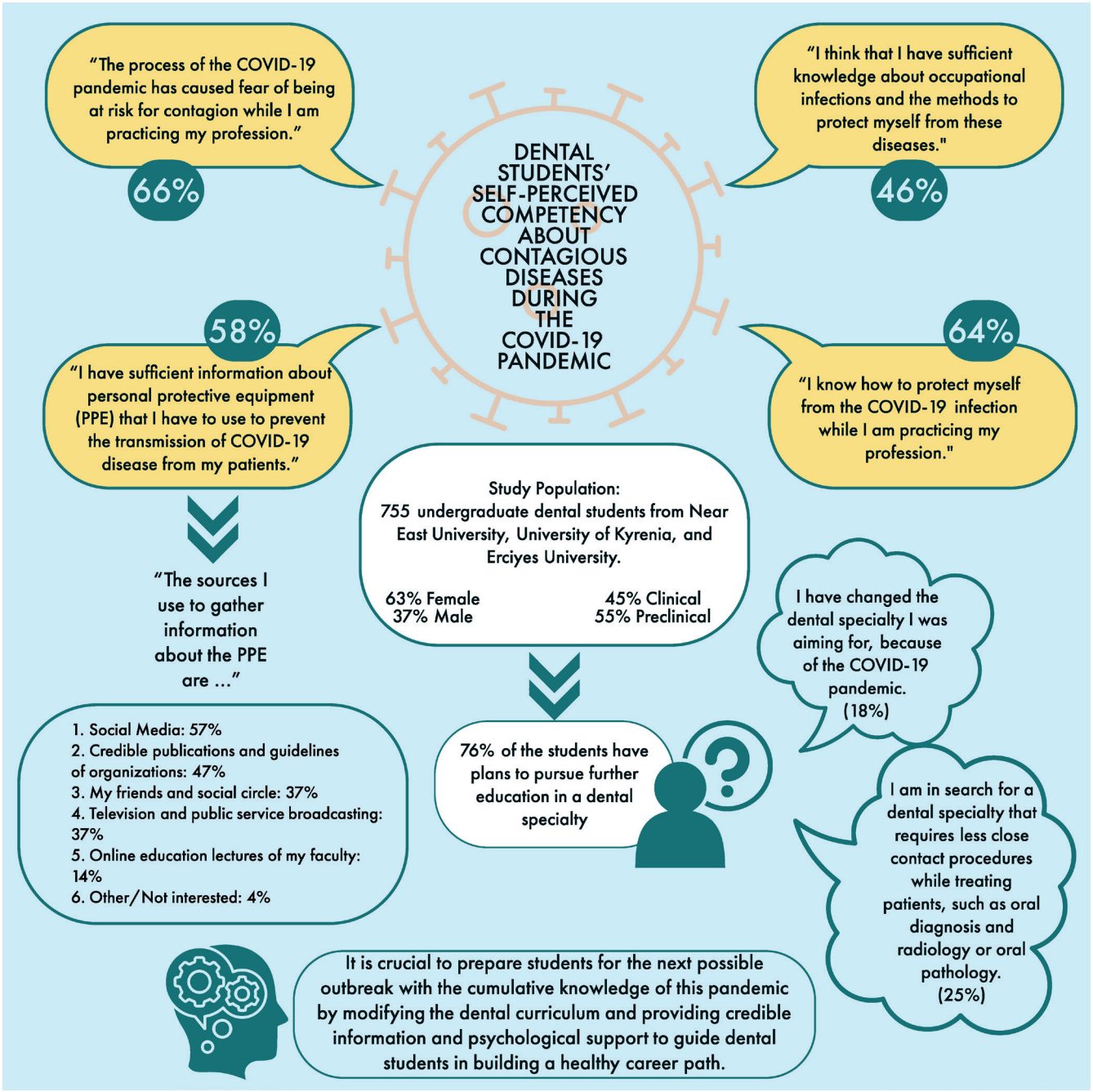

Fig. 6. Graphical abstract for an original paper by Edebal and Doğan[49]

visual means. The other part of such GA provides the necessary context about the thematic field of the analyzed papers and signals the results (and preferably also implications) of the meta-analysis. Examples of GAs using the above concept can be found in articles by Sun and Wu[51] (Fig. 7), Li et al.,[52] and Wang et al.[53]

However, some authors focus solely on the content of the papers subject in the meta-analysis, deducing that the procedure of the meta-analysis is not as important as the discussed problem itself. Therefore, they design a vertical scheme focused on the new perspective on a given issue elucidated in the process of the meta-analysis (the process itself is rather obvious for a professional investigator and does not have to be demonstrated since it is known from the accompanying title of the paper that it is a meta-analysis). Such designs can be simple, like in the papers by Zhuo et al.,[54] Chen et al.,[55] Yang et al.,[56] Zhou et al.,[57] Wang and Zhang,[58] and Chen and Zhang,[59] or more elaborate, somewhat similar to the triptych template proposed by Andrew M. Ibrahim, as in Chen et al.[60] (Fig. 8). Worth mentioning is the outstanding GA submitted by Bi et al., in which high-quality illustrations were combined with a clear, simple composition (Fig. 9).[61]

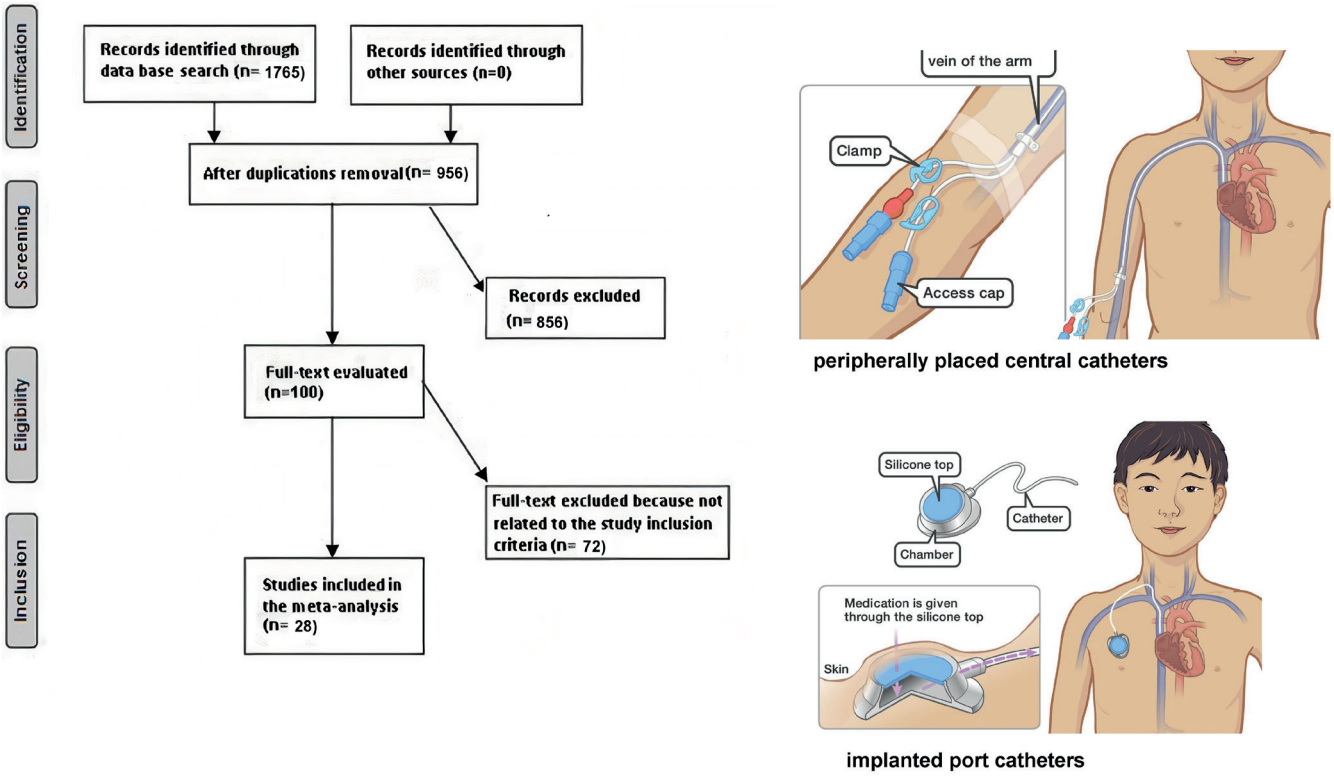

Fig. 7. Graphical abstract for a meta-analysis by Sun and Wu[51]

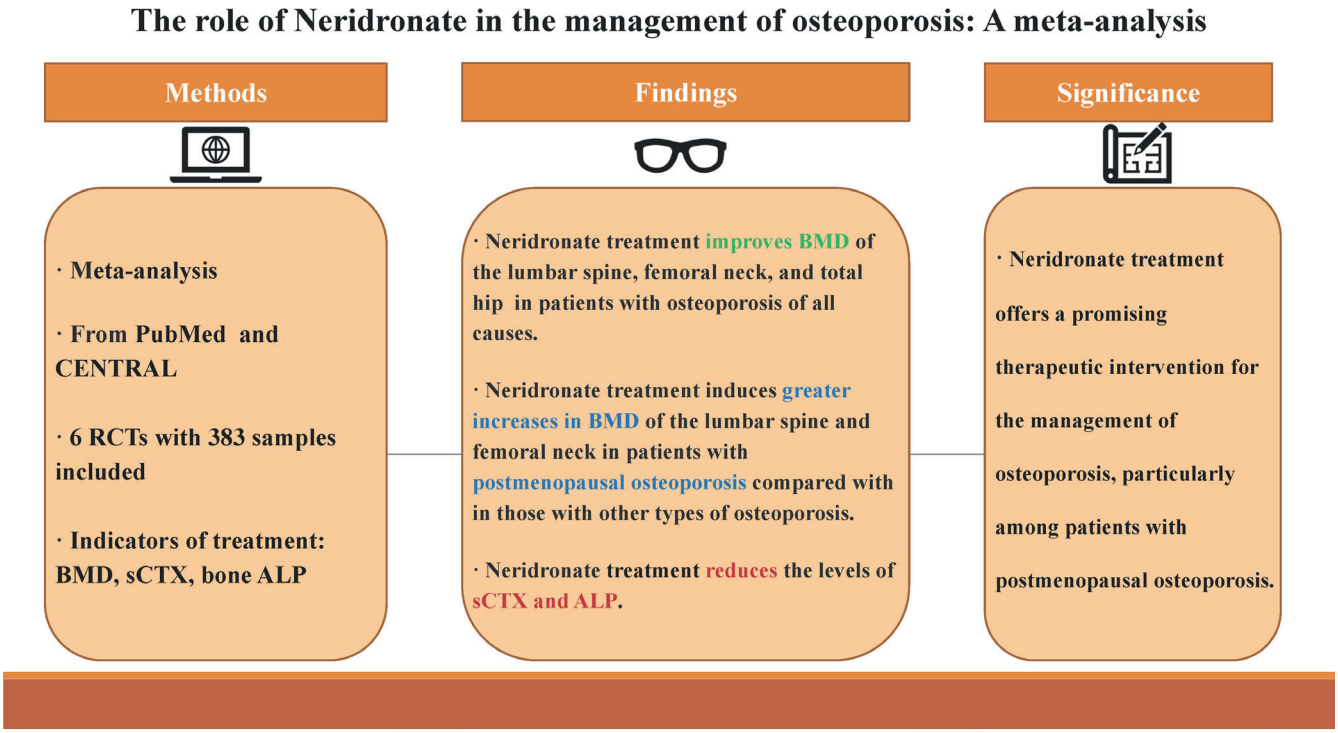

Fig. 8. Graphical abstract for a meta-analysis by Chen et al.[60]

Graphical abstracts in reviews

Authors designing GAs for review papers face problems similar to those encountered regarding meta-analyses: less strictly stipulated structure of the paper is difficult to transpose into visual language. The Introduction and Objectives sections of such papers contain scientific terms that can be represented visually (names of diseases or treatment methods), but the structure of the sections between Objectives and Conclusions (these 2 sections are mandatory

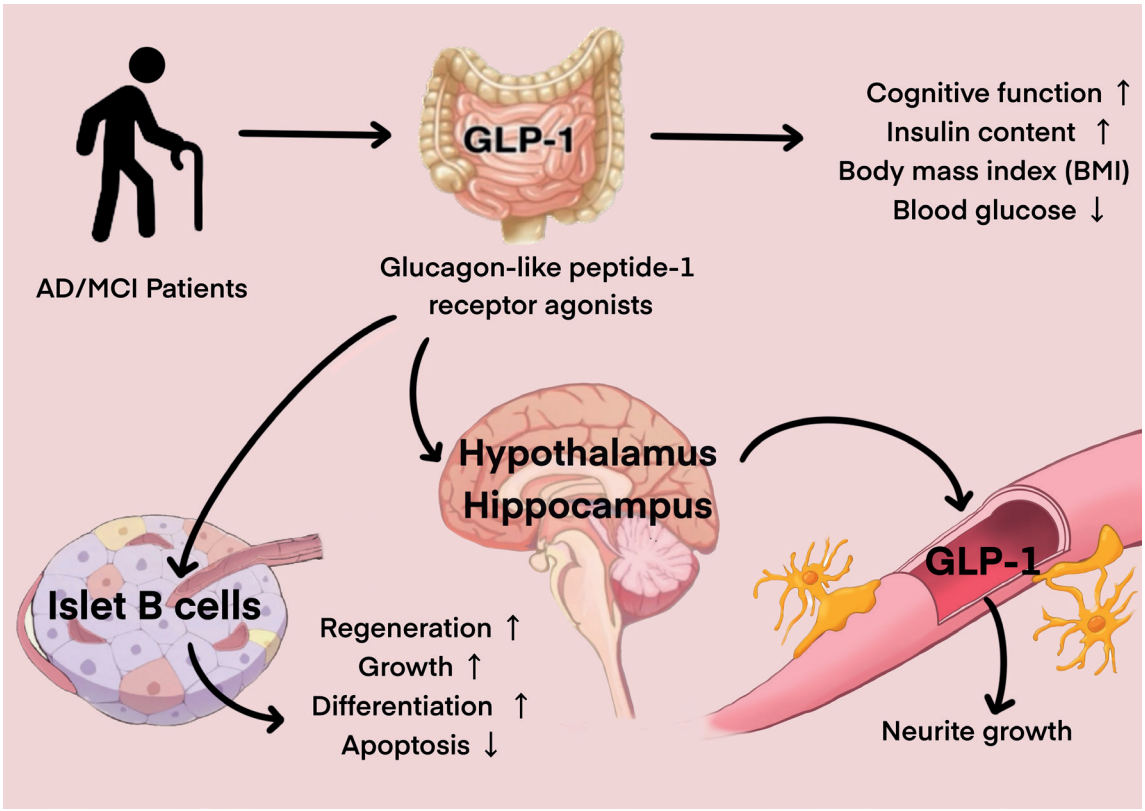

Fig. 9. Graphical abstract for a meta-analysis by Bi et al.[61]

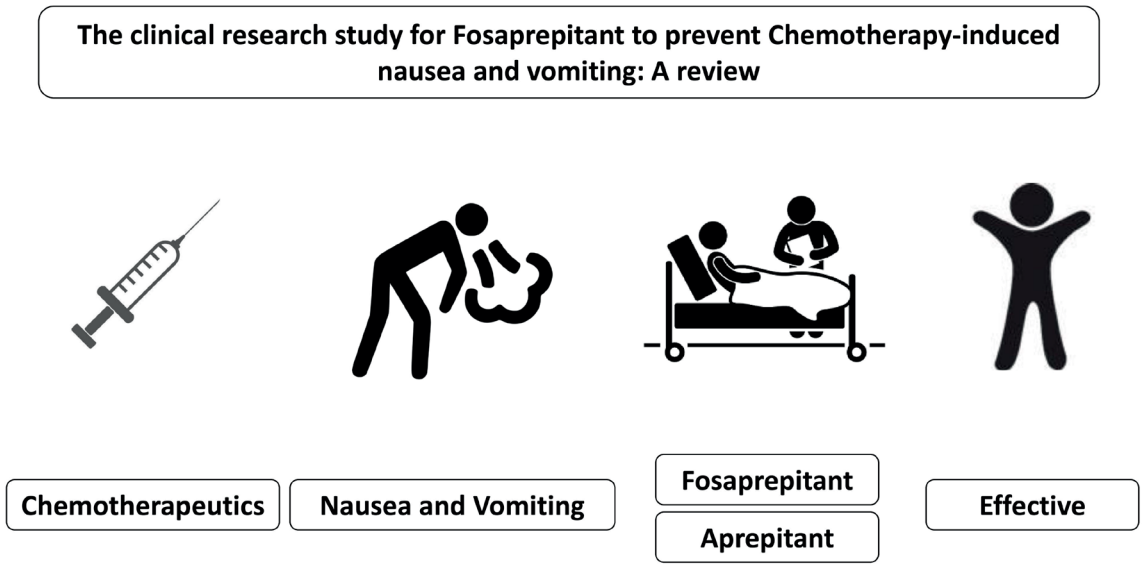

Fig. 10. Graphical abstract for a review by Liu et al.[62]

in reviews published in ACEM) is not stipulated by any checklist or other guideline (Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist does not apply in this regard). In the main part of the paper, several complex aspects of an already complex problem are discussed, and many different articles are cited not only as a reference but as a main topic of discourse. Therefore, a GA simply reflecting the course of the argument would be impossible to design or too intricate to the point of incomprehensibility. Moreover, many papers of this type do not contain any figures, so the authors may not exhibit the appropriate mindset to think more visually about their article.

The only solution seems to be an approach similar to the 2nd one presented in the section on meta-analyses: to focus on the scientific hypotheses and findings presented in the papers discussed in the review and to clearly state what the broader view of the field (obtained through reviewing several articles) tells us about the current state of affairs (and what questions arise or remain open). Various types of content organization are employed. Liu et al. chose a vertical one with schematic, black-and-white yet suggestive pictograms (Fig. 10).[62] Bukłaho et al. proposed a version read from left to right and then towards the lower right corner (Fig. 11),[63] while Szmit et al. prepared an infographic

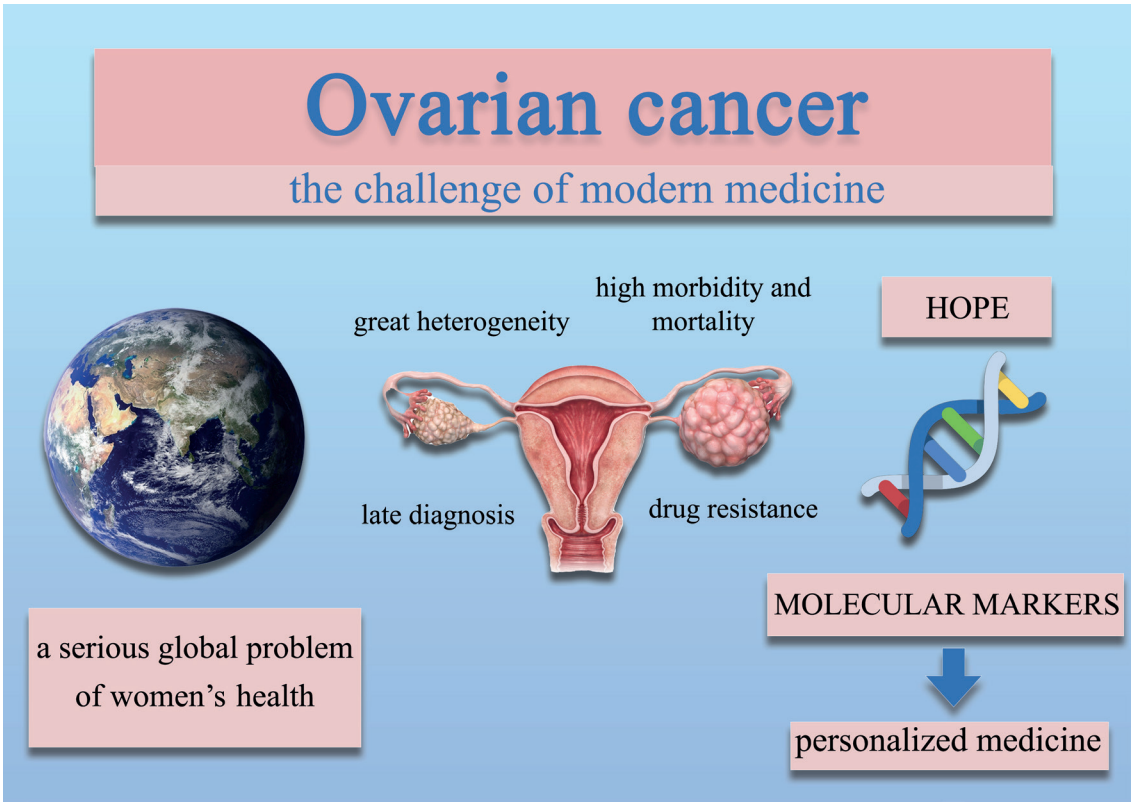

Fig. 11. Graphical abstract for a review by Bukłaho et al.[63]

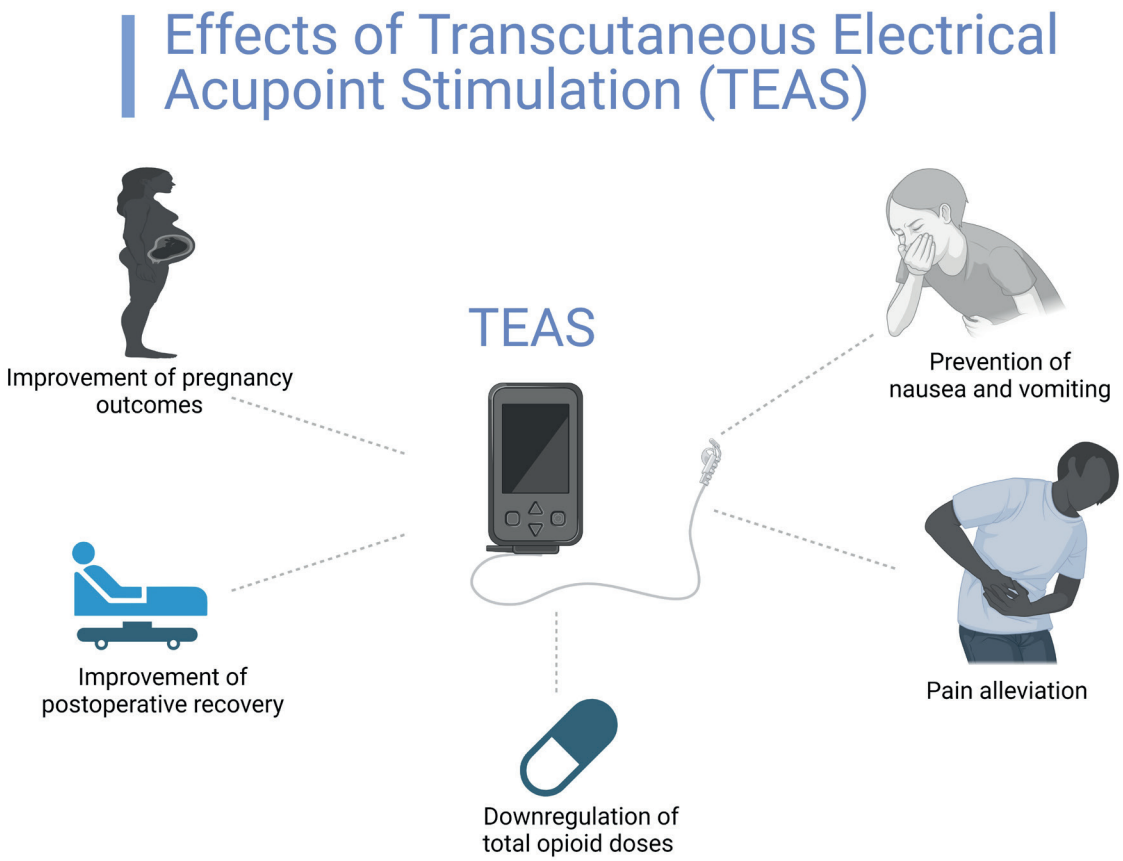

Fig. 12. Graphical abstract for a review by Szmit et al.[64]

in a radial arrangement, which allowed for presenting different aspects and implications of a single therapeutic technique (Fig. 12).[64] On the other hand, we encountered GAs for reviews that reflected the structure of the paper – a good example is the vertical design by Osypko et al., in which the key issues discussed in each section are clearly

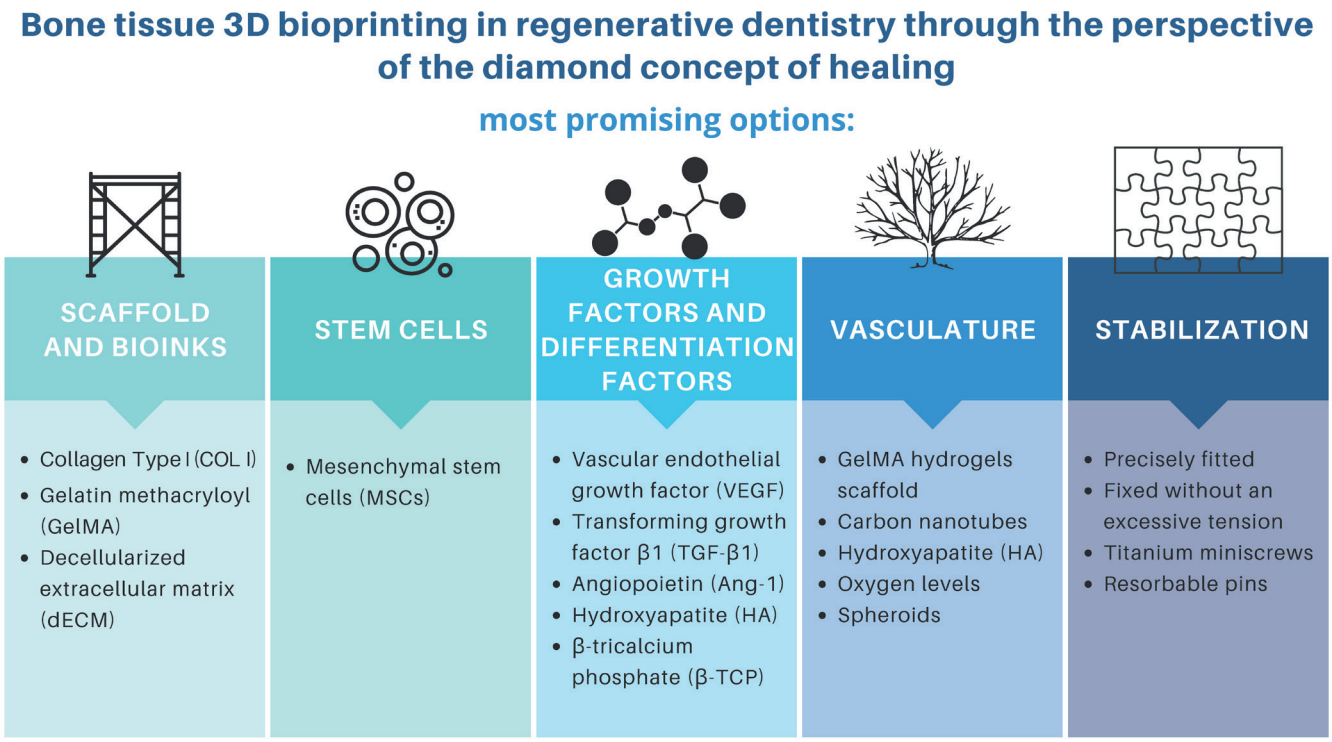

Fig. 13. Graphical abstract for a review by Osypko et al.[65]

summarized in bulleted points and complemented with schematic illustration to enhance remembering (Fig. 13).[65] A completely different approach is represented by the GA for the paper by Nishi et al., which conveys only the general idea of the study by presenting a simplified visual representation of a phenomenon examined in the reviewed studies (Fig. 14).[66]

Graphical abstracts in reviews: strengths and weaknesses

Each of the 5 presented examples have their strengths and weaknesses. The GA by Liu et al. conveys the main concept of the paper clearly and without distracting decorative elements, but the same characteristic is its limitations – the reader learns what the goal was, what medicament was used, and that the results are promising, but no information about the methods employed in different reviewed studies is provided. If the full title of the paper had not been placed on the top of the GA, it would not be clear what type of paper (original or review) this GA summarized.[62]

The main advantage of the GA prepared by Bukłaho et al. is its clear structure (the reader's gaze follows a low arch) and emphasis put on the key issues discussed in the paper. In the focal point of the GA, 4 paramount challenges regarding ovarian cancer treatment are listed, while schematic imagery stimulates memorizing and attracts attention. Moreover, the composition is balanced – on the right, we can see not only that there is hope, but also why. The main disadvantage of this concept is – similar to the design by Liu et al. – its vagueness. This GA does not inform the reader what type of paper it presents.[63]

Szmit et al. decided to focus solely on the benefits for patients and doctors brought about by the discussed medical device. The sheer scope of the content clearly implies that a review or systematic review is being summarized here. Schematic, clipart-style illustrations easily evoke desired associations. However, this GA offers no information on specific studies that were analyzed and says nothing about the study selection process, which is paramount in systematic reviews.[64]

Osypko et al. managed to overcome one of the most common limitations of GAs for various types of reviews. Their design reflects (at least to a large extent) the structure of the main part of the paper (i.e., excluding Introduction and Objectives). It seems that a narrative review, which follows the course of the argument without any predetermined division (a given section ends simply when a given topic is satisfactorily explored), allows for a GA that summarizes the paper in a more detailed way (text elements of this GA provide such details in abundance, given its rather simple composition). The main weakness of the discussed GA is the lack of Discussion and Conclusions sections, which contain crucial content in the paper.[65]

Scoping reviews are commonly undertaken to clarify working definitions and conceptual boundaries of a topic or field. Therefore, Nishi et al. decided to primarily convey in their GA not the contents of the analyzed studies but the core of the discussed field itself, at the same time making sure that a schematic visual representation enhanced memorizing even among non-specialists. The main advantage of their design seems to be the thoughtful use of graphical elements and intertwining them with scarce text. Neither the text elements, comments, nor the graphics are meant

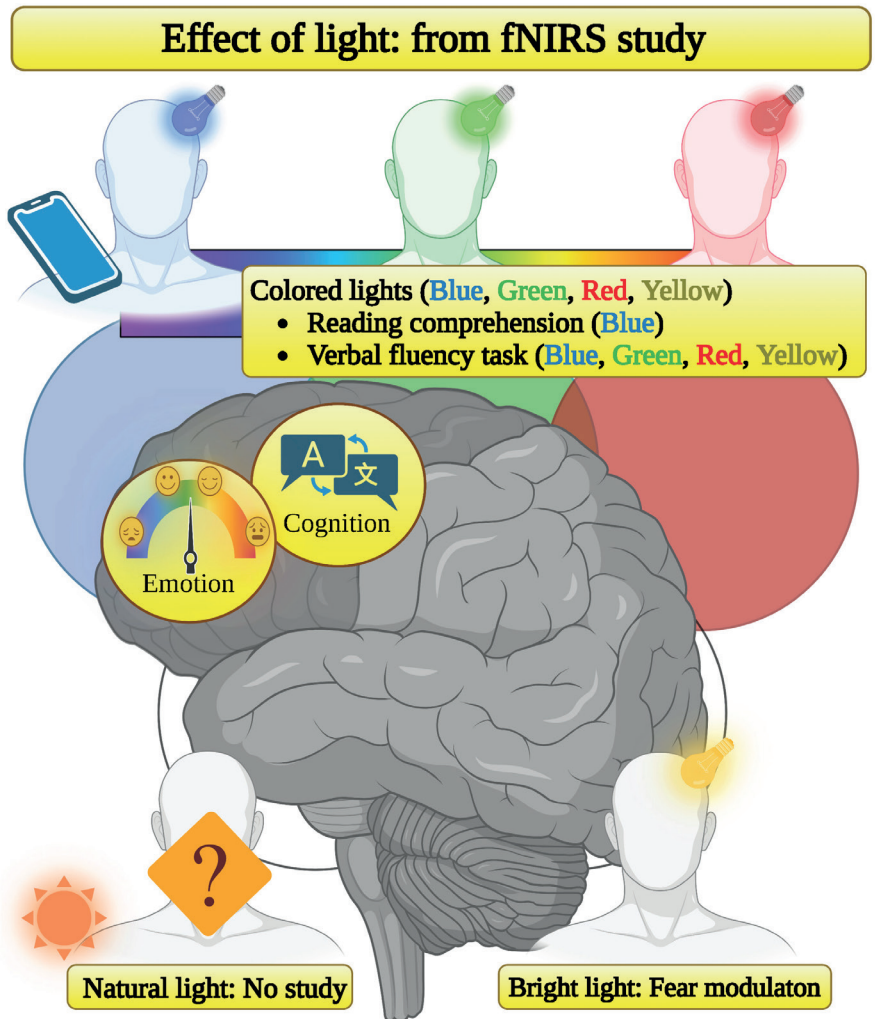

Fig. 14. Graphical abstract for a review by Nishi et al.[66]

to embellish the composition – their GA is a consistent, compact whole. Its main weakness is the usage of only general ideas, which does not reflect the meticulousness with which the authors tackle the subject in the paper.[66]

To sum up, the main weakness of GAs summarizing various types of reviews is the failure to provide any insight (neither detailed nor general) into the differences and specifics of the analyzed studies. Only the results of a summary of their contents are provided, and, in consequence, the specificity of a review as a type of scientific paper is "lost in translation". However, it is possible that a technique to visually represent this aspect of reviews is yet to be proposed – using study selection flow diagrams in GAs of meta-analyses also seems only a half-measure.

Graphical abstracts in other types of papers

Editorials published in ACEM do not follow any particular predefined structure and usually offer some more generalized reflections of an experienced researcher on a given – broader or narrower – field of study. Therefore, GAs for such papers are not expected to reflect the structure of the text and present the general idea and primary notions discussed in the paper in a more cohesive or more linear way. Nevertheless, there are examples of GAs for editorials that are both communicative and well-conceived – e.g., an infographic for a paper by Vinker,[67] a design combining text and pictograms in 4 boxes proposed by Piccoliori et al.[68] (Fig. 15), and a minimalistic GA by Tanaka et al.[69] giving only the general idea of the paper and a handful of key terms (Fig. 16).

A research-in-progress paper does not contain a conclusion but only preliminary remarks, since its main objective is to inform about the progress of the investigation, not its results. Therefore, GAs for such articles focus on the materials and methods used (since some assessment of their feasibility can be offered already at this stage) and on the clinical implications of knowledge acquired so far to point out the practical aspects of the conducted research. A good example is the GA for the paper by Szlenk-Czyczerska and Kurpas.[70]

Feedback from authors and readers

First piece of information regarding the implementation of GAs in the ACEM was disseminated on the journal's website, on social media (Facebook, Twitter and LinkedIn)

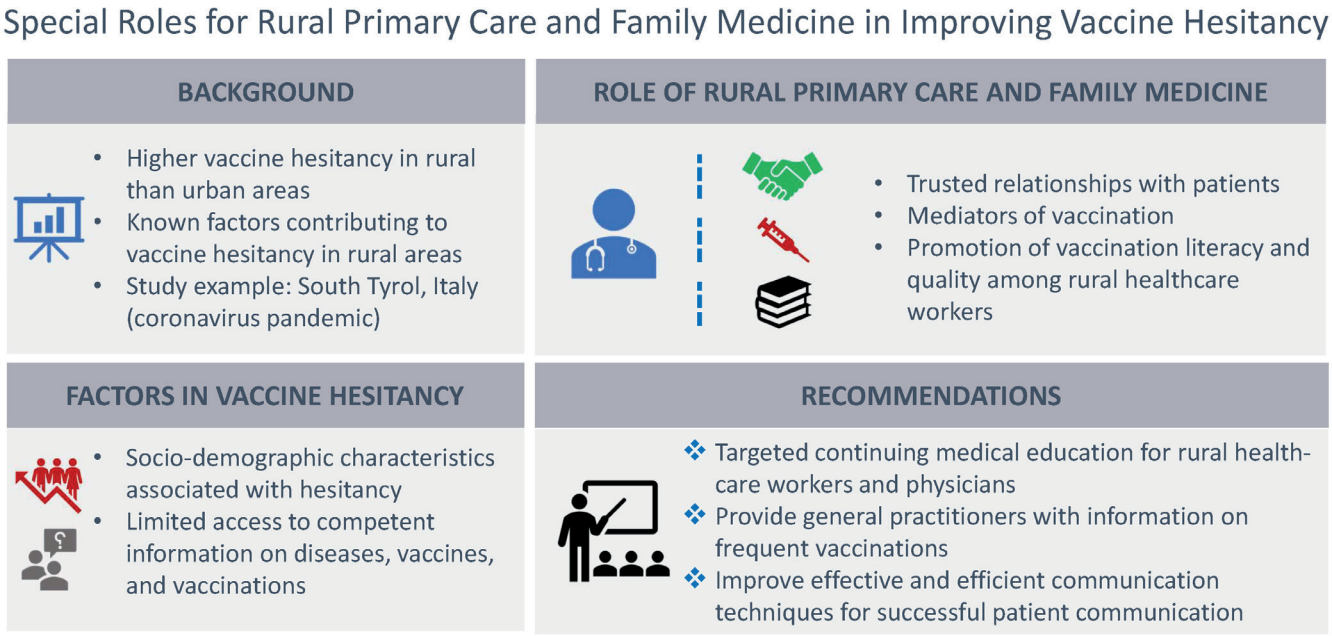

Fig. 15. Graphical abstract for an editorial by Piccoliori et al.[68]

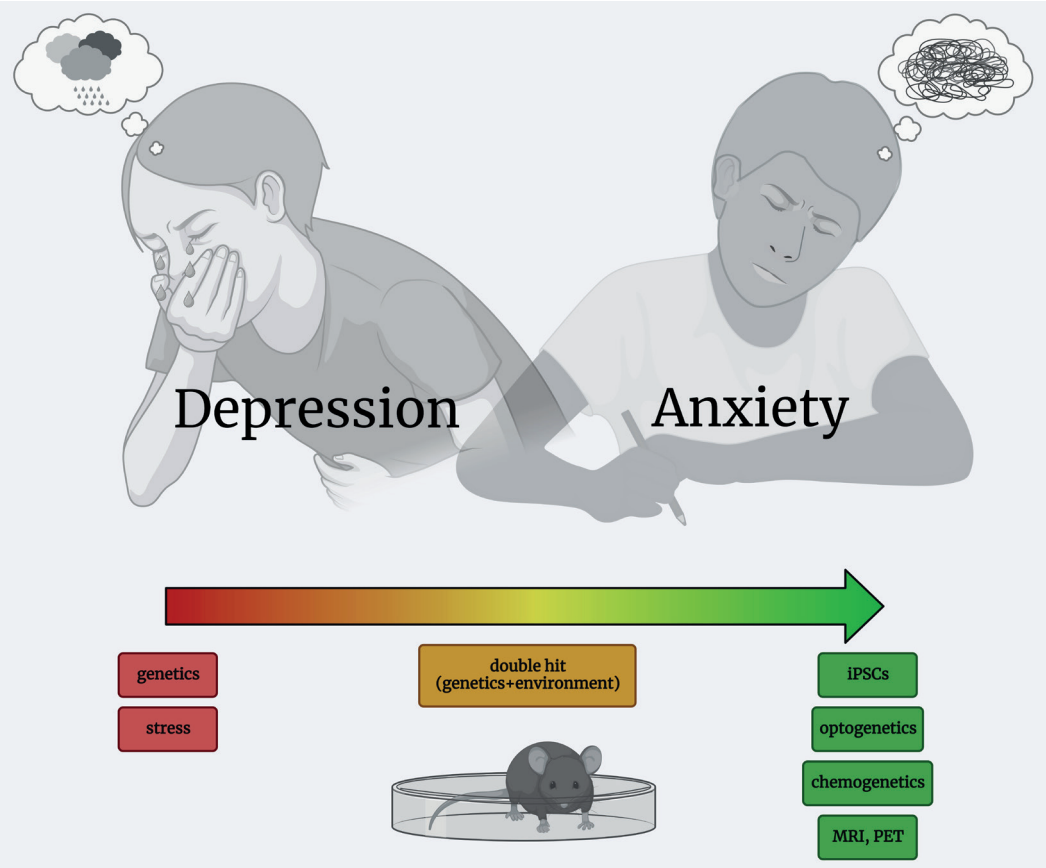

Fig. 16. Graphical abstract for an editorial by Tanaka et al.[69]

and through ACEM's newsletter on June 1, 2022. Since such requirements simultaneously appeared in the system used for manuscript submissions, almost immediately, the editorial staff started to receive feedback from authors. Initially, many researchers were confused about the concept of a GA itself and expressed low confidence about their ability to prepare an appropriate one. Such attitude was a clear indication that GAs had yet to become obvious in scientific medical communication – a significant number of experienced authors from several different countries declared that they encountered such a requirement for the first time in their careers. However, there were only

single cases of refusal to prepare a GA – most authors debuting in such a task faced the challenge, employed a trial-and-error approach and provided acceptable works.

Responses from section editors of the ACEM, responsible for their respective branches of medicine covered in the journal, were more diverse. While a majority of these specialists praised the authors' creativity and declared that, in their opinion, most of the submitted GAs fulfilled their function, some voiced concerns. In their opinion, many submitted GAs presented the main ideas only partially, were misleading regarding the results or conclusions, or (the most often expressed criticism) were incomprehensible without the context of the whole paper.

Reactions from readers have so far been rather lukewarm. In private communications with the editorial office, a handful of readers singled out a small number of GAs which enticed them to read the whole respective papers – in their opinion, after a quick glance at a GA, they were convinced that a given paper is worth their attention, and that reading the whole paper confirmed this conviction. Nevertheless, the experience of the ACEM editors in this regard is scarce and does not allow any firm conclusions to be drawn about the influence of GAs in this journal on its perception within the scientific community.

Rules for designing graphical abstracts

General rules

Ibrahim et al., in their primer about creating GAs, formulated the following general guidelines[25]:

Focus on the user experience. The GA is meant to be informative for the reader, not only to fulfill a submission requirement in a given journal or to impress the editors. Try to imagine what the users will learn from your GA and whether it will entice them to read the whole paper – and design the GA according to your intuition in this regard. It is your paper, and you know best what is important in it.

Clarity of purpose. Particularly regarding complex articles, the GA should present only the key features; otherwise, it will become too complicated and can even significantly distract readers from the key message. It is not always possible to provide all context and retain comprehensibility. Some simplification of the presentation may be necessary to establish a clear focus. Prioritize the key messages over completeness.

Rapid prototyping and iterative improvement. There is no one best GA for a given paper – there are always infinite alternative possibilities. Your 1st, 2nd, or 10th visual abstract will not be your best one. You will improve significantly by trying new formats and seeing what works. Design thinking focuses on what is the next step to make your GA partially better rather than what is best.

Practical tips

From the experience of the editors of the ACEM, the following more detailed tips can be offered (they are discussed at length here and concisely summarized in Table 1):

1. Choose the graphical material wisely. Since the space at your disposal is limited, each picture, pictogram, chart, or table should have informative, not only decorative value. A GA cannot exceed the size of 1 A4 page and should be less complicated than a conference poster.
2. A GA has to have a clear structure, albeit it does not have to reflect the structure of the plain text abstract or the whole paper. If it proves impossible to present materials and methods as well as results and conclusions, it can provide a more general overview of the paper. It can be read horizontally or circularly, can have a radial arrangement, or employ a more eccentric design. It can have a rectangular, square, or round shape, but it must have a clear beginning. What is important is that it cannot be a single column read from top to bottom – such a format will not be useful for a screen display.
3. Regarding text within a GA, its size should be 12–16 points (smaller fonts will not be legible online). Use text sparingly, but if you feel that conclusions should be provided as full sentences or they will be ambiguous – include full sentences in a separate box/section. Do not use abbreviations that can be unclear without reading the text (obvious acronyms, like DNA, are, of course, a good choice).
4. A GA should include the title of the paper – its contents can be incomprehensible without it.
5. Use colors consciously – e.g., to discern different parts of a GA or to highlight key issues.
6. When using materials (e.g., illustrations) prepared by someone who is not a member of your research team, make sure that you have the right to use them to avoid copyright infringement.
7. The aesthetic dimension of a GA is also important, although not paramount. If you have such option, use

Table 1. Summary of the suggestions on how to prepare a proper graphical abstract (GA)

How to prepare a GA?	
General tips	Choose the graphical material wisely – a GA cannot exceed the size of 1 A4 page.
	A GA has to have a clear structure (it can be different from the structure of the paper).
Preparing the GA	The text size within a GA should be 12–16 points.
	A GA should include the title of the paper.
	Use colors consciously – e.g., to discern different parts of a GA or to highlight key issues.
	To avoid copyright infringement, make sure that you have the right to use all material.
External help	If you have such option, use the help of a professional graphic designer.
	Consult specialists in the field, regarding the final GA, who are not members of your team.

the help of a professional graphic designer. If you do not have the opportunity or time for such consultation, do not hesitate to submit your GA as is.

8. When your GA is finished, consult with specialists in the field who are not members of your team. If they rate it as clear and informative, you have succeeded in preparing a professional GA.

Technical issues and further reading

A GA can be designed using various online, free, or more professional software. Ibrahim et al. prepared a detailed primer (57 pages) in which they – in addition to general rules – listed several suggestions on where to seek professional assistance and what software to use.[25] Practical information with multiple examples of inspiring GAs can also be found in non-scientific, albeit reliable sources such as the instructions for authors by Elsevier[26] and Cell Press,[71] guides prepared by the editorial staff of other scientific medical journals (*Diabetologia*[72] and *Journal of Allergy and Clinical Immunology*[73]), and blog entries by professionals – to name a few (among many others), Balbin and Rossi (Animate Your Science),[74] Pamplona (Mind The Graph),[75] Augustus (Researchers' Writing Academy),[76] BioRender,[77] Scribendi,[78] Edanz Learning Lab,[79] and Future Medicine.[80] Thiebes (Simplified Science Publishing) provided a set of diverse examples with free templates to create your own GAs.[81]

Common mistakes

Experience of the ACEM editors amassed during publishing 6 issues and over 50 ahead of print papers with GAs allows for listing several common mistakes made by authors in submitted GAs (since the editorial staff of the ACEM does not offer any assistance in preparing such materials, the authors are solely responsible for both the content and design of the GAs). In the opinion of the editors, the mistakes discussed below are a result of the fact that GAs as a form of disseminating scientific research results are a relatively new phenomenon, and many medical journals – including several prestigious ones – did not implement them at all. In consequence, many authors, even seasoned researchers, are simply not accustomed to such requirements and, when doing it for the first time, adhere to common misconceptions about the GAs or follow their own notions, which sometimes deviate from the stipulations published on the journal's website. The most often encountered mistakes include the following (they are discussed at length here and concisely summarized in Table 2):

1. Submitting one of the figures from the paper as a GA – in original papers, it is usually one of the charts or an RTG/MRI/USG image (if the article contains such materials). In meta-analyses, the flowchart of the meta-analysis (without other, new material) is often used. Some authors, by choosing one of the text figures as a GA, confuse the concept of a GA with the concept of a key figure – this distinction was discussed by Klaassen et al.[14] So far, there are no studies measuring the effectiveness of GAs of different quality since there are no objectives and measurable aspects to assess such quality. However, from a study by Klaassen et al., when comparing the impact of GAs with key figures, it can be deduced that submitting only a chosen figure or a compilation of figures from the paper does not boost the paper's visibility since the effectiveness of key figures proved to be lower than GAs.[14]

Table 2. A summary of common mistakes in graphical abstracts (GAs)

Common mistakes in GAs	
Common misconceptions	Submitting one of the figures from the paper as a GA.
	Compiling several or all figures (and often also tables) from the paper into a single chart.
	A GA containing only a tabulation or even only plain text in the form of highlights.
	A GA consisting only of graphical material (figures, charts, graphs) that were not used in the paper, but their meaning is unclear out of the context of the whole paper.
	A GA so complex that it is incomprehensible even if viewed in full-screen mode.
Technical mistakes	Stark color contrast between the letters and the background.
	Employing several different typefaces, types of text highlighting, and colors.
	Presenting numerical data (like p-values) whose meaning is unclear outside the context of the whole paper.

2. Compiling several (usually 2–6) or even all figures (and often also tables) from the paper into a single chart and submitting it as a GA, or pasting one or more figures and/or tables from the paper into a GA containing original material. It should be strongly stressed that a GA should be less complicated than conference posters. Therefore, it should not contain tables or figures taken directly from the paper, as well as it cannot be compiled from tables and figures from the manuscript. Most of the figures and tables taken directly from the manuscript will be illegible, incomprehensible, or unclear at best when presented out of context and downsized to fit into the GA.

3. A GA containing only a tabulation or plain text in the form of highlights. Sometimes, a flowchart with no graphical material will suffice, but there is usually a possibility to complement it with at least some clipart drawings to attract attention.

4. A GA consisting only of graphical material (figures, charts, graphs) that were not used in the paper, but their meaning is unclear out of the context of the whole paper, and the GA contains no text commentary. If the GA is not comprehensible in the presence of the paper's title and cannot be properly understood after reading the text abstract or even the whole paper, it does not fulfill its role as a GA, which should entice to read the whole article, not only

complement it. Sometimes, authors submit GAs consisting of a single figure without any comment or context, giving only a vague and general idea about the content of the paper. Such illustrations would be more suitable for a book cover than a GA.

5. A GA so complex that it is incomprehensible even if viewed in full-screen mode and requires panel-for-panel examination. In such cases, the risk of the reader becoming confused increases with each new section of the GA read. In a similar vein, some authors employ overly long text field sets with very small letters to fit into a GA viewed on screen and perceived in a single glance.

6. Stark color contrast between the letters and the background (e.g., red letters against a green background), which often makes the text – even very short – virtually illegible.

7. Employing several different typefaces, types of text highlighting (bold, italics, underlining) and colors within a relatively small space of a single GA – such visual chaos distorts any structure.

8. Presenting numerical data (like p-values) whose meaning is unclear outside the context of the whole paper.

Conclusions

Graphical abstracts exhibit a potential to improve scientific communication that remains largely unexplored. Every year, more scientific journals announce their adoption. It can be expected that GAs appearing in these journals will be more and more professionally prepared, and their impact will be deeper. There is currently no universal standard for GAs, which can lead to inconsistencies in their formats and contents.[23] Therefore, more detailed guidelines to standardize GAs for scientific research are warranted.[19] We are still at the beginning of the journey – GAs are a relative novelty, but new types of abstracts have already started to emerge: video abstracts, GAs involving GIFs, interactive GAs, etc. Science evolves – and ways of disseminating it evolve with it.

The main take-home message for the authors is as follows: 1) a GA cannot contain figures or any other material from the paper – it must be an original work, 2) the main challenge in preparing a GA is avoiding oversimplification while at the same time reflecting the content of the paper as fully as possible, 3) a GA should entice the reader to read the whole paper, not only complement it, and as literature shows, it can fulfill such a role when designed properly.

Future directions

Potential future advancements in GA design involve 2 paths of development (which are complementary, not exclusive) concerning the medium and the content of the GAs.

Video abstracts have been mentioned in the paper, albeit only in passing. However, the potential of social media and other web services based on posting video releases (YouTube, TikTok) and video materials on social media enabling such posting as one of several modes of disseminating content (Facebook, Twitter) is still largely unexplored. Zong et al. discussed the usage of video abstracts in the *New Journal of Physics*,[82] while Ferreira et al. presented their experiences from *Ecology and Environmental Sciences*.[83] The advent of broader implementation of video abstracts in medical journals seems to be only a matter of time.

The possibility of incorporating brain imaging techniques or data visualization methods to represent neural substrates (NS) directly in GAs could boost their informative capabilities. A "neural substrate" is a term used in neuroscience to indicate the part of the central nervous system (i.e., brain and spinal cord) that underlies a specific behavior, cognitive process, or psychological state. The potential of NS in general has been discussed by Tanaka et al.,[69] Tanaka et al.,[84] and Di Gregorio and Battaglia.[85] Neural network functioning in health and disease is being studied in more and more different contexts, and such graphical material could prove useful in GAs of papers concerning diverse topics from various fields of medicine.

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Does autologous fat grafting serve the need for reconstructive surgery in oral cancer patients? A prospective evaluation in cosmetic surgery patients

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Abstract

Background. The prevalence of cancer is growing daily. Oral cancer, which is primarily triggered by tobacco use, can have detrimental effects on facial appearance. Despite significant advances in the molecular underpinnings of cancer, surgery, chemotherapy and radiotherapy have become standard cancer treatment methods. These treatments remove the tumor but can significantly alter patient's appearance, which can impact their physical and mental wellbeing. The soft tissue augmentation technique of autologous fat grafting (AFG), commonly referred to as lipofilling, is frequently used in cosmetic and reconstructive surgery to promote facial rejuvenation and body form remodeling. The advantages of AFG include its biocompatibility, low immunogenicity and allergenicity, as well as its capability to heal wounds.

Objectives. To explore the advantages of and patient satisfaction with the AFG technique as a potential facial restoration procedure in oral cancer patients.

Materials and methods. We examined the effects of facial AFG in cosmetic surgery patients and investigated the prevalence of postoperative problems. Patient satisfaction and potential complications after autologous fat filling in different areas of the facial space were investigated using clinical evaluations, patient-reported outcomes and photographic assessments.

Results. All of the patients were satisfied with the results in terms of improved facial shape, skin glossiness, skin elasticity, ptosis, and facial expressions. More than 80% of the patients and surgeons reported overall satisfaction.

Conclusions. Based on these findings, we hypothesize that the AFG approach may be beneficial as a reconstructive therapy for patients with oral cancer following treatment. This technique will improve the patient's physical appearance, confidence and mental wellbeing.

Key words: reconstructive surgery, oral cancer, autologous fat grafting, fat fillers, lipofilling

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Background

Cancer is one of the leading causes of death worldwide and was responsible for approx. 10 million deaths in 2020.[1] Oral cancer is the 6th most common type of cancer and has a high incidence in South Asia.[2] Oral cancer is caused by various physical, chemical and biological carcinogens, which have drastically hampered prophylactic measures. To prevent metastasis and disease progression, chemotherapy, radiotherapy and surgery are commonly employed. Even though these treatments are effective, they can cause tissue damage, aesthetic defects, irregularities, atrophic skin, scars, and so on.[3] These side effects can significantly alter patient's appearance and have an impact on their physical and mental wellbeing.

A common soft tissue augmentation procedure used in cosmetic and reconstructive surgery to achieve body shape remodeling and facial rejuvenation is autologous fat grafting (AFG), commonly referred to as lipofilling.[4,5] The AFG is known for its benignity, reliability, predictability, and biocompatibility, and is an affordable technique used to improve tissue deformities and for reconstruction. Fat cell grafts are considered the closest acceptable autologous soft tissue, and these cells can be easily harvested with low donor-recipient morbidity, immunogenicity and allergenicity. In addition, these cells promote wound healing due to the presence of cytokine profiles and certain extracellular matrices.[6,7]

Moscatello et al.[8] showed that an increase in the important surface area of the recipient bed's troubled fat lobules led to improved fat survival after infusion. Li et al. examined facial fat filling under the anatomical structure of the facial fat chamber.[9] They proposed that injecting a small amount of fat into the subcutaneous layer was enough to make the transition between the zygomatic arch and the buccal and mandibular regions look natural, which provided a personalized approach based on the facial fat compartment theory.[10,11] The aforementioned technique overcomes the disadvantages of other grafting cosmetic surgery techniques and artificial materials, such as prostheses, hyaluronic acid and botulinum toxin type A.[12]

The current study aims to evaluate the feasibility of utilizing the AFG technique to improve facial aesthetics in oral cancer patients after cancer treatment. We compared the impact of autologous fat in the face area before and after the surgery, as well as the occurrence of postoperative problems. After the autologous fat filling in various areas of the facial space, patient satisfaction and potential problems were examined further.

Objectives

The aim of this research was to explore the advantages of utilizing the AFG technique as a potential facial restoration procedure in oral cancer patients to promote physical and mental health after treatment.

Materials and methods

Ethical approval

This study was approved by the Institutional Review Board at Shandong Provincial Hospital, Jinan, China (approval No. SDPH202139002), and was performed in accordance with the guidelines of Good Clinical Practice and the Declaration of Helsinki. Written informed consent was obtained from all patients after explaining the limitations, benefits and realistic surgical outcomes. Preoperative images were also obtained.

Inclusion and exclusion criteria

A total of 52 patients who underwent AFG for facial rejuvenation between January 2016 and December 2020 were enrolled in the study, and were prospectively followed up at 3, 6, 8, 12, 24, and 36 months after the procedure. The inclusion criterion was age ≥18 years. Patients who had a positive pregnancy test or were breastfeeding were excluded from the study. Other exclusion criteria were current infection at the treatment site and systemic diseases (such as diabetes, hypertension, iatrogenic coagulation disorders, disorders of lipid metabolism, and history of connective tissue disorders). Preoperative laboratory tests, including a complete blood routine, comprehensive metabolic panel and prothrombin time/partial thromboplastin time, were performed to assess current health status of the patients.

Surgical procedure

Donor site and marking

Considering the availability of accessible adipose tissue and the individual facial features of each patient, the outer thighs (saddlebags) were mainly selected as the donor site. However, the lower abdomen, inner thighs, periumbilical region, and buttocks were occasionally used. Preoperative marking of the surgical site was done with the patient standing before administering general anesthesia.

Tumescence and fat harvesting

Harvesting and subsequent fat injection were performed using a previously published method.[5] A tumescent solution (500 mL) containing normal saline, 0.5 mL of 0.1% epinephrine (1:100,000) and 10 mL of 2% lidocaine was administered through a multi-hole infiltration cannula (Wells Johnson, Tucson, USA; diameter: 1.5 mm). In addition, 15 mL of 2% lidocaine was injected as a local anesthetic. The entire procedure was carried out with extreme caution. The tumescent solution was injected gently from one site to another until maximum vasoconstriction was achieved at the fat donor site. After waiting for

10 min, a 10-milliliter Luer-Lock syringe (Sigma-Aldrich, St. Louis, USA) connected to a liposuction needle with a blunt 3-millimeter tip was used to extract the adipose tissue under negative pressure, minimizing potential oxidation and injury to the adipose tissue.

Fat purification

After collection, the fat was allowed to stand to eliminate excess water and blood. For this procedure, the 10-milliliter syringe that was utilized for fat harvesting was used. Then, the syringe was capped and centrifuged at 3000 rpm for 3 min, resulting in a separation of the fat grafts into 3 distinct layers: oil in the upper layer, intact adipose aspirates in the middle layer and cell debris in the lower layer.[6] Finally, the deep portion of the middle layer was collected in a 1-milliliter syringe, capped and stored in an upright position until subsequent use.

Fat implantation

A disposable 18-gauge needle was used to treat the temporal, frontal and orbital facial regions (at the forehead, upper anterior space of masseter muscles, corner regions of the mouth, zygomatic arch, and areas surrounding the eye). Before fat grafting, all patients (regardless of the anesthetic modality) were injected with the tumescent fluid (same composition as described above) to fill the facial spaces. The surgeon decided on the graft volume after considering individual characteristics of a patient. An injecting cannula was used to transfer fat from a 1-milliliter syringe to the recipient's facial site. Special attention was paid to homogenously deposit the fat. Finally, oral antibiotics were administered to the injected area of the face to avoid microbial infection.

Aesthetic outcome evaluation

At the end of each follow-up visit, aesthetic improvement was evaluated by the operating surgeon. A 4-point Likert scale was used to assess the results, with 1 = excellent (for utterly satisfactory results), 2 = good (for substantially satisfactory results), 3 = fair (for somewhat satisfactory results), and 4 = poor (for unsatisfactory results). Patient satisfaction was measured using a questionnaire based on 5 evaluation indicators: correction of facial expressions, alleviation of facial droop, improvement of fine facial lines, and naturalness of facial expression, with each given a score out of 20. A score over 90 points was considered excellent, 81–90 was considered good, 70–80 was considered moderate, and ≤70 was considered poor. The degree to which patients were satisfied with their surgical outcomes was derived based on the following formula (Equation 1):

$$\text{patient satisfaction index (PSI) \%} = \frac{(\text{excellent score} + \text{good score}) \times 100}{\text{maximum possible score}} \quad (1)$$

Regular postoperative care was administered for the first 7 days after the surgery. In addition, the injected fat volume, follow-up period, observed complications, treatment site, and patient satisfaction scores were recorded.

Results

In the present study, most of the participants were female (female-to-male ratio: 16:1). Several facial regions, including the superior temporal space, anterior zygomatic space and buccal clearance, were used as filling sites. The patients' age ranged from 28 to 62 years, with a mean age of 41.6 ±9.2 years. The mean follow-up period was 18.3 months (range: 6–36 months, excluding a 28-year-old female patient who was followed up for 40 months). Table 1 summarizes the quantity of fat that was injected into each site (range: 5–20 mL), which was based upon the facial morphology and the desired corrections. The anterior zygomatic, upper anterior masseter, superior temporal, and frontal reticular tissues were the most frequent injection locations. The frontal reticular tissue received the maximum fat volume (20 mL).

During follow-up visits, cosmetic evaluation of preoperative and postoperative photographs showed 100% patient

Table 1. Injected volumes per facial site and their effects

Filling position	Number, n (%)	Filling amount [mL]	Effects
Frontal reticular tissue layer	43 (82.7)	20	projecting forehead
Superior temporal space	48 (92.3)	4–6	projecting forehead
Zygomatic anterior space	52 (100)	2–3	correcting the central sag, projecting apple muscle
Upper anterior space of masseter	49 (94.2)	4–8	correcting the depression under the zygomatic arch on both sides of the cheeks and lifting the saggy jaw
Buccal clearance	23 (44.2)	2	correcting the sag outside the corner of the mouth
Anterior inferior orbital space	10 (19.2)	0.5–1	correcting the tears ditch
Superior anterior orbital space	4 (7.7)	0.5–1	correcting the upper eye socket depression

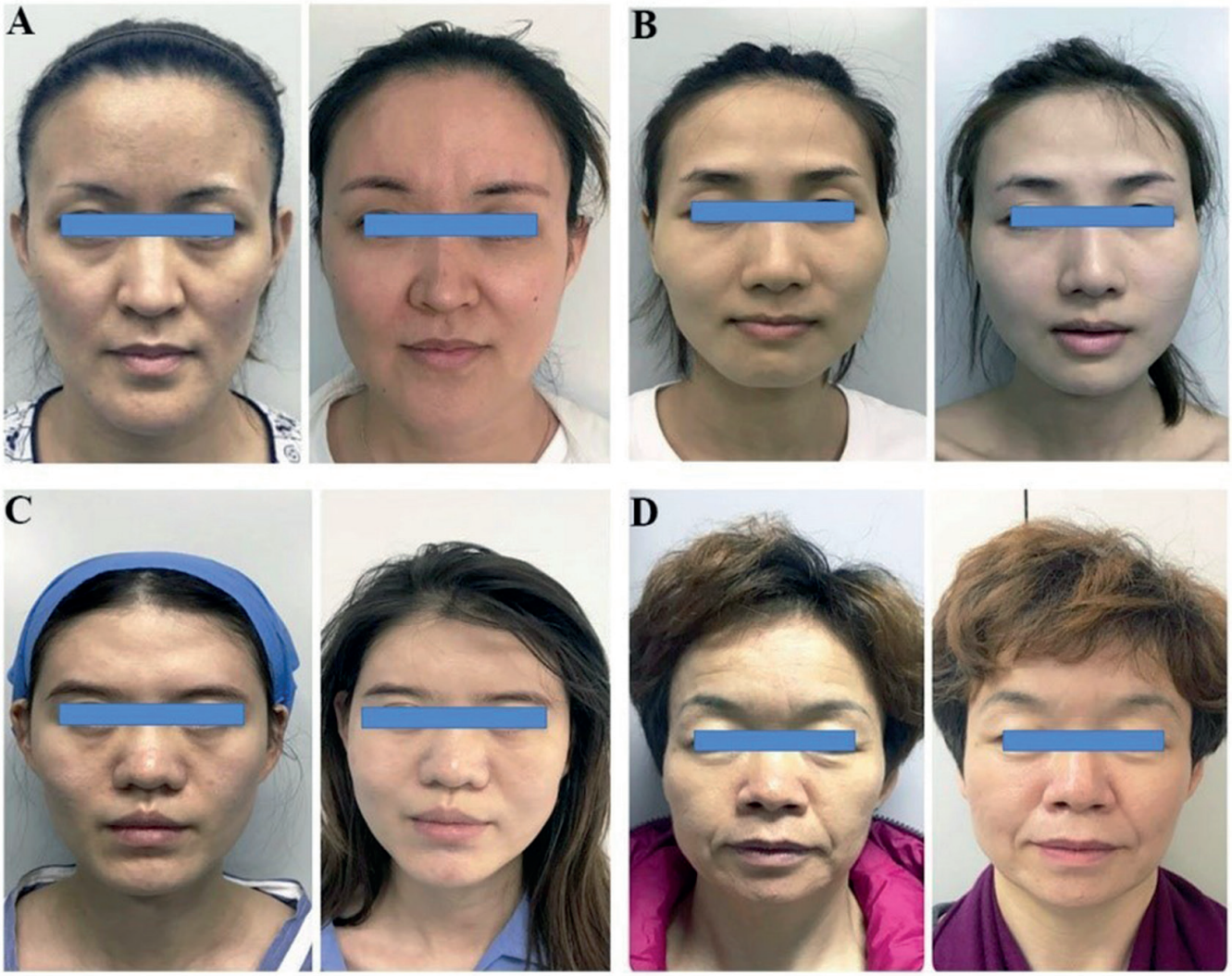

Fig. 1. Representative patients who received fat grafts for facial rejuvenation. A. Preoperative (left) and 6-month postoperative (right) images of a 54-year-old female. The frontal reticular tissue layer, anterior zygomatic region, the upper part of the anterior masseter, buccal area, and anterior inferior orbital septum showed aesthetic improvements (case 1); B. Diagonal view (right and left) of a 36-year-old female. Preoperative (left) and 6-month postoperative (right) images. Filled areas included the anterior superior and inferior orbital septa (case 2); C. Right and left diagonal view of a 40-year-old female. Preoperative (left) and 12-month postoperative (right) images. The anterior zygomatic area, the upper part of the anterior masseter and the buccal regions received cosmetic enhancement (case 3); D. Frontal view of a 46-year-old female. Preoperative (left) and 36-month postoperative (right) images. Filled areas included the frontal reticular tissue layer, the anterior zygomatic region (2 grafts), the upper part of the anterior masseter (2 grafts), the buccal region, the anterior superior and the anterior inferior orbital septum regions (case 4)

satisfaction in terms of improved facial contours, skin glossiness, skin elasticity, ptosis, and facial expressions. More than 80% of both patients and surgeons reported feeling generally satisfied, as shown in Table 2. Figure 1 illustrates 4 representative cases of cosmetic improvement achieved after 6, 12 and 36 months of follow-up. Standardization and facial portion analysis through meticulous photography are critical steps in assessing the postoperative results of fat grafting, and are typically achieved through 2D photography.[13] The 2D imaging was used to evaluate the aesthetic results of the surgery, which showed a high level of satisfaction among patients and surgeons.

Table 2. Evaluation of treatment

Evaluation	Insufficient correction, n (%)	Sufficient correction, n (%)
Patient	–	52 (100.0)
Surgeon	9 (17.3)	44 (84.6)

Discussion

In this study, although we could not estimate the amount of fat retained at each facial site and only knew the total amount of fat grafted, the atrophy corrections for different facial compartments were significant and lasted for the entire follow-up period. Despite this, 9 patients underwent additional fat grafting sessions to correct asymmetries, likely due to differences in graft vascularization, delaying the healing of the initial graft.

As the human body ages, numerous facial changes occur. Gravity causes face soft tissue to descend, forming unattractive folds and shadows (e.g., nasolabial folds, tear troughs, marionette lines) that contribute to an aged appearance. In addition, as patients age, their facial volume decreases due to a combination of skin thinning, muscular atrophy, fat volume loss, and bone thinning.[13] While gravity-related changes are traditionally treated with resuspension procedures such as face lifting, brow lifting, midface lifting, and neck lifting, the loss of facial volume is treated with a variety of filler materials, which in some cases may produce results comparable to surgical lifting. Due to their effectiveness, fat grafting and dermal filler injections have recently gained popularity as supplementary facial rejuvenation treatments.[14] Fat grafting requires a comprehensive knowledge of facial anatomy and the interactions between various structures and fascial planes in order to obtain satisfactory results and reduce complications.[15]

In the past, a number of dermal fillers have been tested with varying degrees of success. However, fat fillers have gradually become a cheaper and more effective replacement for augmenting soft tissue for cosmetic indications.[16] Other than being employed either as a standalone technique or as an adjunct to other facelift surgeries, AFG has also been used in breast reconstruction, periocular facial rejuvenation, and for the correction of congenital deformities (Parry–Romberg syndrome, Poland syndrome, Dupuytren's and Raynaud's diseases, and pectus excavatum), treatment of burn scars, hand rejuvenation, augmentation rhinoplasty, and breast and gluteal augmentation.[17,18]

Clinical observation studies have demonstrated long-term differences in the outcomes of fat implantation at various anatomical facial region planes.[16] In addition, it has been shown that the quantity of the fat graft is connected with the volume of adipose tissue in the patients. Lipomodelling increases the volume of body regions. Moreover, the presence of mesenchymal stem cells in the adipose tissue plays a crucial role in repairing tissue deformities.[19]

The harvested fat, which is often extracted from the flank, inner thigh or abdomen, is injected into the recipient locations only after careful planning. It is crucial to identify the proper position and depth of the fat compartments that will receive the grafts. The primary fat compartments of the face are separated by the superficial musculoaponeurotic system into the superficial and deep fat spaces.[20] Each of these regions can be further subdivided within the same plane, and the identification of suitable recipient sites requires substantial skill. The superficial fat space is comprised of the nasolabial fat pad, superior, inferior and lateral orbital fat pads, temporal and forehead fat pads, jowl fat pad, and medial, middle and lateral cheek fat pads. The medial and lateral suborbicularis oculi fat pads, the suborbicularis oris fat pad, the deep medial and lateral cheek fat pads, and the buccal fat pad make up the deep fat space.[21] Understanding these subdivisions enables injectors to choose ideal areas for fat transfer and to determine the necessary volume and the most effective injection procedures and equipment.

The AFG for facial rejuvenation improves facial volume and revitalizes skin by enhancing its tone and texture. Despite the fact that fat grafting is typically utilized for cosmetic purposes, it is a significant, minimally invasive technique that can be used for a number of reconstructive indications.

Age-related facial alterations that can be treated with AFG include forehead hollowing, sagging cheeks, thinning lips, deep rhytides (especially on the forehead and glabella), temporal depressions, supraorbital depressions, deformities of the tear trough, a deep nasojugal groove, malar bags, nasolabial creases, marionette lines, poor jawline contour or jowls, and a receding chin. In addition to facial aging, AFG can be performed alone or in conjunction with facial rejuvenation to address a number of additional issues.[22] Those include nasal contour anomalies, scars, burns, radiation dermatitis, human immunodeficiency virus-associated lipodystrophy, facial deformities (congenital, acquired or traumatic), facial asymmetry, and flap augmentation.

In many cases, fat grafting may not be the most effective technique for rejuvenation and may even be dangerous, especially if general anesthesia is used. The AFG may be contraindicated when there is a significant probability of fat volume instability, such as in cases of targeted weight loss or continuous weight increase, or if the patient has a history of complete or partial resorption of previously grafted fat. Patients receiving fat injections for breast reconstruction or recontouring should also be informed that the injected fat may produce nodules or calcifications that may seem malignant on subsequent imaging.

Patients with local, regional or systemic disorders that impede blood flow or wound healing are also susceptible to complications and unsatisfactory outcomes. Examples include significant burn scarring, radiation therapy, keloid scarring, coagulopathies, the necessity for immunosuppressant drugs, and other metabolic or chronic disorders.

Previously, Li et al. proposed a fat grafting method based on a compartment distribution model, delineating functional zones from transitional zones for the distribution of fat in different anatomical recipient sites.[9] In this study, the authors advocated grafting fat tissue deep into the tissue layer for the premaxillary area and into the subcutaneous layer for the nasal labial groove, brow tail and temporal region. When fat grafts were implanted in the frontal region or the cheek area, the sub-galea layer and below the zygomatic arches were preferred locations. Similarly, other researchers have explored different strategies to graft fat cells in various facial layers (single or multiple planes) for more satisfactory patient outcomes. While lipofilling is generally considered a safe procedure, several publications have reported various sequelae, ranging from minor skin irregularities, asymmetries and prolonged edema to devastating complications, such as systemic infections, bleeding, graft hypertrophy, fat necrosis, fat embolism, and

cerebral infarction, resulting from the intravascular injection, improper intraoperative disinfection and liposuction contamination.[12–17]

Despite thorough collection, processing and grafting processes, fat grafting is associated with various complications. The most commonly observed sequelae are bruising, edema, ecchymosis, overcorrection due to the transfer of extra fat, and undercorrection caused by inadequate fat transfer or high resorption of the grafted fat. Other complications include pyogenic granulomas or cellulitis due to infection and/or persistent inflammation. Bleeding, scar tissue bunching or palpable abnormalities may be caused by superficially placed injections. Further problems may include contour abnormalities and the presence of nodules or calcifications. Pain at the donor site may also result from insufficient anesthesia. In addition, deformities caused by excessive or uneven fat harvesting or violations of an adhesion zone may occur. Blindness may also result from ocular artery occlusion, stroke from internal carotid artery occlusion or fat emboli.

In the current study, swelling and edema were the most common patient complaints, which is an expected outcome of the normal postoperative course. However, 2 patients developed complications, including a bulged superior orbital septum (adjusted using fat aspiration after 6 months) and a soft tumor deep into the right cheek (corrected by graft manipulation). No other complications (e.g., donor site hematoma, nerve or vascular injury, necrosis, calcification, or surgical site infections) were observed.

Autologous fat joining is thus an integral tool that can be used by plastic surgeons for facial rejuvenation. Notwithstanding its volumizing impacts and skin rejuvenation, facial fat joining is an optimal filler due to its long lifespan, low cost, compatibility with living tissue, low incidence of complications, and high fulfillment rates.[23,24] A more comprehensive understanding of facial maturing mechanisms, including fat decay and ptosis of the distinctive facial compartments, has permitted fat joining to be regarded as a potential procedure for facial restoration.[25,26]

Limitations

This study included participants with a female-to-male ratio of 16:1. As the number of male participants was limited, we were unable to determine the male satisfaction level with the AFG technique. In future studies, more male oral cancer patients should be recruited to ensure a female-to-male ratio of 1:1. In addition, the patients' age ranged from 28 to 62 years, which excluded teenagers. Adolescents may show different satisfaction levels or recover faster than adults and the elderly. Future studies should divide the participants into smaller age groups (e.g., 18–27 years old, 28–37 years old, etc.). As only Asian patients were involved in this study, the results may not be representative of other races.

Conclusions

We investigated the impact of autologous fat injections into the facial area in cosmetic surgery patients and examined the prevalence of postoperative problems. In addition, patient satisfaction and complications after autologous fat filling in different areas of the facial spaces were investigated through clinical evaluations, patient-reported outcomes and photographic assessments.

Autologous fat filling in the facial spaces positively affected facial rejuvenation and improved aesthetics. This technique is a safe and effective surgical procedure, worthy of clinical application. In addition to these effects, the presence of stem cells in the adipose tissue can provide additional support for tissue repair. Thus, the AFG technique may offer significant advantages for oral cancer patients to improve their facial appearance after surgery. The present study provides insights into the use of the AFG technique as a cheap and effective surgical procedure to improve deformities, irregularities, facial lines, and depressions, thereby enhancing the quality of life and the physical and mental health of oral cancer patients.

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Suicide among doctors in Turkey: Differences across gender, medical specialty and the method of suicide

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Abstract

Background. Doctors have higher rates of mental illness and suicide than the general population worldwide. Suicides of doctors are known to be underreported in developing countries. To the best of our knowledge, there are no studies investigating suicides among medical students and doctors in Turkey.

Objectives. To investigate the characteristics of suicides in medical school students and doctors in Turkey.

Materials and methods. In this retrospective study, newspaper websites and Google search engine were searched for information on medical school student and doctor suicides in Turkey between 2011 and 2021. Cases of suicide attempt, parasuicide or deliberate self-harm were not included in the study.

Results. Sixty-one suicides were reported between 2011–2021. There was a male predominance (45 (73.8%)), and more than half of the suicides (32 (52.5%)) occurred among specialist doctors. Self-poisoning, jumping from heights and firearms were the most common methods of suicide (18 (29.5%), 17 (27.9%) and 15 (24.6%), respectively). Cardiovascular surgery, family medicine, gynecology, and obstetrics specialties had the highest numbers of suicide deaths. Depression/mental illness was the most common speculated etiology. These results show that suicides among medical students and doctors in Turkey have characteristics that differ from both suicided among the general population in Turkey and doctor suicides in other countries.

Conclusions. In this study, we identified the suicidal characteristics of medical students and doctors in Turkey for the first time. The results help us to better understand this understudied topic and provide an avenue for future studies. The data also indicate that it is important to monitor the individual and systemic difficulties experienced by doctors, starting from the medical education stage, and to provide individual and environmental support to help decrease the risk of suicide.

Key words: suicide, medical student, doctor

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Background

Although being a medical doctor is still one of the most respected and desired professions, medical education and working in the medical field can be both stressful and risky. Doctors generally work under stressful conditions, facing the increasing demands of patients and governments. The professionalism required for this job and some structural factors within the work environment increase the pressure on doctors, and this pressure continues to rise.[1] In recent years, the number of studies on mental health and suicide among physicians has increased.[2,3]

Similarly to the general population, the most common mental disorders reported among physicians are depression and anxiety. In a meta-analysis that included over 17,500 resident physicians from 18 different countries, it has been reported that the rate of clinically significant depressive symptoms was 28.8%.[4] It has also been reported that 13% of medical students were depressed, as compared to 7.8% in an age-matched control sample.[5] Moreover, while depressive symptoms are reported by approx. 30% of the members of the general population, this rate is observed to be at 41% in doctors, and while anxiety is present in approx. 26% of the members of the general population, it has been reported at a rate of 40% in physicians.[6]

However, it has been questioned how accurate it is to compare the mental health of doctors with the general population, and it was decided that comparing it with other professional groups would yield better results.[7] Indeed, studies have shown that estimates of depression and anxiety are higher when measured in occupational groups than in the general population, and that mental disorders in doctors are seen at rates close to other occupational groups.[8,9]

While the frequency of mental disorders is consistently higher in doctors than in the general population, findings on suicide rates seem to be a little more complex. In a recent study, it has been reported that doctors have a significantly higher suicide rate (average of 1.3%) than the general population (average of 0.8%).[10] Other studies comparing suicide rates in doctors with the general population have yielded varying results; many studies have reported that suicide rates among doctors are 2.5–7 times higher than in the general population.[11,12] In contrast, several studies have reported that doctor suicide rates are indistinguishable from the general population.[13] There are also studies showing lower suicide rates among doctors compared to the general population.[14] Although the findings are mixed, a systematic review and meta-analysis calculated an overall standardized mortality rate of 1.44 for suicides among physicians,[11] and another meta-analysis showed a significantly higher suicide mortality ratio (SMR) in female doctors compared with women in general, and a significantly lower SMR in male doctors compared with men.[15] The risk of suicide was higher in junior trainees compared to senior ones, in divorced doctors and in doctors without children.[9,11]

There is also emerging evidence for a link between the area of specialty and the risk of suicide.[15] Although an older study reported no differences between specialties,[16] many studies have shown that suicide rates are higher in anesthesiology, psychiatry, general practice, and general surgery.[1,15] It is unclear if these findings are due to these specialists having higher rates of poor mental health, increased access to suicide means (e.g., highly lethal drugs), more barriers to seeking help, or a combination of these and other factors.[17]

Many risk factors related to increased mental problems and suicide rates have been identified in the literature. It is emphasized that both individual and environmental risk factors contribute to the high incidence of mental disorders in doctors.[1] Individual risk factors for doctors include genetic predisposition, adverse childhood experiences, experiences of loss, and preexisting psychiatric disorders, which are similar in the general population.[2,3] Neurobiological and neurophysiological abnormalities have also been shown to increase the incidence of suicidal behaviors.[18–25] Abnormalities in the serotonergic system and aberrant functioning in specific brain areas such as the prefrontal cortex, orbitofrontal cortex, amygdala, and nucleus accumbens have been reported.[8,26–28] A dysregulation of the immune system and inflammatory processes have also been linked to the pathophysiology of suicidal behavior.[29–34] Moreover, relationships between neurodegenerative processes and both mental disorders and suicide are well known. In addition, it has been shown that coronavirus disease 2019 (COVID-19) increases complaints of anxiety, depression and suicidality in almost all age groups, especially in young people.[35]

Traits that can be more common in doctors, such as perfectionism, obsessiveness, increased drive, feeling of inadequacy, individualism, and ambition, can also be associated with an increased risk of mental disorders.[36] In addition to these traits, situations such as an excessive workload, short rest periods, a lack of support, increasing demands, increased paperwork, financial difficulties, disruptions in the healthcare system, inability or lack of possibility to take time off or to relax, or a lack of safe spaces where doctors can refresh themselves also create the basis for mental disorders.[1,3,37] It has been proposed that the modern lifestyle can decrease mental resilience and lead to the development of mental disorders.[38] In addition, the strain placed on doctors across the world during the COVID-19 pandemic has worsened this situation. Many studies have shown an increase in acute self-reported symptoms of depression and anxiety, as well as high rates of insomnia and distress.[39] Moreover, in many countries, physicians have faced increasing bureaucratic and administrative burdens and healthcare system constraints that

have tended to increase year over year, resulting in an erosion of their income and social status.[40]

It has also been suggested that the increased suicide risk in doctors is associated with the increased access to more dangerous means of suicide.[41] For example, an increased use of drugs for suicide by doctors has been reported in different countries.[16] While it is known that the methods of suicide can vary according to geographical location and gender,[15,42] the existing literature has also sought to identify the potential etiologies of doctor suicides.[43]

It has been found that doctors who have died by suicide tended to have multiple complex problems, including mental health issues. Although mental disorders and suicidal thoughts in doctors are more prevalent than in the general population, it is also known that the likelihood of searching for professional help is relatively low.[44] Both medical students and doctors are usually resistant to help-seeking and treatment, and are unwilling to disclose their mental health problems.[45–47] Barriers to accessing care include the fear of stigma, the perception that they should be able to look after themselves and that seeking help is often seen as a sign of weakness, as well as confidentiality issues and potential problems with licensing.[1,48] These barriers constitute a risk for medical students, trainees, doctors, their patients, and the public as a whole.[49] Medical professionals usually prefer nonformal ways to receive support by informally consulting their friends, families, peers, and colleagues.[50] This generally results in a lack of care or delays in accessing healthcare, as well as in self-medication and, for some, harmful behaviors.[51]

Although the number of studies on suicide in Turkey is substantial, there are hardly any studies examining specific occupational groups. In a study covering the years 2009–2018, it was observed that the crude suicide rate in the general Turkish population was 3.61–4.37 for a population of 100,000, and that this rate was higher in men than in women (male: 5.03–6.30, female: 1.81–2.43).[52] Despite the information on the general population, there are no known statistics or information about suicides among medical students or doctors in Turkey.

Objectives

Suicide rates vary between countries and studies regarding the suicide of medical doctors remain rare.[53,54] It has also been reported that doctor suicides are widely underreported in developing countries.[2,53] Although the topic has been widely covered in the Turkish press and media, to the best of our knowledge, there is no scientific study investigating suicide among Turkish doctors. There may be a lack of knowledge on this subject due to cultural factors, fear of the stigma related to suicide, and the lack of an association that systematically collects such data. Therefore, this study aimed to examine the descriptive data on the suicides of medical school students and doctors in Turkey by using information reported in the national press and found on Google search engine. We aimed to determine demographic parameters such as age, gender, medical specialty, mortality rates, and the possible etiology of suicides.

Materials and methods

This retrospective descriptive study was conducted in adherence to the Declaration of Helsinki. The researchers were provided with fully anonymized data. The study was approved by the Marmara University Clinical Research Ethics Committee (approval date 05.11.2021, approval No. 09.2021.1275), and the need for written informed consent was waived.

Study design

The Turkish Statistical Institute (Türkiye İstatistik Kurumu (TÜİK)) has been collecting data on suicide events in the country since 1962; all the relevant data are available on the Institute's website. However, it is not possible to extract the suicide rates for doctors from these statistics. As it is not possible to obtain such data from official sources, and the literature mentions the use of newspaper news for this purpose,[12,55,56] the use of online news portals seemed to be the only viable alternative to achieve the aim of this study.

After obtaining the approval from the ethics committee, the online data of doctors who were reported to have committed suicide in the national Turkish press over the last 11 years were collected retrospectively.

Setting

To obtain relevant data, the news reports found on Google and in the online archives of 10 major Turkish national newspapers based on circulation numbers were analyzed. The search was performed retrospectively using pertinent search words individually or in combination, and reports published in Turkish from January 2011 to December 2021 were taken into account. Both Google database and newspaper websites were thoroughly searched for applicable news using the following key words: "doctor suicide", "doctor death", "medical student suicide", and "medical student death." Three authors initially reviewed the links (MY, BA and SHE) and 4 authors (KDB, BÖ, HŞ, and MSE) extracted the data from relevant links. All news links were screened using eligibility criteria. Repetitive cases were examined and the repetition of data was prevented. Due to ethical concerns, name of cases, places of suicide and hospital information were not included in this study. A thorough content analysis of each suicide report was done.

Cases

Completed suicides committed by medical students and doctors reported on Google search engine or on newspaper websites between 2011 and 2021 were included in the present study. Our target population included suicide cases involving only medical students and doctors. Cases with a suicide attempt, parasuicide or deliberate self-harm were not included. We also excluded cases in which autopsy ruled out suicide, and dubious cases where a suicide, homicide or accident was suspected but not confirmed. In line with the literature, self-poisoning was defined as taking a drug overdose or ingesting substances never intended for human consumption, and self-injury was defined as causing physical injury by means such as cutting. It should be noted that the underreporting of suicide cases in medical students and doctors may be a major confounding factor in the present study.

Variables

The following information was taken from each report: sociodemographic variables (age, gender, specialty, and degree of expertise), suicide method, place of suicide, and other related variables.

Statistical analyses

All descriptive variables (sociodemographic and suicide-related variables) were analyzed using R software v. 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria). Percentages were calculated and the Pearson's χ^2 test was used for comparisons. The level of significance was fixed at $p < 0.05$, at a 95% confidence interval (95% CI).

An estimated mortality rate (per 100,000 medical students/year or per 100,000 doctors/year) for deaths by suicide was calculated using publicly available total medical school student (https://istatistik.yok.gov.tr/) and doctor (https://data.tuik.gov.tr/ and https://sbsgm.saglik.gov.tr/) data. In Turkey, between 2011 and 2021, the mean number of medical students was approx. 74,976 per year and the mean number of doctors was approx. 158,492 per year. For the calculations, annual country-specific data were obtained from https://www.theglobaleconomy.com/rankings/doctors_per_1000_people/ and yearly population data were obtained using the Google search engine.

Results

Sociodemographic characteristics of suicide cases

While 16 (26.2%) of the 61 cases in our study were female, 45 (73.8%) were male. Sixteen (26.2%) of the cases were married, 1 (1.6%) was single and without a partner, 1 (1.6%) was single and had a partner, and 4 (6.6%) were widows or widowers. Data on marital/relationship status could not be obtained in 39 (63.9%) cases. With regard to age, 24 (39.3%) cases were under the age of 35 years, 17 (27.9%) were between the age of 35 and 49 years, 11 (18%) were between the age of 50 and 64 years, and 3 (4.9%) were over the age of 65 years (Table 1).

The entire study group was divided according to their academic rank into students (8 (13.1%)), assistants (10 (16.4%)), practitioners (32 (52.5%)), specialists (32 (52.5%)), and academics (6 (9.8%)) (Fig. 1).

Among all of the groups, drug/chemical ingestion emerged as the most common method of suicide (18 (29.5%)). Jumping from heights was the 2nd most common method of suicide (17 (27.9%)), followed by 15 cases (24.6%) involving firearms, 7 cases of hanging (11.5%), and 1 case

Table 1. Distribution of the age of healthcare providers who died by suicide

Age group	n (%)
<35	24 (39.3)
35–49	17 (27.9)
50–64	11 (18.0)
>65	3 (4.9)
No data	6 (9.8)
Total	61 (100.0)

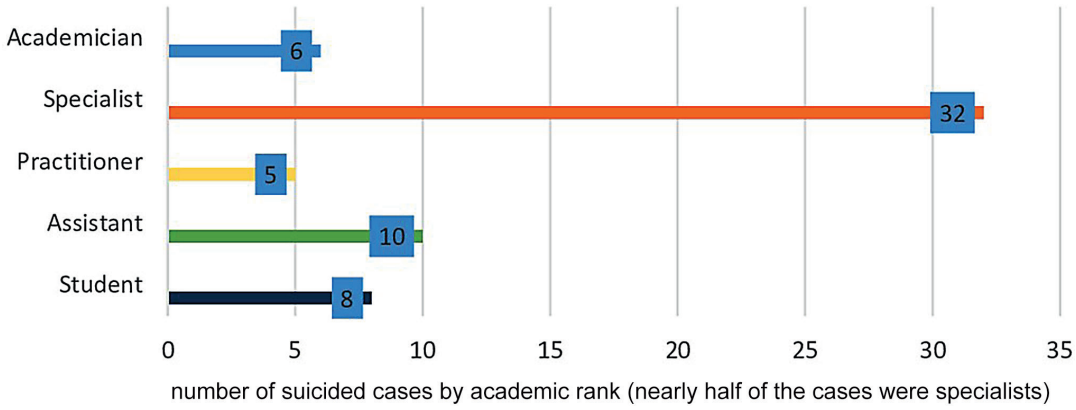

Fig. 1. Distribution of academic ranks across the medical doctor suicide cases

Table 2. Distribution of suicide methods

Method	n (%)
Drug/chemical use	18 (29.5)
Jumping from heights	17 (27.9)
Firearms	15 (24.6)
Hanging	7 (11.5)
Poisoning with natural gas	1 (1.6)
No data	3 (4.9)

(1.6%) of poisoning with natural gas. No data could be obtained in 3 (4.9%) cases (Table 2).

Specialty distribution

Of all the suicide cases, there were 7 (11.5%) cardiovascular surgeons, 7 (11.5%) family doctors, 6 (9.8%) gynecologists and obstetricians, 5 (8.2%) general practitioners and 5 (8.2%) pediatrics doctors, as well as 4 (6.6%) internal medicine and 4 (6.6%) emergency doctors. There were also 2 (3.3%) doctors of each of the following specialties: anesthesiology, orthopedics, psychiatry, radiology, and urology, and there was one case (each: 1.6%) from each of the following specialties: biochemistry, infection, physical therapy and rehabilitation, general surgery, and neurosurgery (Table 3).

Estimated suicide-specific mortality rates

Between 2011–2021, there were 8 student deaths and 53 doctor deaths resulting from suicide. The estimated suicide rate was 0.97^{-5} for students and 3.04^{-5} for doctors.

Table 3. Distribution of medical specialties

Specialty	n (%)
Cardiovascular surgery	7 (11.5)
Family medicine	7 (11.5)
Obstetrics and gynecology	6 (9.8)
General practitioner	5 (8.2)
Pediatrics	5 (8.2)
Emergency medicine	4 (6.6)
Internal medicine	4 (6.6)
Anesthesiology	2 (3.3)
Orthopedics	2 (3.3)
Psychiatry	2 (3.3)
Radiology	2 (3.3)
Urology	2 (3.3)
Biochemistry	1 (1.6)
Infectious diseases	1 (1.6)
Physical therapy and rehabilitation	1 (1.6)
General surgery	1 (1.6)
Neurosurgery	1 (1.6)
Student	8 (13.1)
Total	61 (100.0)

Table 4. Variability of suicide methods between surgeons and other groups

Method	Surgeon	Not surgeon	Total
Self-injury	10	29	39
Self-poisoning	10	9	19
Total	20	38	58

When we look at the relationship between the method of suicide and the victim being a surgeon or nonsurgical doctor, 10 of the 20 surgeons committed suicide by self-poisoning, while 9 out of the 38 nonsurgical doctors used this method (χ^2 test, $p = 0.042$; Table 4).

When we look at the environment in which the suicide occurred, there were 16 (26.2%) doctors who committed suicide in the hospital and 37 (60.7%) who committed suicide at home. While the place of suicide for 4 doctors was listed as a public space, there was 1 (1.6%) doctor who committed suicide in prison. For 3 (4.9%) cases there were no available data regarding location.

Speculated etiologies of suicide

After the examination of the news articles about the suicides, speculated etiology for 21 (34.4%) cases was listed as depression/mental illness. This was followed by family problems (6 (9.8%)) and mobbing (6 (9.8%)). The other listed etiologies were financial problems (1 (1.6%)), serious illness (1 (1.6%)) and other (1 (1.6%)). No etiology was speculated for 25 cases (41%) (Table 5).

Table 5. Distribution of speculated etiologies of suicides

Etiology	n (%)
Depression/mental illness	21 (34.4)
Family problems	6 (9.8)
Workplace problems/mobbing	6 (9.8)
Financial problems	1 (1.6)
Serious illness/cancer	1 (1.6)
Other	1 (1.6)
No etiology determined	25 (41)
Total	61 (100.0)

Discussion

The main objective of the present study was to examine the sociodemographic variables associated with cases of doctor suicides in Turkey. In this study, we describe the general characteristics of 61 suicide cases among medical doctors and students over an 11-year period (2011–2021). The majority of the cases included in our study were male, and more than half of them were specialist doctors. The 3 most commonly used suicide methods were self-poisoning, jumping from heights and firearms. The medical specialties with the highest number of suicides were

cardiovascular surgery, family medicine, and obstetrics and gynecology. Depression/mental illness was the most commonly speculated etiology for suicide cases among medical students and doctors.

While discussing the data, separating the student group from the doctors will make it easier to compare the current findings with other studies. In our study, it has been observed that there were 8 medical student suicides over a period of 11 years. Although there are few studies examining suicide in medical students, a study in the USA reported 6 suicides over 5 years,[57] a study in Austria reported 6 suicides over a 5-year period,[58] and a study in Canada reported 6 medical student suicides over 10 years.[59]

Previous studies have reported rates of 0 to 39.6/100,000 completed suicides among undergraduate medical students.[59] When calculated over the the 11-year period, the suicide rate among undergraduate medical students in Turkey was 0.97/100,000 medical students/year. When looking at relevant age comparison groups for medical students, the suicide rate for the age of 15–24 years was 5.58 in Turkey.[60] Based on our results, it is not correct to conclude that the suicide rate among medical school students is lower than that of the general population. The lower rate of suicide in medical faculty students compared to similar age groups could be due to an underreporting of suicides or due to the methodology of our study.

Similar studies have reported lower suicide rates in medical school students compared to their age-matched counterparts.[57,59] However, the major limitations of these 2 studies are that both used surveys which include questions directed only to deans of medical schools and had low participation rates.

In the current study, it was observed that there were 53 doctor suicides over 11 years, excluding the student group. Similar studies have reported 51 cases of suicide over 8 years in China, 233 cases of suicide over 10 years in India, 203 cases over 5 years in the USA, and 430 suicide cases in the UK over 5 years.[59,61] The number of cases reported in the current study is comparable to the number of suicide cases reported in China over 8 years, but it is far below the numbers observed in the other studies. However, considering that the durations of the studies are different and that the number of doctors by country varies widely, the numbers across studies are not completely comparable. Thus, more valid measures of suicide rates are necessary for more meaningful comparisons across countries.

In this respect, when the results from the studies cited above are compared with the current study, the countries with the highest doctor suicide rates are the UK, the USA, Turkey, India, and China (47.7^{-5}, 5.0^{-5}, 3.0^{-5}, 1.83^{-5}, and 0.24^{-5}, respectively). It should be kept in mind that officially recorded data were used in the studies undertaken in the UK and the USA, and online information and newspapers were used in the studies performed in China and India, as well as in the present study.

The crude suicide rate in Turkey varies between 3.61/100,000 and 4.37/100,000.[52] In the current study, it was observed that the suicide rate among doctors over over the 11-year period is 3.04/100,000. Based on this finding alone, it cannot be concluded that doctors are at a lower risk of suicide compared to the general population. The lower suicide rate in the current study could be associated with the methodology used, the lack of publicly available data on doctor suicides or an unwillingness of relatives to report doctor suicides. In addition to many studies reporting a higher suicide rate in doctors than in the general population,[11,12] there are also studies reporting lower suicide rates in doctors compared to the general population, similar to our study.[14]

Despite the limitations of our data collection methods, one remarkable finding is that the estimated suicide rate among doctors is higher than among students. The higher suicide rates of doctors compared to students may be attributable to many factors, including the stress of the transition from medical school to residency, training difficulties, economic distress, a heavy workload, or many other general suicidal risk factors.

In the present study, there appears to be a male predominance (73.8% male, 26.2% female). In similar studies, it has been found that male suicide cases made up 57–85% of all cases. It has been suggested that the higher incidence of suicides among male doctors may be due to the fact that the number of male doctors is higher than that of women-doctors and that the financial burden on men is higher.[61] Although the number of male doctor suicides is higher than for their female counterparts, a systematic review and a meta-analysis have shown that the relative risk of suicide for female doctors is higher than for male doctors.[62,63]

Considering the age groups, the current study showed that suicide is most common in the group below the age of 35 years and that the number of suicides decreases with increasing age. In addition, when suicide cases are grouped by academic rank, it is seen that more than half of the cases are specialist doctors. A study exploring doctor suicides in England and Wales found no differences in terms of academic rank and period of employment.[64] However, in a meta-analysis that examined doctor suicides, the authors mentioned that the risk of suicide in male doctors is increased during early training – a time following competitive schooling when they have a heavy workload, when they are isolated, and when they are most professionally productive.[65]

With regard to suicide methods, drug/chemical ingestion was the most commonly used method, followed by jumping from heights, firearms, hanging, and poisoning with natural gas. Studies in Turkey have shown that the most common suicide method in the general population is hanging, followed by firearms, poisoning, and jumping from heights.[52] This difference between doctors and the general population may be associated with many factors and seems to change across different cultures. Since doctors have

knowledge regarding the use of lethal drugs and better access to them, self-poisoning has been suggested to be the most common suicidal means for doctors.[65,66] However, it has also been reported that poisoning is the most common choice in Europe, while firearms and poisoning are the most common methods in the USA, Brazil and South Africa.[15] Hanging is also used as a suicide method in different countries but it is never mentioned as the most common method.[15]

Interestingly, in the current study, working as a surgeon was associated with greater use of self-poisoning as compared to non-surgeons. This may be because surgeons may have easier access to lethal drugs in the operating room.

In the present study, the medical specialties with the highest suicide numbers were cardiovascular surgery (7 (11.5%)), family medicine (7 (11.5%)), obstetrics and gynecology (6 (9.8%)), general practitioners (5 (8.2%)), pediatrics (5 (8.2%)), emergency medicine (4 (6.6%)), and internal medicine (4 (6.6%)). While an older study reported no differences in the suicide rates between specialties,[16] more recent studies have shown that certain branches of medicine have higher risks of suicide. Specialties associated with a higher risk of suicide include psychiatry and anesthesiology, followed by radiology, rehabilitation medicine, community health, and general practitioners.[15] While anesthesia and psychiatry have been identified as riskier specialties in the literature, these branches are not among the first 6 places in the current study. Turkey has the lowest number of psychiatrists in the Organisation for Economic Co-operation and Development (OECD) countries compared to the overall population (Turkey: 3/100,000, OECD countries: 10–20/100,000).[67] Similarly, the number of anesthesiologists per population in Turkey is low compared to other European countries.[68] Therefore, varying results in different studies in terms of the specialties with the highest suicide rates may be due to the differences in the distribution of specialists across countries. Interestingly, the fact that the number of cardiovascular specialists per population in Turkey is twice that of Europe provides a better understanding of the numerical distributions in the present study.[69] Providing psychoeducation about suicide to doctors working in specialties with higher suicide rates and implementing supportive measures in the workplace may be effective in preventing self-inflicted deaths. Considering the vulnerability of medical students and doctors, some countries require an annual assessment and report from medical trainees, trainers and general practitioners on many issues, including mental health.[70] The development of a similar program suitable for the conditions of our country could help prevent suicide cases.

It is noteworthy that the number of suicides appears to be higher in the specialties of cardiovascular surgery, family medicine, and obstetrics and gynecology, which have not been identified as related to particular suicide risk in the previous studies. It is not known whether these differences are due to methodological differences between studies, the healthcare system differences between countries (workload, etc.), or cultural differences across different populations.

It was observed that the majority of suicide cases (37 (60.7%)) described in the present study took place at home. However, one point to be considered is that 16 cases (26.2%) occurred at the workplace. Thus, it may be argued that hospital environments should be reviewed in terms of the safety of healthcare workers.

In terms of the speculated etiologies for suicide, the 3 most common were depression/mental illness, family problems and workplace problems/mobbing. Having a psychiatric disorder (mainly depression) has been reported as a risk factor for doctor suicide in different studies.[66,71] Although it has not been specified as a specific risk factor for doctor suicides, it is known that family problems are one of the main psychosocial risk factors that increase the risk of suicide. Additionally, a heavy workload and isolation are risk factors for doctor suicides.[71] However, it is necessary to keep in mind that the results reported here are only speculated etiologies that have been reported in the news.

We believe that the current study makes an important contribution to the literature and increases the knowledge in this area. Among the most striking findings in this study are that more than half of the suicides were committed by specialist doctors, and, although younger doctors are stated to be at a higher risk for suicide in the current literature, the suicide rate among specialist doctors in our study was higher than that of students. These findings may be due to the high patient burden of specialist doctors in our country, the psychosocial changes that come with age, or the loss of reputation and the financial standing of doctors that has occurred in our country over the years. Future research should be carried out to identify the factors that contribute to poor mental health during the transition from student to specialist and to determine what measures can be taken to mitigate these effects.

In this study, the suicide methods used by doctors in our country and the specialties with the highest number of suicides were identified for the first time. As the current findings are different from the previous literature regarding suicides across medical branches, the high number of suicides identified in specialties such as cardiovascular surgery, family medicine, and obstetrics and gynecology needs explanation. Although we consider cardiovascular surgery and obstetrics and gynecology to be specialties related to higher suicide risk in our country, the numbers in family medicine in particular need to be further examined. Future studies may help to better understand factors that contribute to the differences in the number of suicides across these specialties.

The current results indicate that it is important to develop programs to increase doctors' self-awareness and help them cope with stressful situations during and after

medical school education, and to take steps to reduce the stigma associated with mental disorders to facilitate getting help. Perhaps, instead of developing theoretical courses, the development of online channels (e.g., online support portals) where doctors could get support and protect their privacy should be considered. Special mental health services for doctors have been created in some countries and it has been proven that those who make use of these services show positive results.[72,73]

Thus, both follow-up and supportive studies should be carried out at universities to help medical students and doctors with mental health screening and the development of preventive measures. However, it may not be easy to establish the necessary infrastructure and overcome bureaucratic obstacles. Moreover, in some studies, it has been emphasized that administrators may be reluctant to provide data about their students.[59] In addition, instead of getting these data from newspapers and Google, collecting them in a pool as it is done in developed countries (e.g., via common software) may allow for better follow-up studies and may help to identify changes over the years.

Lastly, in addition to increasing individual mental resilience and facilitating support, it seems necessary to review policies in the healthcare system in order to develop new measures and institute necessary improvements.

Limitations

The number of doctor suicides identified in this study is not accurate, as all recorded suicides were collected from websites or the media. The data in the present study were collected from 10 major Turkish newspapers and Google, thus other online media or newspapers that do not have websites were excluded from the study. Although the number of suicide cases not included in major newspapers or Google is thought to be relatively low, such exclusion is still a limitation. It should also be taken into account that news sites may not accurately reflect the facts, especially with regard to the etiology of the suicide.

A common limitation of studies in this area is the fact that suicide is generally less often reported on in developing countries such as Turkey, both for the general population and among doctors. In addition, some of the cases listed as suicide attempts in newspapers or online platforms may turn into completed suicides during follow-up, and the difficulty in accessing these data should be taken into account. Moreover, it was not possible to retrieve the sociodemographic information or other variables for some suicide cases, which will probably be a common limitation for similar studies in this field. Although the study included data from the last 11 years, it should be kept in mind that these data may not fully reflect the whole doctor population in Turkey. In the future, a prospective study involving all students and doctors may enable us to obtain more reliable data on this subject.

Conclusions

To the best of our knowledge, this is the first study investigating the characteristics of suicides in medical students and doctors in Turkey. There was a male predominance in the suicide cases and more than half of the suicides occurred among specialist doctors. Self-poisoning, jumping from heights and firearms were the most common methods of suicide. Cardiovascular surgery, family medicine, and obstetrics and gynecology were the specialties with the highest number of suicides. The most common speculated etiology for suicide was depression/mental illness. Considering the current findings and the existing literature and taking into account that medical training is relatively stressful as well as the increasing intensity of working conditions for doctors in recent years, it seems reasonable that some precautions should be taken to protect healthcare providers. Providing individual support to medical students and doctors and making systemic adjustments will reduce the pressure on physicians, can help support them in terms of mental difficulties, and can prevent undesirable events such as suicide.

Despite its limitations, the current study provides valuable descriptive information about this understudied topic. As the present study is a retrospective study that included online newspapers and Google searches, there is a need for further studies to clarify the rates of suicides for medical students and doctors.

Data availability

The dataset is available from the corresponding author upon request.

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Large unstained cell (LUC) count as a predictor of carotid artery occlusion

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Abstract

Background. Carotid artery stenosis is often considered a stable clinical condition, and the underlying atherosclerosis is thought to have an inflammatory background.

Objectives. The aim of the study was to assess the value of different parameters obtained from whole blood counts for the prediction of advanced carotid artery atherosclerosis, including vessel occlusion, irrespective of symptom occurrence.

Materials and methods. The study group comprised 290 patients (84 (29%) females and 206 (71%) males) with a mean age of 68 ±8 years, who were admitted to the Vascular Surgery Department due to significant carotid artery disease. Patients were retrospectively divided into 2 subgroups regarding the presence or absence of artery occlusion. The demographic, clinical and laboratory preoperative data were compared between both groups.

Results. We found significant differences in preoperative large unstained cell (LUC) counts between patients with and without carotid artery occlusion ($p = 0.003$), when analyzed with the Mann–Whitney test for independent samples. The receiver operating characteristic (ROC) curve showed that LUC count has prognostic properties for carotid artery occlusion, with an area under the curve (AUC) of 0.637 ($p = 0.033$), yielding a 69.70% sensitivity and a 51.75% specificity.

Conclusions. Large unstained cells represent an acute inflammatory state related to artery occlusion. An LUC count below the cutoff value of 0.16×10^9/L may be a predictor of carotid artery occlusion. Therefore, carotid artery occlusion should not be regarded as a chronic state, but as a clinical challenge being promoted by active inflammatory processes.

Key words: inflammation, atherosclerosis, occlusion, carotid stenosis, large unstained cells

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Background

Carotid artery disease is a challenging clinical problem that has recently been recognized as one of the most common causes of stroke.[1] Instead of viewing age as the traditional risk factor, clinical considerations have assumed a greater significance for carotid plaques and stroke prediction.[2] Asymptomatic carotid artery disease is diagnosed in less than 1% of those aged below 50 years, and in over 3% of patients over 80 years.[3,4] The monitoring of atherosclerotic plaques plays an important role in stroke prevention,[5] with the prevalence of symptomatic intracranial stenosis being higher in elderly patients than in those younger than 70 years.[6]

Numerous clinical factors have been associated with the formation of atherosclerosis, including components of the metabolic syndrome, such as diabetes, obesity and hyperlipidemia, all of which show an elevated inflammatory response. In obesity, perivascular adipose tissue which surrounds blood vessels, where it becomes dysfunctional and secretes pro-inflammatory molecules, promotes the infiltration of inflammatory cells, and furthers the development of atherosclerosis.[7–9]

There is an increased body of evidence suggesting that the size of carotid artery atherosclerotic plaques, more so than their composition, plays a significant role in the clinical presentation of carotid artery disease.[10] However, studies examining the makeup of plaques and subsequent remodeling and influence on mechanical forces should be taken into consideration with the use of ultrasound Doppler, computed tomography (CT) and positron emission tomography (PET), or magnetic resonance imaging (MRI) for predicting possible complications.[11–13]

Atherosclerosis is considered a lipid-derived disease with an inflammatory background.[14] The inflammatory reactions initiate the formation of plaques if the endothelium becomes dysfunctional, while also facilitating disease progression.[15–17] The infiltration of inflammatory cells has previously been presented as a hallmark feature of plaque instability,[18] while a reduction of the inflammatory response was associated with plaque reduction in animal models.[19] In our previous reports, we found a significant relationship between simple inflammatory indices obtained from the whole blood counts and overall mortality.[20–22]

Objectives

The current study aimed to assess the value of different morphological parameters obtained from whole blood counts for the prediction of advanced carotid artery atherosclerosis, including vessel occlusion and irrespective of symptom occurrence.

Materials and methods

Study patients

Three hundred ninety-one patients were admitted to the Department of Vascular Surgery at the Poznan University of Medical Sciences (Poland) between January 2018 and December 2020 due to significant carotid artery disease. Of this group, 290 patients (84 (29%) females and 206 (71%) males) with a mean age of 68 ±8 years underwent detailed laboratory evaluation and were enrolled in the final retrospective single-center analysis. The laboratory tests were performed upon hospital admission. The study group received carotid artery treatment including percutaneous (50 (17%)) and surgical (239 (82%)) interventions (Fig. 1). One patient was disqualified due to a high perioperative mortality risk. Patients requiring unplanned intervention or concomitant surgery were also excluded from the study. Additional exclusion criteria encompassed inflammatory, autoimmune, oncological, or hematological proliferative diseases.

Thirty-one (11%) patients admitted for vascular intervention presented with carotid artery occlusion, while 259

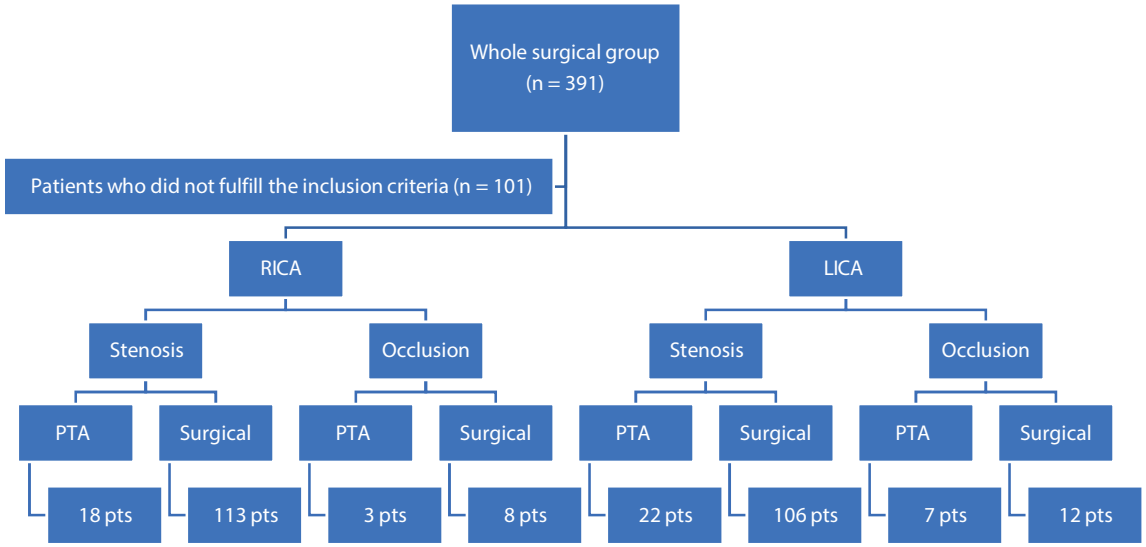

Fig. 1. Flowchart of conducted procedures

LICA – left internal carotid artery; PTA – percutaneous carotid angioplasty; pts – patients; RICA – right internal carotid artery.

(89%) had significant stenosis. Comorbidities included hypercholesterolemia 196 (68%)), arterial hypertension (178 (61%)), a history of stroke (146 (50%)), tobacco use (96 (33%)), diabetes mellitus (73 (25%)), and permanent atrial fibrillation (AF) (19 (7%)) (Table 1).

Table 1. Demographic and clinical data

Parameters			Carotid stenosis (n = 259)	Carotid occlusion (n = 31)	p-value
Demographic parameters		gender M/F, n (%)	183 (71)/76 (29)	23 (74)/8 (26)	0.682
		age (M ±SD) [years]	68.2 ±9.1	68.4 ±7.8	0.906[t]
		BMI (M ±SD) [kg/m^2]	27.0 ±4.4	26.9 ±4.1	0.904[t]
		obesity, n (%)/available data	34 (26)/131	4 (18)/22	0.4349
Comorbidities		arterial hypertension, n (%)	158 (61)	20 (65)	0.507
		diabetes mellitus, n (%)	63 (24)	10 (32)	0.470
		stroke, n (%)	130 (50)	16 (52)	0.886
		hypercholesterolemia, n (%)	174 (67)	22 (68)	0.181
		chronic kidney disease, n (%)	15 (5)	5 (16)	0.892
		AF, n (%)	14 (5)	5 (16)	0.033*
		tobacco use, n (%)	84 (32)	12 (39)	0.299
		coexisting CAD	61 (24)	13 (42)	0.027*
		coexisting peripheral artery disease (non-carotid)	31 (12)	7 (23)	0.098
Symptoms		all, n (%)	140 (54)	15 (48)	0.8131
		visual disturbances, n (%)	25 (18)	4 (13)	0.2947
		vertigo, n (%)	26 (10)	5 (16)	0.3782
Laboratory parameters	whole blood count	WBC × 10^9/L, median (Q1–Q3)	8.1 (7.0–9.9)	8.6 (6.8–9.7)	0.936
		neutrophil × 10^9/L, median (Q1–Q3)	5.3 (4.3–6.7)	5.7 (4.3–7.1)	0.353
		lymphocyte × 10^9/L, median (Q1–Q3)	1.9 (1.5–2.4)	1.7 (1.4–2.1)	0.107
		monocyte × 10^9/L, median (Q1–Q3)	0.5 (0.4–0.6)	0.5 (0.3–0.6)	0.369
		MLR, median (Q1–Q3)	0.3 (0.2–0.3)	0.3 (0.2–0.3)	0.658
		NLR, median (Q1–Q3)	2.8 (2.1–3.7)	3.3 (2.7–3.8)	0.053
		PLR, median (Q1–Q3)	124 (92–169)	128 (101–165)	0.423
		SII, median (Q1–Q3)	654 (457–928)	745 (479–993)	0.295
		SIRI, median (Q1–Q3)	1.4 (0.9–1.9)	1.2 (0.99–2.1)	0.615
		AISI, median (Q1–Q3)	312 (192–490)	304 (219–467)	0.820
		eosinophils × 10^9/L, median (Q1–Q3)	0.1 (0.1–0.2)	0.1 (0.01–0.2)	0.451
		basophils × 10^9/L, median (Q1–Q3)	0.05 (0.03–0.05)	0.04 (0.02–0.05)	0.492
		LUCs × 10^9/L, median (Q1–Q3)	0.17 (0.13–0.22)	0.14 (0.11–0.18)	0.010*
		RBC × 10^9/L, median (Q1–Q3)	4.5 (4.2–4.8)	4.6 (4.3–4.9)	0.195
		Hb [mmol/L], median (Q1–Q3)	8.7 (8.1–9.1)	8.6 (8.2–9.4)	0.563
		hematocrit (%), median (Q1–Q3)	41 (38–43)	41 (39–44)	0.721
		MCH × 10^9/L, median (Q1–Q3)	1.9 (1.9–2.0)	1.9 (1.9–2.0)	0.926
		MCHC × 10^9/L, median (Q1–Q3)	21.3 (20.9–21.8)	21.2 (21–21.8)	0.677
		RDW × 10^9/L, median (Q1–Q3)	13.5 (13.0–14.1)	13.1 (12.9–13.9)	0.172
		platelets × 10^3/μL, median (Q1–Q3)	233 (188–294)	222 (201–286)	0.717
	lipidemic profile	total serum cholesterol [mmol/L], median (Q1–Q3)	3.99 (3.41–4.73)	3.84 (3.35–4.36)	0.306
		HDL fraction [mmol/L], median (Q1–Q3)	1.30 (1.05–1.60)	1.22 (1.08–1.50)	0.555
		LDL fraction [mmol/L], median (Q1–Q3)	2.06 (1.66–2.75)	1.90 (1.50–2.70)	0.566
	kidney function tests	creatinine [mg/dL], median (Q1–Q3)	85.2 (71.7–104.6)	78.8 (71.5–111)	0.999
		GRF, median (Q1–Q3)	75 (60–90)	77 (57–90)	0.679
	thrombotic risk	fibrinogen [mg/dL], median (Q1–Q3)	335 (287–407)	325 (273–376)	0.413

Table 1. Demographic and clinical data – cont.

Parameters		Carotid stenosis (n = 259)	Carotid occlusion (n = 31)	p-value
Preoperative pharmacotherapy	β-blockers, n (%)	87 (34)	13 (42)	0.008
	statins, n (%)	174 (67)	22 (71)	0.083
	ACE-I, n (%)	80 (31)	13 (42)	0.361
	antiplatelet therapy, n (%)	259 (100)	31 (100)	1.000
Performed procedures	surgical, n (%)	215 (83)	20 (64)	0.136
	percutaneous, n (%)	40 (15)	10 (3)	0.021
	disqualified, n (%)	4 (2)	1 (3)	0.504

AF – atrial fibrillation; AISI – aggregate index of systemic inflammation; BMI – body mass index; CAD – coronary artery disease; F – female; GFR – glomerular filtration rate; HDL – high-density lipoprotein; LDL – low-density lipoprotein; LUCs – large unstained cells; M – male; MCH – mean hemoglobin concentration; MCHC – mean corpuscular hemoglobin concentration; MLR – monocyte-to-lymphocyte ratio; NLR – neutrophil-to-lymphocyte ratio; PLR – platelet-to-lymphocyte ratio; RBC – red blood cells; RDW – red blood cell distribution width; M ±SD – mean ± standard deviation; SII – systemic inflammatory index; SIRI – systemic inflammatory response index; WBC – white blood count; * statistically significant; ^{t} p-value of the Student's t-test; ACE-I – angiotensin-converting-enzyme inhibitor.

Study design – laboratory analysis

The blood samples for whole blood analysis were collected upon patient admission. The study group was divided retrospectively into 2 subgroups, namely those with carotid artery stenosis and those with carotid artery occlusion. The blood morphological results were compared between both subgroups, and the ability of various blood markers to predict carotid artery occlusion was analyzed.

Inflammatory indices were calculated, including neutrophil-to-lymphocyte ratio (NLR), monocyte-to-lymphocyte ratio (MLR), platelet-to-lymphocyte ratio (PLR), the systemic inflammatory index (SII) (the quotient of neutrophils and platelets divided by the lymphocyte count), the systemic inflammatory response index (SIRI) (the quotient of neutrophils and monocytes divided by the lymphocyte count), and the aggregate index of systemic inflammation (AISI) (the proportion of neutrophils, monocytes and platelets divided by the lymphocyte count).

Analysis

We analyzed demographic, clinical and laboratory data comprising whole blood count parameters with the use of a routine hematology analyzer (Sysmex Europe, Norderstedt, Germany).

The researchers adhered to the principles of good clinical practice and the Declaration of Helsinki, and the study was approved by the Local Ethics Committee of the Poznan University of Medical Sciences (approval No. 784/21, October 13, 2021).

Statistical analyses

Numerical data were presented as mean ± standard deviation (M ±SD) when they followed normal distribution (Shapiro–Wilk test). Otherwise, data were reported as medians and interquartile range (Q1–Q3), where Q1 is the lower quartile and Q3 is the upper quartile. Categorical variables were presented as counts and percentages. The comparison between carotid stenosis patients and occlusion patients was performed with the Student's t-test when data followed normal distribution (Table 2) and variation between groups was homogenous (Levene's test). When data did not follow normal distribution, the Mann–Whitey test was used. Categorical data were compared using the χ^2 test. The receiver operating characteristic (ROC) curve analysis was used to find the parameters that have prognostic properties for the presence of occlusion. The cutoff point was estimated with the Youden's index. The uni- and multivariable logistic regression with backward stepwise selection was performed to find factors which increased the occlusion risk. The results are presented as odds ratio (OR) and 95% confidence interval (95% CI). The inclusion criteria for the study was whole blood count results in the normal range to prevent outliers. The lack of multicollinearity of explanatory variables was checked by assessing the correlation and by estimating variance inflation factor (VIF). For all analyzed predictors, the VIF < 1.7. The assumption regarding the linear relationship between explanatory variables and the logic of the response variable was checked using the Box–Tidwell test. The statistical analysis was performed using MedCalc® Statistical Software v. 20.027 (MedCalc Software Ltd., Ostend, Belgium). The assumptions regarding logistic regression were performed using Stata 17 software (StataCorp, College Station, USA). A p-value of 0.05 was considered statistically significant for all tests.

Results

A total of 290 patients were analyzed, including 155 (53%) who presented with clinical symptoms (visual disturbances in 29 (10%) patients and vertigo in 31 (11%) patients). Stroke was reported in 146 (50%) patients, and there were

Table 2. Shapiro–Wilk normality test results

Parameter	Carotid stenosis			Carotid occlusion		
	N	W	p-value	N	W	p-value
Age	259	0.9904	0.5035	31	0.9391	0.1895
BMI	259	0.9959	0.9713	31	0.9703	0.7184
WBC	259	0.5363	<0.0001	31	0.9739	0.5960
Neutrophils	259	0.9750	0.0002	31	0.9820	0.8437
Lymphocytes	259	0.1743	<0.0001	31	0.9379	0.0590
Monocytes	259	0.9351	<0.0001	31	0.9570	0.2125
MLR	259	0.7287	<0.0001	31	0.9154	0.0137
NLR	259	0.9131	<0.0001	31	0.9187	0.0169
PLR	259	0.9362	<0.0001	31	0.8484	0.0003
SII	259	0.8481	<0.0001	31	0.7705	<0.0001
SIRI	259	0.8153	<0.0001	31	0.8343	0.0002
AISI	259	0.6565	<0.0001	31	0.6506	<0.0001
Eosinophils	259	0.6633	<0.0001	31	0.7695	<0.0001
Basophils	259	0.4971	<0.0001	31	0.8010	<0.0001
LUCs	259	0.2135	<0.0001	31	0.9830	0.8719
RBC	259	0.9850	0.0084	31	0.9365	0.0538
Hb	259	0.9460	<0.0001	31	0.9758	0.6559
HCT	259	0.0690	<0.0001	31	0.9355	0.0502
MCH	259	0.9338	<0.0001	31	0.1756	0.0000
MCHC	259	0.9515	<0.0001	31	0.9516	0.1483
RDW	259	0.8790	<0.0001	31	0.8525	0.0004
PLT	259	0.9546	<0.0001	31	0.8182	0.0001
CHOL	214	0.2269	<0.0001	31	0.9035	0.0066
HDL	214	0.4782	<0.0001	31	0.9705	0.4938
LDL	214	0.8959	<0.0001	31	0.9028	0.0063
Creatinine	237	0.7715	<0.0001	31	0.9182	0.0164
GFR	237	0.8872	<0.0001	31	0.8475	0.0003
Fibrinogen	259	0.9720	0.0001	31	0.9333	0.0435

BMI – body mass index; WBC – white blood count; MLR – monocyte-to-lymphocyte ratio; NLR – neutrophil-to-lymphocyte ratio; PLR – platelet-to-lymphocyte ratio; SII – systemic inflammatory index; SIRI – systemic inflammatory response index; AISI – aggregate index of systemic inflammation; LUCs – large unstained cells; RBC – red blood cells; MCH – mean hemoglobin concentration; MCHC – mean corpuscular hemoglobin concentration; RDW – red blood cell distribution width; HDL – high-density lipoprotein; LDL – low-density lipoprotein; GFR – glomerular filtration rate; PLT – platelets; CHOL – total serum cholesterol; Hb – hemoglobin; HCT – hematocrit; GRF – glomerular filtration rate; Hb – hemoglobin.

178 (61%) and 112 (39%) patients diagnosed with significant (more than 70% of lumen narrowing) right and left carotid artery disease, respectively. Collateral atherosclerosis of carotid arteries was present in 108 (37%) patients.

A total of 169 (58%) and 16 (6%) patients underwent surgery, while 29 (10%) and 8 (4%) underwent angioplasty procedures, in the stenosis and occlusion groups, respectively. It was found that 13 (5%) episodes of perioperative neurological complications were reported, including 11 (4%) transient ischemic attacks (TIA) and 2 (1%) strokes.

The Mann–Whitney test for independent samples revealed significant differences in preoperative large unstained cells (LUCs) between patients with carotid artery occlusion and stenosis (p = 0.003). The ROC curve analysis showed that LUC count has prognostic properties for carotid artery occlusion featuring an area under the curve (AUC) = 0.637, 95% CI: 0.58–0.69, and a p-value of 0.033 with a 69.70% sensitivity and a 51.75% specificity (Fig. 2).

Univariable analysis

According to the univariate logistic regression analysis (Table 3), significant preoperative factors obtained from the whole blood analysis included LUC count below the cut-off value of 0.16 (OR = 2.47, 95% CI: 1.13–5.39, p = 0.024).

Moreover, the univariable analysis of demographical data including gender (OR = 1.58, 95% CI: 0.66–3.81, p = 0.300) or obesity (OR = 0.63, 95% CI: 0.20–2.01, p = 0.438) did not reveal significance of these factors towards an increased risk of coronary artery disease (CAD). Neither preoperative

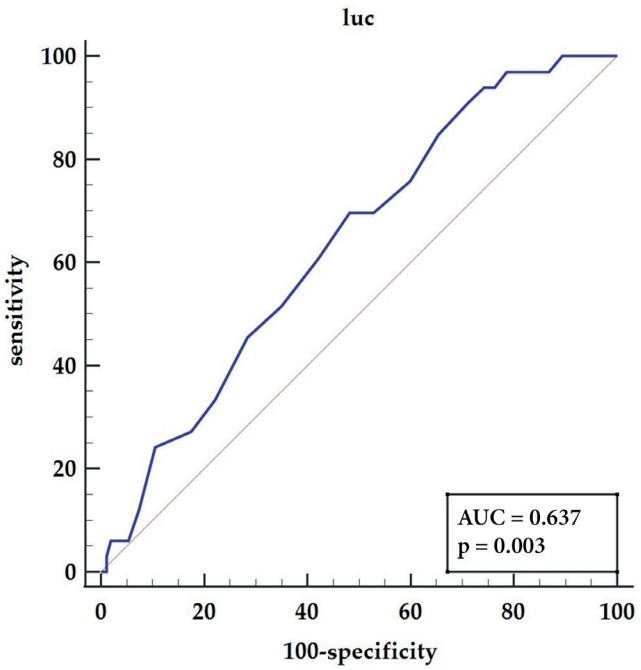

Fig. 2. Receiver operating characteristic (ROC) curve of preoperative large unstained cells (LUCs) for predicting carotid artery occlusion

AUC – area under the curve.

symptoms (OR = 1.06, 95% CI: 0.38–2.91, p = 0.915) nor standard risk factors including smoking (OR = 1.06, 95% CI: 0.38–2.91, p = 0.915), diabetes mellitus (OR = 1.33, 95% CI: 0.60–2.96, p = 0.472), hypercholesterolemia (OR = 1.27, 95% CI: 0.78–2.11, p = 0.611), hypertension (OR = 1.29, 95% CI: 0.60–2.78, p = 0.508), or body mass index (BMI; OR = 0.99, 95% CI: 0.90–1.11, p = 0.992) showed statistical significance for either coexisting CAD (OR = 2.08, 95% CI: 0.98–4.42, p = 0.058) or peripheral artery disease (OR = 1.95, 95% CI: 0.78–4.88, p = 0.151) (Table 3). The only significant factor discovered in our analysis was AF (OR = 3.09, 95% CI: 1.03–9.25, p = 0.043). Similarly, laboratory results were not predictive, including serum cholesterol (OR = 0.86, 95% CI: 0.62–1.19, p = 0.363), low-density lipoprotein (LDL; OR = 0.93, 95% CI: 0.63–1.36, p = 0.698), or high-density lipoprotein (HDL; OR = 0.67, 95% CI: 0.28–1.63, p = 0.383).

Multivariable analysis

According to the multivariate logistic regression analysis (Table 3), the only significant preoperative factor was LUC count below the cutoff value of 0.16 × 10^9/L (OR = 2.70, 95% CI: 1.22–6.03, p = 0.015) and AF (OR = 3.75, 95% CI: 1.22–11.65, p = 0.022).

Moreover, the logistic regression with the Hosmer–Lemeshow test for goodness-of-fit, log-likelihood ratio test p-values, and Nagelkerke pseudo R^2 test of analyzed parameters were performed (Table 4). The tests revealed significance of LUC count and CAD co-existance for carotid artery occulsion.

Discussion

To the best of our knowledge, our study is the first to reveal the predictive value of LUC count for carotid artery occlusion. We present the results of a multivariable analysis of preoperative whole blood count, with a cut-off value of 0.16 × 10^9/L as a predictive factor, regardless of symptoms.

Contrary to popular belief, our analysis revealed neither predictive value of potential comorbidities of atherosclerosis, nor predictive values of laboratory results of serum cholesterol fractions for carotid artery obstruction. Moreover, while some studies[23,24] have shown gender dependence of peripheral atherosclerotic disease, we did not find such

Table 3. Univariate and multivariate analysis of clinical and laboratory parameters in occluded arteries

Parameters		Univariate analysis			Multivariate analysis		
		OR	95% CI	probability	OR	95% CI	probability
Clinical parameters	sex	1.58	0.66–3.81	0.300	–	–	–
	obesity	0.63	0.20–2.01	0.438	–	–	–
	coexisting CAD	2.08	0.98–4.42	0.058	–	–	–
	coexisting PAD	1.95	0.78–4.88	0.151	–	–	–
	AF	3.09	1.03–9.25	0.043	3.75	1.22–11.65	0.022
Whole blood count parameters	platelets	1.0	0.99–1.01	0.943	–	–	–
	LUCs below cutoff value of 0.16	2.47	1.13–5.39	0.024	2.70	1.22–6.03	0.015
	hematocrit	0.68	0.04–13.86	0.805	–	–	–
	MPV	1.09	0.79–1.49	0.597	–	–	–
	MHC	1.09	0.76–1.57	0.626	–	–	–
	MCHC	1.23	0.74–2.05	0.408	–	–	–

AF – atrial fibrillation; CAD – coronary artery disease; 95% CI – 95% confidence interval; OR – odds ratio; LUCs – large unstained cells; MCHC – mean corpuscular hemoglobin concentration; MHC – mean hemoglobin concentration; MPV – mean platelet volume; OR – odds ratio; PAD – peripheral artery disease (non-carotid).

Table 4. Hosmer–Lemeshow logistic regression, log-likelihood ratio test p-values and Nagelkerke pseudo R^2 of analyzed parameters

Parameters	Univariate analysis			Multivariate analysis		
	Hosmer–Lemeshow test probability	log-likelihood ratio test probability	Nagelkerke R^2	Hosmer–Lemeshow test	log-likelihood ratio test	Nagelkerke R^2
Clinical parameters						
Gender	–	0.283	0.008	–	–	–
Obesity	–	0.421	0.008	–	–	–
Coexisting CAD	–	0.063	0.023	–	–	–
Coexisting PAD	–	0.171	0.013	–	–	–
AF	–	0.061	0.024	0.571	0.007	0.067
Whole blood count parameters						
Platelets	0.054	0.943	0.000	–	–	–
LUCs below cutoff value of 0.16	–	0.018	0.037	0.571	0.007	0.067
Hematocrit	0.249	0.652	0.001	–	–	–
MPV	0.562	0.601	0.002	–	–	–
MHC	0.249	0.035	0.029	–	–	–
MCHC	0.608	0.402	0.005	–	–	–

AF – atrial fibrillation; CAD – coronary artery disease; OR – odds ratio; LUCs – large unstained cells; MCHC – mean corpuscular hemoglobin concentration; MHC – mean hemoglobin concentration; MPV – mean platelet volume; PAD – peripheral artery disease (non-carotid).

correlation with carotid artery occlusion. While our study examined the whole blood count analysis and compared its results to diagnostic tools such as ultrasound imaging in patients with defined carotid disease, our rationale was to find predictive indicators for carotid artery disease progression that could be performed during routine check-ups. Based on our results, we believe that LUCs can be regarded as a simple marker within whole blood that can help distinguish patients with more advanced stages of carotid artery disease.

The LUC population reflects activated lymphocytes and peroxidase-negative large cells that do not contain morphological features of lymphocytes, eosinophils, basophils, or neutrophils.[25,26] This population can include virally activated lymphocytes, plasma cells, pediatric lymphocytes, hairy cells, and peroxidase-negative blasts. Those cells are beyond clear classification but have been postulated to be clinically relevant during inflammatory states, viral infections and hematological malignancies.[27–29]. Their increased amount in whole blood analysis was found to be correlated with immunological activation.[27] Vanker and Ipp have indicated LUCs as a valuable marker of both innate immunity and $CD8^+$ lymphocyte activation.[30] Previously, LUCs have only been analyzed in a small number of studies related to leukemia, myelodysplastic syndromes and viral infection.[31] Though LUCs reflect organism activation to variable factors, we are the first to present their association with atherosclerosis.

Since LUCs represent activated lymphocytes, they can also be regarded as a carotid stenosis progression indicator and the underlying inflammatory activation indicator.[32] The inflammatory origin of atherosclerosis has been previously presented and suggests this origin is involved in both disease initiation and progression.[33–35] Simple parameters from whole blood count, including NLR, MLR and SIRI, were postulated to be related to atherosclerosis progression.[36–42] However, in our analysis, none of these indices were related to carotid artery occlusion, although we previously presented the prognostic value of MLR for collateral carotid artery involvement.[32] The significant difference between LUCs and lymphocyte, monocyte and neutrophil counts is based on the characteristics of the cells; LUC counts take into account activated cells, while other cell counts measure the concentration of the cells.

The standard inflammatory markers such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were analyzed in patients with carotid disease as possible simple markers. In the study by Liu et al., CRP combined with lipoprotein-associated phospholipase A2 was found to be a significant marker for carotid atherosclerosis.[43] Moreover, Boaz et al. showed the relationship between intimal thickening and CRP.[44] However, Schmidt et al. did not reveal any interactions between vascular risk factors for carotid atherosclerosis and CRP, as CRP was found to be related to the severity of small brain vessel injury.[45] Finally, the CRP concentration as a serological determinant of carotid plaques vulnerability was postulated by Fittipaldi et al., who demonstrated the relation between atherosclerotic plaques vulnerability evaluated in histological examination and CRP values >5 mg/L.[46]

Large unstained cells have been described as peroxidase-negative cells. Myeloperoxidase (MPO) is a protein of 140 kDa molecular weight, composed of a tetramer from 2 α chains (60 kDa) and 2 β chains (14 kDa).[47] This protein is commonly used for the classification of acute leukemia during diagnosis.[48] The MPO deficiency has been reported

as of either primary (genetic) or secondary origin as a disease consequence,[49,50] and has been described in renal failure, cardiovascular disease and diabetes mellitus.[51–53] According to previous reports, MPO deficiency can be related to chronic disease development, including the extent of brain damage during stroke.[54] An increased number of LUCs as peroxidase negative cells in peripheral blood test, especially in patients with carotid artery occlusion, may be regarded as a secondary type of deficiency. This cell type is activated by various factors, although not those typically associated with monocyte or lymphocyte activation, and may be associated with vasculitis.[55] Moreover, LUCs were related to acute inflammatory reaction.[31] According to previous reports, LUCs should be regarded as a mixture of activated lymphocytes, monocytes and lymphoblasts, and therefore we concluded that chronic carotid artery occlusion induces an active inflammatory response.[32,56]

The role of monocytes in atherosclerosis has been described as facilitating increased cytokine release and being involved in plaque destabilization.[57,58] The inflammatory indices have been gaining scientific attention in recent years due to their low cost and easy availability as a possible predictive tool in cardiovascular disease.[59,60]

The role of lymphoblasts in atherosclerosis has not been previously investigated in a general population, although their presence in premature atherosclerosis in hematological diseases has been postulated.[61] The results of our study may shed new light on the role of lymphoblasts, especially in the narrowing of arteries during the atherosclerotic processes.

The second factor which appeared predictive for carotid artery occlusion was AF. Obviously, inflammatory activation, with the co-occurrence of AF, may serve as a trigger and result in carotid artery disease, as AF may enhance the inflammatory response.[62] However, the inflammatory activation related to several conditions and diseases, such as carotid artery disease, heart failure and acute coronary syndrome, may trigger AF occurrence.[63,64] It has already been observed in patients after cardiac surgery that AF in the early postoperative period may occur even without the arrhythmia in the patient's history.[65]

Limitations

The study was performed as a single-center, retrospective study and involved only patients with advanced stages of carotid artery disease referred for surgical intervention. Future studies including patients with a wide spectrum of carotid artery severity would be beneficial. The results of the study may be relevant to subgroups of patients with carotid disease, irrespectively of clinical symptoms or comorbidities, indicating those who present with artery occlusion. Second, our study lacked a healthy control group. Although the results of logistic regression model are significant, the pseudo R^2 is low, possibly due to the relatively small sample size and lack of control group.

Conclusions

Large unstained cells represent an acute inflammatory state related to artery occlusion, and their concentration below a cutoff value of 0.16×10^9/L may predict carotid artery obstruction. Carotid artery occlusion should not be regarded as a chronic state, but as a clinical challenge promoting an active inflammatory process.

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Expression of micro-ribonucleic acids in thyroid nodules and serum to discriminate between follicular adenoma and cancer in patients with a fine needle aspiration biopsy classified as suspicious for follicular neoplasm: A preliminary study

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Abstract

Background. Approximately 10% of thyroid nodules undergoing fine needle aspiration biopsy (FNAB) receive a suspicious for follicular neoplasm (SFN) classification. Currently, there is no diagnostic tool to preoperatively discriminate between follicular adenoma (FA) and thyroid cancer (TC), and most patients require surgery to exclude malignancy.

Objectives. To characterize the micro-ribonucleic acid (miRNA) signature of tumors assessed as SFN and define circulating miRNA patterns to distinguish FA from follicular cancer in patients with thyroid nodules biopsied using FNAB.

Materials and methods. The study included excised tumor and thyroid tissue samples from 80 consecutive patients collected by a pathologist in the operating theater. The miRNA was isolated from specimens at the Center for Medical Genomics OMICRON, and next-generation sequencing (NGS) was used to obtain target miRNAs. In addition, miRNA expression was detected in serum using polymerase chain reaction (PCR).

Results. Well-differentiated thyroid cancer (WDTC) samples had significantly higher expression levels of hsa-miR-146b-5p ($p = 0.030$) and hsa-miR-146b-3p ($p = 0.032$), while the expression levels of hsa-miR-195-3p were significantly lower ($p = 0.032$) in WDTC samples compared to FA specimens. The serum of TC patients showed markedly higher expression of the unique miRNA hsa-miR-195-3p ($p = 0.039$).

Conclusions. The overexpression of hsa-miR-146b-5p and hsa-miR-146b-3p, and the downregulation of hsa-miR-195-3p expression could be used as biomarkers to distinguish FA from WDTC in patients with FNAB results classified as Bethesda tier IV. In addition, hsa-miR-195-3p could act as a serum biomarker for differentiating patients with FA from those with WDTC, and preoperative measurement of its expression would help avoid unnecessary surgeries. However, this concept needs further verification in a more substantial prospective study.

Key words: thyroid cancer, follicular adenoma, suspicious for follicular neoplasm, next-generation sequencing

Background

Thyroid cancer (TC) is the most common endocrine malignancy. It accounts for more than 95% of endocrine cancers. Over the last 3 decades, the incidence of TC in the USA has been growing rapidly and has increased 2.3-fold.[1,2] Much of this rise appears to be due to more sensitive diagnostic procedures, such as computed tomography (CT) and magnetic resonance imaging (MRI), often undertaken for unrelated medical problems. These modalities can detect small incidental thyroid nodules that might have gone undetected previously. The death rate for TC slightly increased from 2009 to 2018 (0.6% per year), though it appears to have stabilized in recent years. Nonetheless, there were more than 4100 new cases of TC diagnosed in Poland in 2018, which resulted in 338 deaths.[3]

Thyroid nodules are highly prevalent and occur in up to 65% of the healthy adult population.[4] Ultrasound (US) is currently the best detection tool available for the initial work-up of thyroid nodules, and the primary goal of thyroid US examination is to discriminate benign nodules from lesions comprising malignant features that require advanced diagnostics.[5,6] Particular sonographic features of thyroid nodules associated with a high risk of malignancy include microcalcifications, irregular margins, absence of elasticity, and a taller-than-wide shape.[7–10] Nevertheless, no US feature can efficiently detect or disregard malignancy.[11–14]

The main examination in preoperative diagnostics of thyroid nodules is fine needle aspiration biopsy (FNAB), which has an accuracy of >95%. Fine needle aspiration biopsy demonstrates benign cytological features in more than half of the cases (60–70%), and the risk of malignancy in less than 3%.[15–19] Recent retrospective studies at high-volume centers revealed TC in 3–15% of biopsies.[20–22] Despite high confidence levels, up to 30% of FNABs are reported as indeterminate and unsatisfactory in terms of certainty for malignancy.[23–25]

The malignancy rate increases to 40% in nodules verified as suspicious for follicular neoplasm (SFN), also known as Bethesda tier IV. Indeterminate cytopathology often requires diagnostic surgery for a definitive diagnosis, based on the presence of tumor cells within the lumen of blood vessels or tumor capsular invasion.[26] On the contrary, patients undergoing thyroid lobectomy for an indeterminate biopsy may require completion surgery if the final pathological report confirms cancer.

Significant efforts have been made to combine FNAB results with immunocytochemical and molecular markers, clinical information, and ultrasonography to enhance the accuracy of thyroid biopsies.[27–32] Numerous mutations have been analyzed for their applicability to FNAB diagnosis in different settings,[33–36] though there is not enough confidence in the results for this approach to be widely implemented in clinical practice.

Several studies have appraised the potential of microribonucleic acids (miRNAs) as diagnostic markers for TC. These tiny noncoding molecules are endogenous, single-stranded and highly conserved, with lengths ranging from 18 to 25 nucleotides. Furthermore, miRNAs are involved in many biological and pathological processes, including proliferation, differentiation and apoptosis, and can alter gene expression at the post-transcriptional messenger RNA (mRNA) level. In addition, miRNAs are very stable and remain intact in tissues, whether fresh, frozen or formalin-fixed paraffin-embedded (FFPE),[37] a property exploited in the development of several commercially available miRNA-based molecular tests.[38,39] Moreover, miRNAs have demonstrated influence on the activity of TC-related signaling pathways such as mitogen-activated protein kinase (MAPK) and the *RET* gene.[40]

The hypothesis explored in this pilot study was that miRNA signatures in malignant tumors assessed as SFN using FNAB differ from signatures in benign tumors, and circulating miRNA distinguishes between the 2 tumor types.

Objectives

The purpose of this study was twofold and included characterizing the miRNA signature of tumors assessed as SFN and defining circulating miRNA patterns in order to distinguish follicular adenoma (FA) from follicular cancer in patients with thyroid nodules verified as SFN using FNAB.

Materials and methods

Patients

This noninterventional study included 80 consecutive patients operated on in the Department of Endocrine Surgery (Third Chair of General Surgery, Jagiellonian University Medical College, Kraków, Poland) between January 2016 and October 2018. The indication for surgery was an FNAB result of SFN. Patient baseline demographic and clinical characteristics were extracted from de-identified patient history, physical examination records, US, and pathology reports. This information included patient age, gender, tumor size, presence of Hashimoto's disease, and an aggregate of increased malignancy risk factors, including tumor phenotype based on preoperative US malignancy risk stratification using the European Thyroid Imaging and Reporting Data System (EU-TIRADS) classification.

Blood samples were drawn in the operating theater before the skin incision and were transferred immediately to the Center of Medical Genomics OMICRON of Jagiellonian University Medical College.

An experienced pathologist excised specimens from the tumor and normal thyroid tissues in the operating theater, with samples of normal thyroid tissue obtained

from the contralateral thyroid lobe. The specimens were immediately placed into RNAlater® (Invitrogen, Thermo Fisher Scientific, Waltham, USA) and stored at –80°C until RNA extraction. Based on histopathological examination, patients were assigned to TC (study group) or FA (control group) group matched for age, sex and body mass index (BMI). All TC patients and 14 FA patients were eligible for further analysis of complete clinical data and uncontaminated serum samples with detectable miRNA.

All participants provided written informed consent on the day of recruitment. The Institutional Review Board (Bioethical Committee of Jagiellonian University, Kraków, Poland) approved the study (approval No. 122.6120.17.2016 granted on January 28, 2016), which adhered to the Declaration of Helsinki.

RNA extraction

The extraction of tumor RNA from 50–100 mg of disrupted tissue using TissueLyser LT (Qiagen, Hilden, Germany) with zirconium beads (A&A Biotechnology, Gdańsk, Poland) and RNAzol® (Molecular Research Center, Cincinnati, USA) resulted in an RNA fraction of less than 200 base pairs (bps) long. The quality and concentration of RNA were determined using TapeStation (Agilent, Santa Clara, USA) and NanoDrop (Thermo Fisher Scientific), respectively. The RNA was extracted from 0.5 mL of serum using the total RNA protocol provided by RNAzol®.

Next-generation sequencing

A next-generation sequencing (NGS) library was generated using 100 ng of small RNA. After adapter ligation (NEBNext Small RNA Library Prep Set for Illumina; New England Biolabs, Ipswich, USA), the library was enriched using polymerase chain reaction (PCR) (12 cycles), and the size was selected using BluePippin (Sage Science, Beverly, USA) to cut out 141-bp fragments. In total, 48 samples were multiplexed and individually barcoded. The pooled library was run on NextSeq 500 (Illumina, San Diego, USA) using 75 cycles and a single read mid-output cartridge, with the addition of an artificial PhiX library (Illumina) at a final concentration of 20%.

Bioinformatics pipeline

A local server housed raw miRNA reads. The bioinformatics pipeline and miRNA identification were previously described.[41] The reads were de-multiplexed based on the 5'-nucleotidase barcode sequence at the beginning of the read, and FastQC software (Illumina)[42] was used to assess read quality, with adapters removed at the 3' end using Cutadapt (https://cutadapt.readthedocs.io/en/stable/).[43] For quality purposes, all reads with a length below 15 nucleotides or without an adapter were removed. The cleaned reads were then aligned using miRBase 22.1[44] and counted using miRDeep2 software.[45] The raw counts were normalized for library size using miRDeep2 software and represented as counts per million (CPM) for use in further analysis.

Multidimensional scaling (MDS) used miRNA read counts normalized for library size and outlier selection based on dimensions 1 and 2 (Fig. 1).

Quantitative polymerase chain reaction

Confirmation of the presence of the most reliable miRNAs in serum was peformed using quantitative PCR (qPCR) with SYBR Green intercalating dye (Qiagen/Exiqon, Vedbæk, Denmark). Briefly, 5 µL of total RNA underwent reverse transcription (RT) using the miRCURY™ LNA™ Universal RT miRNA PCR system (Qiagen/Exiqon). The generated complimentary deoxyribonucleic acid (cDNA) was diluted 30 times and applied in triplicate to the qPCR reaction, with a specific primer set and spike-in standard (reference gene: *UNI SP6*), using the CFX384 thermal cycler (Bio-Rad Laboratories, Singapore) and a standard Exiqon protocol.

Ultrasound malignancy risk assessment

The risk of malignancy index was derived from a mean value of clinical factors listed in the Guidelines of Polish National Societies Diagnostics and Treatment of Thyroid Carcinoma and sonographic features according to EU-TIRADS for each group.[46,47] Patients received 1 point for the presence of each clinical risk factor and from 2 to 5 points for the US risk category. Clinical risk factors included lymph node metastases, distant metastases, history of previous neck exposure to radiation, rapid tumor growth, a hard thyroid nodule fixated to surrounding tissues, tumor >4 cm in diameter, nodule occurrence before 20 years of age, nodule occurrence after 60 years of age, and paresis of recurrent laryngeal nerves, particularly the unilateral nerves. The EU-TIRADS categories are presented in Table 1.

Statistical analyses

Phenotype data were analyzed statistically using Statistica v. 13.0 software (TIBCO Software, Palo Alto, USA). The χ^2 test of independence was applied to determine if a dataset was well-modeled by a normal distribution. Data did not follow a normal distribution. Therefore, mean, median and range values were calculated. Mann–Whitney U tests and the Fisher's exact tests were used to determine statistical significance, as appropriate. A value of $p < 0.05$ was considered statistically significant.

Statistical and exploratory analyses of miRNome data used the limma package in R (R Foundation for Statistical Computing, Vienna, Austria).[48] The CPM values of miRNA expressions were graphed on a two-dimensional scatterplot to find and remove outliers (Supplementary Fig. 1), which included miRNAs with low CPM values (median below

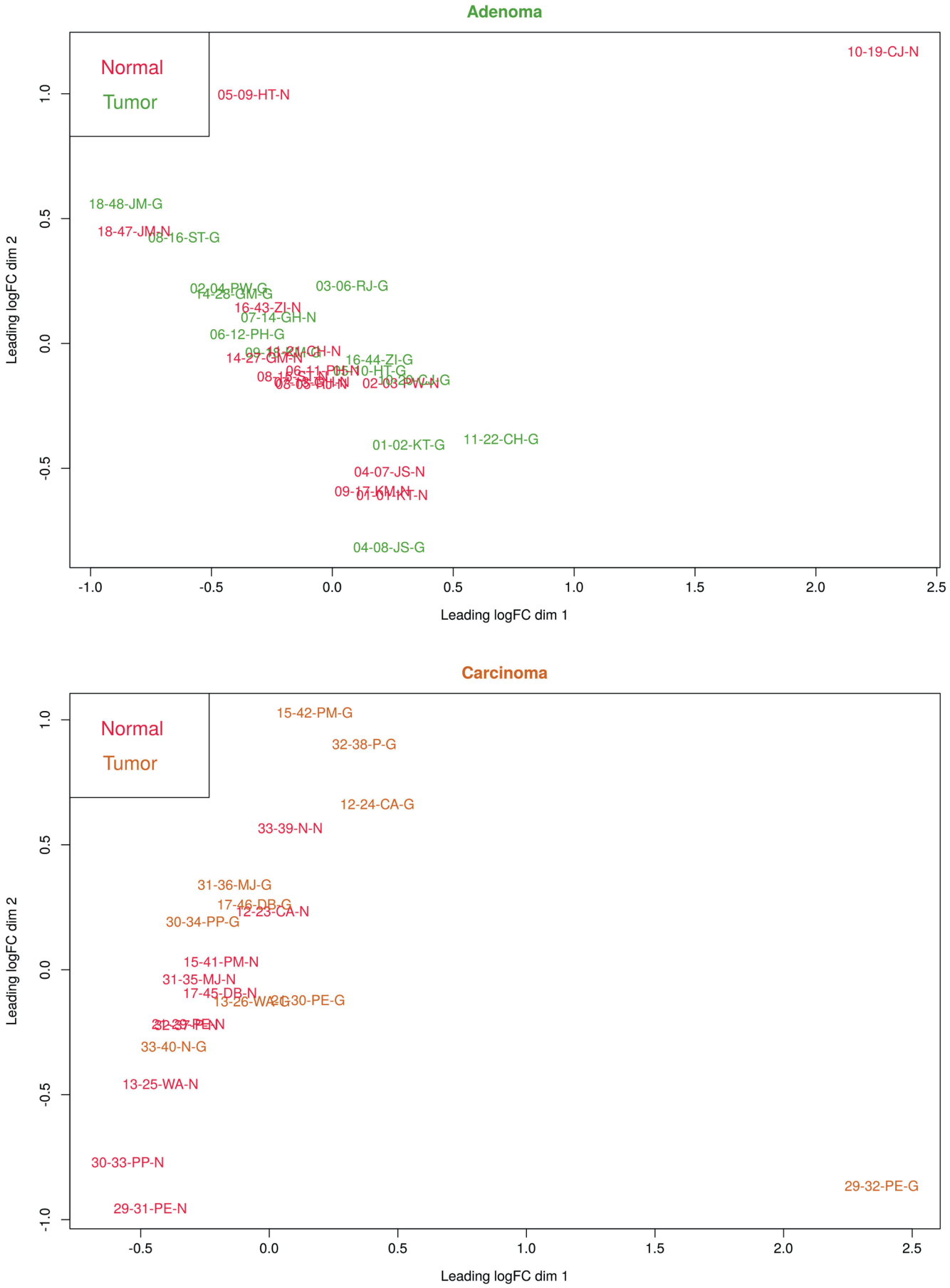

Fig. 1. Multidimensional scaling analysis of adenomas and carcinomas

Table 1. EU-TIRADS categories and risk of malignancy

Category	US features	Malignancy risk [%]
EU-TIRADS 1: normal	no nodules	none
EU-TIRADS 2: benign	pure cyst entirely spongiform	0
EU-TIRADS 3: low risk	ovoid, smooth isoechoic/hyperechoic no features of high suspicion	2–4
EU-TIRADS 4: intermediate risk	ovoid, smooth, mildly hypoechoic no features of high suspicion	6–17
EU-TIRADS 5: high risk	at least 1 of the following features of high suspicion: – irregular shape – irregular margins – microcalcifications – marked hypoechogenicity (and solid)	26–87

EU-TIRADS – European Thyroid Imaging and Reporting Data System; US – ultrasound.

5 reads). Values were then log-transformed using the voom function. The duplicateCorrelation function created gene-wise mean cell models, and, since the study contained paired samples (tumor and healthy tissue samples from the same patient), patientID randomization generated block variables. The lmFit function was applied for each gene to fit the linear model, and a contrast matrix was created for individual comparisons. The control of the false discovery rate of multiple testing was performed using the Benjamini–Hochberg correction method. Quality measures of the regression model are presented in Supplementary Table 1. Diagnostics plots (voom function plot) for routine check mean–variance relationships of the count data after linear model fitting are shown in Supplementary Fig. 2.

The tests were performed as moderated t-statistics, assuming moderated standard errors across genes and effectively borrowing information from the ensemble of genes. In most cases, the distribution of gene expression data was normal, and this assumption still holds for certain technologies, such as microarrays and qPCR. Regardless, the limma package used log-CPM values (log2 of counts per million), for which the normal distribution is assumed. The mean–variance relationship was accounted for using precision weights calculated with the voom function.[48] The analysis followed all transformations recommended as necessary by the package developers (conf. Supplementary Fig. 2). The variance dropped at the low end of the expression scale due to very small counts.[49] Nonetheless, the output was interpreted as a typical voom plot, with a decreasing trend towards higher expression genes (count size) and a very high level of biological variation.[50] Genes with significant differential serum expression between FA and well-differentiated thyroid cancer (WDTC) patients were processed using GenEx software (MultiD Analyses AB, Gothenburg, Sweden). Raw quantification cycle (Cq) values were prepared according to protocol by removing missing data and applying an efficiency correction. The following steps included sample amount normalization, the calculation of qPCR replicate mean values and log2 transformation to relative quantities. After the normalization of the relative quantities against serum level (each patient provided the same volume of serum for miRNA isolation), the differences between the FA and WDTC groups were analyzed using Student's t-test.

Results

Group characteristics

Pathology reports determined the final diagnoses in cases that underwent total thyroidectomy or thyroid lobectomy. There were no significant differences between the characteristics of both groups, and no patient had a family history of TC. The characteristics of study cohort are shown in Table 2. The WDTC was diagnosed in 10 patients, though there were no significant differences in clinical features or EU-TIRADS scale stratification between the groups. Thyroid nodule phenotypes classified according to EU-TIRADS and pathology reports are presented in Supplementary Table 2.

Exploratory analysis of miRNA profiles

Matched tumor and healthy samples were sequenced in 1 NextSeq run. A total of 48 samples were multiplexed, which resulted in over 130 million reads (130857304), with a mean of 2,726,194 reads per sample. Alignment to mirBase identified 1610 miRNA transcripts with at least 5 read genes expressed. The most abundant transcript was miR-26a with 16.6 million reads (7.87%).

Data were explored using MDS analysis (Fig. 1). Meanwhile, sample identifications highlighted outliers and the distribution of particular samples (patients). A dot plot of these findings is shown in Supplementary Fig. 1A–D, from which a minor separation between carcinoma and healthy samples was evident, based on the second dimension. One outlier was identified and removed from the FA and WDTC samples, and these patients were excluded from further analysis. Interestingly, the FA outlier had the highest US features

Table 2. Characteristics of the groups

Category	FA, n = 14 (range; median)	WDTC, n = 10 (range; median)	p-value
Female/male cases	12/2	9/1	0.066 (Fish)
Mean age [years]	55.7 (30–80; 60)	51.4 (37–71; 49.5)	0.371 (U-MW)
Presence of Hashimoto's disease [%]	5/9	3/7	0.166 (Fish)
Tumor diameter [mm]	16.21 (6–40; 15)	12.75 (5.5–25; 9.5)	0.114 (U-MW)
Index of malignancy risk of thyroid tumor (EU-TIRADS)	4.57	4.5	0.593 (U-MW)

EU-TIRADS – European Thyroid Association Thyroid Imaging and Reporting Data System; FA – follicular adenoma; WDTC – well-differentiated thyroid cancer; Fish – Fisher's exact test; U-MW – Mann–Whitney U test. This table shows characteristics of the group with FA and the group with thyroid cancer. In case of nominal variables (sex and presence of Hashimoto's disease), Fisher's exact test has been applied. The distribution of quantitive variables was determined using χ^2 test of independence. The p-value has been calculated using Mann–Whitney U test.

Table 3. Differential expression miRNAs between FA and WDTC samples

miRNA	Precursor	logFC	AveExpr	p-value	adj. p-value
miR-146b-5p	mir-146b	3.16	11.25	0.000078	0.031
miR-195-3p	mir-195	–1.36	5.41	0.00022	0.032
miR-146b-3p	mir-146b	3.03	4.87	0.00024	0.032

FA – follicular adenoma; WDTC – well-differentiated thyroid cancer. Table presents 3 significant differentally expressed (DE) miRNAs between studied groups with annotation of fold change (logFC), average expression (AveExpr), p-value, and adjusted p-value (adj. p-value). The analysis of DE miRNAs was conducted using limma package in R with Benjamini–Hochberg correction method.

of malignancy sum (number 4) among the entire cohort. Four mean–variance graphs corresponding to miRNA signature analysis were plotted separately for adenoma and carcinoma (Supplementary Fig. 2A,B), as were findings on genes differentially expressed between the 2 groups (Supplementary Fig. 2C,D). A high level of biological variation was evident in both groups due to patient differences.

Follicular adenoma and well-differentiated thyroid cancer miRNA signatures: the analysis of matched tumor and healthy samples

The primary analysis determined the miRNA signatures of the FA and WDTC separately, using the healthy samples as a baseline. Compared to the healthy tissue, FA samples had 9 differentially expressed miRNAs. At the same time, 20 miRNAs were differentially expressed in the WDTC tissue compared to the healthy samples (Fig. 2 and Supplementary Table 3). Interestingly, the downregulation of 1 miRNA only, miR-873-5p, was shared between the FA and WDTC signatures.

Differential miRNA expression in follicular adenoma and well-differentiated thyroid cancer samples

The main purpose of this study was to identify differences between FA and WDTC. In this regard, the expression levels of miR-146b-5p ($p = 0.031$) and miR-146b-3p ($p = 0.034$) were significantly higher in patients with WDTC than in those with FA. At the same time, miR-195-3p ($p = 0.032$) expression was significantly lower in patients with WDTC than in those with FA (Table 3, Fig. 3).

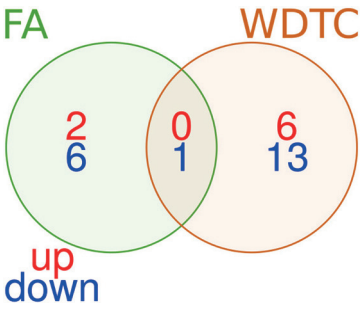

Fig. 2. Venn diagram of the upregulated, downregulated and overlapping miRNAs in follicular adenoma (FA) and well-distinguished thyroid cancer (WDTC)

Validation of main findings using The Cancer Genome Atlas data

External data from The Cancer Genome Atlas (TCGA) were used to validate the study findings. However, the TCGA repository lacked FA data, and the analysis was limited to papillary thyroid carcinoma (PTC) (follicular type: 14 tumors and matched healthy controls; classical type: 114 tumors and matched healthy controls). The miRNA signatures were determined using healthy tissue samples as a baseline, and the comparison to the current study cohort provided a WDTC signature with 20 significant miRNAs. The analysis of the data extracted from TCGA used methods identical to the current study and resulted in the identification of 188 significant miRNAs (Supplementary Table 4), from which the 2nd most significant hit was miR-146b, and the 15th most significant was miR-195. Overall, the WDTC signature had a 90% overlap (18 of 20 miRNAs) with the TCGA signature. Moreover,

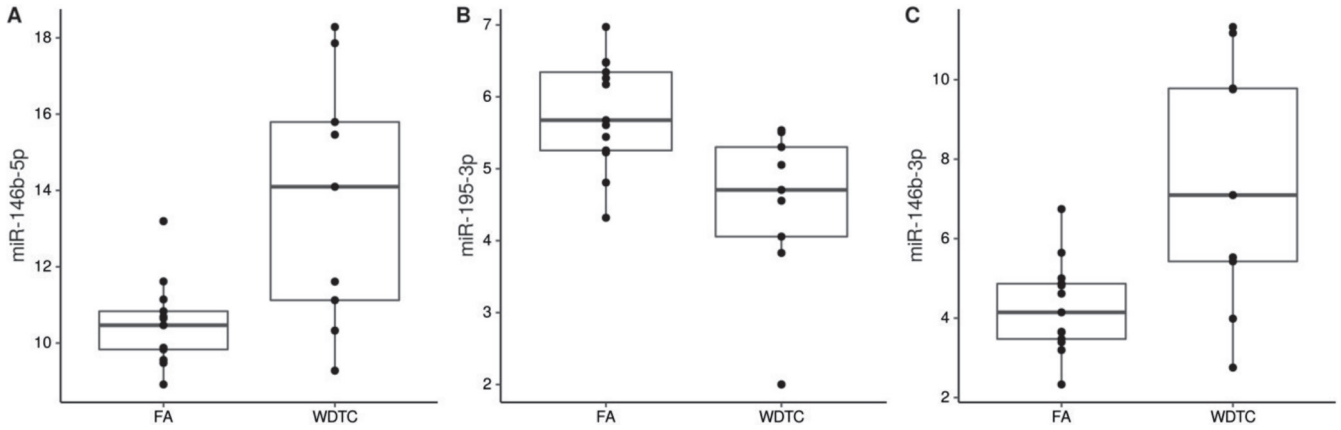

Fig. 3. Differential expression of 3 miRNA acids in follicular adenoma (FA) and well-distinguished thyroid cancer (WDTC) patient tissues. The differentially expressed miRNAs were miR-146b-5p (A), miR-195-3p (B) and miR-146b-3p (C)

the direction of change (upregulation or downregulation) matched perfectly, and the first hit in both cohorts was miR-221.

Serum expression of miR-195

The presence of miR-195 was confirmed in serum using qPCR. The WDTC patients had higher expression levels (log fold change: 4.13, p = 0.039) of circulating hsa-miR-195-3p than FA patients, contrary to findings of tissue miRNA expression analysis (Fig. 4). However, the serum analysis of the WDTC group used only 3 samples.

Discussion

The current study evaluated miRNA signatures in patients with indeterminate FNAB cytology (Bethesda tier IV). According to the literature, such an approach has not been used in a clinical setting before. The miRNA signature in surgically excised specimens differed between WDTC and FA patients. Indeed, the differential expression analysis between these groups highlighted 3 miRNAs (146b-5p, 146b-3p, 195-3p) which could act as biomarkers for thyroid malignancy testing.

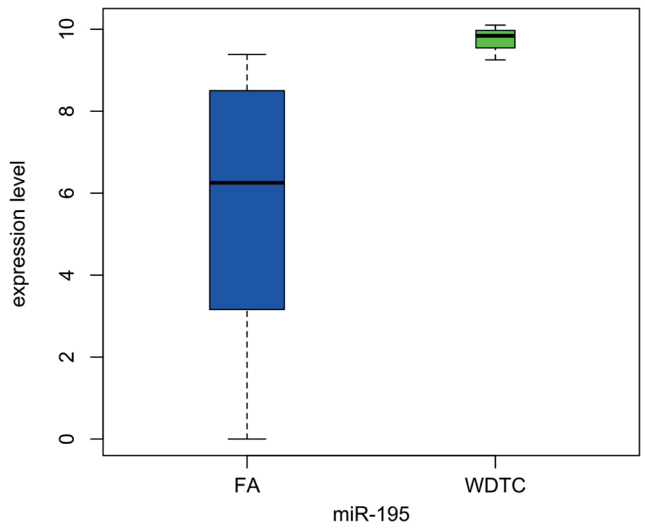

Fig. 4. Serum expression of miRNAs in follicular adenoma (FA) and well-distinguished thyroid cancer (WDTC) patients

Due to the absence of specific US features that can definitively predict malignancy, a standardized system for reporting US features, TIRADS, was developed[51] in 2009 by Horvath et al. and Park et al.[52,53] In 2017, the European Thyroid Association created the new and simplistic EU-TIRADS, in which classified scores stratify thyroid lesions in adults according to the risk of malignancy into benign, low-risk, intermediate-risk, and high-risk.[47,52,53] The current EU-TIRADS evaluates 5 lesion patterns and classifies the nodules into 1 of the 5 categories based on the number of suspicious features.

The NGS-based miRNA profiling is very sensitive and detects samples with different phenotypes (see outliers in Fig. 1). Numerous miRNA profiles have also been reported in distinct cancer types.[54] Recent studies demonstrated miRNA extraction from blood, urine and saliva samples,[55,56] and their expression levels provide information about ongoing physiological and pathological conditions.[56] This study differentiated between FA and WDTC based on 3 miRNAs, namely miR-146b-5p, miR-146b-3p and miR-195-3p.

The molecule miRNA-195 engages in various malignant cellular processes via complex mechanisms, including proliferation, apoptosis, invasion, angiogenesis, and metastasis. Indeed, the molecule may be involved in various signaling pathways, such as retinoblastoma-early region 2 binding factor (Rb-E2F) and phosphatidylinositol 3-kinase/protein kinase B (PI3K/AK).[57] In a study by Wang et al., miR-195 was downregulated in thyroid tumor tissues. Moreover, the proto-oncogene *Raf1* is a target of miR-195, although its regulatory mechanisms are unclear.[58–60] In addition, the overexpression of miR-195 markedly suppressed the growth of TC cells.[57,61] The current study demonstrated the differential expression of hsa-miR-195-3p in tumor and thyroid tissues, which was also demonstrated in PCR analysis of serum samples.

The miRNA-146b plays a role in post-transcriptional gene silencing,[62,63] and its expression demonstrated a disparity between PTC tissue and normal thyroid tissue.[64–66] At the same time, several studies have implicated miRNA-146b dysregulation in the different variants of PTC, and recent investigations have revealed that diversity in miRNA-146b expression may be associated with advanced malignant tumor characteristics,[67–69] including extrathyroidal invasion and metastases by suppressing interleukin-1 receptor-associated kinase 1 (*IRAK1*) expression.[70,71] In addition, other thyroid neoplasms such as follicular thyroid carcinoma (FTC) and poorly-differentiated thyroid cancer (PDTC) encompass miR-146b, and its expression in these subtypes of TC has been described.[72]

The crucial point of this study was to confirm differences in miRNA expression between FA and WDTC tissues and matched serum. Among the 3 identified miRNAs, only miR-195-3p was expressed in serum, and its levels were higher in TC patients. However, the results should be interpreted carefully due to the low number of patients in both groups. There was a paradoxical hsa-miR-195-3p expression pattern between tissue and serum, as it was upregulated in serum and downregulated in the tissue of patients with WDTC compared to FA patients. A similar phenomenon has been documented in studies on other cancer variants.[73–75]

The precise mechanisms of miRNA release into the extracellular environment remain only partially explored. According to one hypothesis, damaged cells release miRNAs via microvesicles or directly through different types of proteins.[76–79] Another potential mechanism suggests that extracellular miRNAs originate from immunocytes in the tumor microenvironment.[80]

The focus of studies has shifted to altered miRNA expression in PTC, as FTC prevalence has decreased in recent decades.[81] The current study concentrated on discriminating between miRNAs in FA and FTC, though PTC occurred 4 times more frequently than FTC. Consequently, identifying miRNAs independently associated with PTC and FTC was impeded. Nonetheless, from our clinical experience in the surgical treatment of thyroid disorders, distinguishing between PTC and FTC in thyroid nodules verified as SFN is not obligatory. Also, the most recent recommendations for TC treatment allow to resign from elective central neck dissection in low-risk patients, including those with tumor size up to 4 cm. Indeed, differentiating FA from WDTC should be sufficient to apply an appropriate treatment.[46,82]

Detection of circulating miRNAs may be a useful diagnostic tool for TC. Circulating miRNAs appear to be a more promising biomarker than other RNAs due to their serum stability and tissue specificity. In this regard, serum-based identification of miRNAs released from the tumor during disease progression could lead to early cancer detection. The expression of miRNAs varies at different stages of malignant disease. Therefore, evaluating serum miRNA levels after thyroid resection may be a viable noninvasive patient follow-up method.[83] As such, improved standardization of methods used to assay circulating miRNAs may result in an optimal miRNA diagnostic biomarker. In addition, miRNAs could become a promising strategy in cancer research, as several studies have demonstrated the silencing of overexpressed miRNAs and their downregulation using synthetic oligonucleotides.[84–90]

Next-generation sequencing miRNA profiling provides time- and pathophysiological state-specific miRNome information. As such, miRNA expression is an obvious biomarker, and a systems biology approach can delve deeper into the molecular pathophysiology of certain types of cancer and benign lesions. For example, the current study found miRNA upregulation in WDTC related to interleukin-7 (IL-7) regulation processes involving 2 genes, IL-7 receptor (*IL7R*) and IL-2 receptor subunit gamma (*IL2RG*). So far, no studies have described *IL7R* in the context of WDTC, although its connection with other thyroid pathologies, such as thyroid lymphoma and thymoma, has been demonstrated.[91,92] Therefore, the *IL7R* gene is a potential marker for differentiating FA from WDTC.

A literature search found no studies demonstrating differences in miRNA expression in thyroid nodules verified as SFN. Here, a set of 3 miRNAs were found, as was the differential serum expression of 1 miRNA in patients with SFN biopsy results. Further studies encompassing larger groups of patients are needed to verify these differential expression patterns. Hopefully, molecular diagnosis based on circulating miRNA will avoid unnecessary surgeries or even FNAB.

Limitations

The study was limited by the low number of WDTC samples. It assessed and evaluated only 2 FTC patients due to low FTC incidence. Of these 2 patients, MDS analysis led to the exclusion of one. The group of patients with benign FA lesions was more numerous and histopathologically homogeneous compared to the cancer patient group, which had 3 subtypes of WDTC. Also, sampling was limited to the population of Lesser Poland Voivodeship. As such, the conclusions do not apply to other populations due to genetic differences.

Conclusions

Conclusions and future perspectives:

1. The FA and WDTC had different miRNA signatures, with only miR-873 overlapping.
2. Three miRNAs, namely 146a, 146b and 195, could be used to diagnose patients as SFN.

Supplementary files

The supplementary files are available at https://doi.org/10.5281/zenodo.7576125. The package contains the following files:

Supplementary Table. Pearson's χ^2 test results.

Supplementary Table 1. Measure of the quality of the regression model.

Supplementary Table 2. Phenotype of thyroid nodules according to EU-TIRADS scoring as well as pathology reports.

Supplementary Table 3. The miRNAs differentiate FA and WDTC from normal tissue.

Supplementary Table 4. Analysis of data extracted from TCGA compared with results obtained as a signature of 188 significant miRNAs.

Supplementary Fig. 1. Multidimentional scaling analysis.

Supplementary Fig. 1A. MDS_tumor.

Supplementary Fig. 1B. MDS_group.

Supplementary Fig. 1C. MDS_WDTC.

Supplementary Fig. 1D. MDS_FA.

Supplementary Fig. 2. Mean–variance trend of log-CPM values of data on adenoma and carcinoma.

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Helicobacter pylori infection is associated with increased accumulation of advanced glycation end products in the skin in patients with type 1 diabetes: A preliminary study

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Conflict of interest
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Abstract

Background. *Helicobacter pylori* infection (HPI) is more frequently diagnosed in patients with diabetes. Insulin resistance in patients with type 1 diabetes (DMT1) is associated with the accumulation of advanced glycation end products (AGEs) in the skin and progression of chronic complications.

Objectives. Assessment of the relationship between the incidence of HPI and skin AGEs in patients with DMT1.

Materials and methods. The study included 103 Caucasian patients with a DMT1 duration >5 years. A fast qualitative test was performed to detect the HP antigen in fecal samples (Hedrex). The content of AGEs in the skin was estimated using an AGE Reader device (DiagnOptics).

Results. The HP-positive (n = 31) and HP-negative (n = 72) groups did not differ in terms of age, gender, duration of diabetes, fat content, body mass index (BMI) and lipid profile, metabolic control, and inflammatory response markers. The studied groups differed in the amount of AGEs in the skin. The relationship between HPI and increased AGEs in the skin was confirmed in a multifactor regression model taking into account age, gender, DMT1 duration, glycated hemoglobin A1c (HbA1c), BMI, low-density lipoprotein cholesterol (LDL-C) and the presence of hypertension, and tobacco use. The studied groups also differed in serum levels of vitamin D.

Conclusions. Increased accumulation of AGEs in the skin of patients with DMT1 with coexisting HPI suggests that eradication of HP may significantly improve DMT1 outcomes.

Key words: vitamin D, *H. pylori*, advanced glycation end products, type 1 diabetes, diabetic complications

Background

In recent years, a significant increase in the incidence of *Helicobacter pylori* infection (HPI) has been observed.[1] According to the World Health Organization (WHO), about 30% of the population in developed countries and 70% of the population in developing countries carry this Gram-negative anaerobic bacilli.[2] *Helicobacter pylori* (HP) is the principal etiological agent of diseases such as gastritis, peptic ulcers and gastric cancer.[3] The infection may aggravate gastric symptoms of patients treated with chronic steroid therapy or nonsteroidal anti-inflammatory drugs (NSAIDs), and plays an important role in the exacerbation of certain autoimmune diseases, such as hypothyroidism. Furthermore, chronic inflammation associated with infection and secretion of pro-inflammatory cytokines may cause many extragastric symptoms such as iron deficiency anemia, insulin resistance, ischemic heart disease, and neurological disorders. Meta-analysis data from 2013 confirm that HPI is recognized significantly more often in patients with diabetes, and patients infected with HP are more frequently diagnosed with diabetes.[4] The relationship between the infection and insulin resistance of peripheral tissues is yet to be determined.

Type 2 diabetes (DMT2) patients infected with HP showed significantly increased Homa Insulin Resistance Index (HOMA-IR) values than those without any coexisting infection. Similar observations have been made in patients with type 1 diabetes (DMT1).[5] Insulin resistance in DMT1 patients is associated with thickening of the intima-media complex (IMT), accumulation of advanced glycation end products (AGEs) in the skin, and development of chronic complications. The assessment of the association of HPI with anthropometric parameters and insulin resistance indices may bring significant clinical benefits. Enhanced accumulation of AGEs in the skin and, thus, an increased risk of chronic complications of diabetes in patients with coexisting HPI, may suggest that eradication of the bacteria might alleviate some of the complications of DMT1.

Objectives

The aim of this study was to evaluate the relationship between the presence of HPI and the accumulation of AGEs in the skin of patients with DMT1.

Materials and methods

The study involved 103 patients with DMT1 treated in the Department of Gastroenterology, Dietetics, and Internal Diseases and in the Department of Internal Medicine and Diabetology (Poznan University of Medical Sciences, Poland) in 2017–2020, who met the criteria for inclusion. All patients gave informed consent.

The inclusion criteria were:

1. DMT1 (diagnosis supported by the presence of autoantibodies ICA, IA2 and GAD) treated with intensive functional insulin therapy or multiple injections of fixed insulin doses;
2. Age > 18 years;
3. Caucasian;
4. Diabetes duration > 5 years;
5. Informed consent for study participation.

Exclusion criteria were:

1. Diabetes other than DMT1;
2. Persons treated with chronic proton pump inhibitors, H_2 blockers and bismuth preparations;
3. Subjects receiving antibiotics within 1 month before study entry;
4. Persons treated with chronic steroids, NSAIDs;
5. Pregnancy;
6. Other chronic gastrointestinal diseases (gastric cancer, inflammatory bowel disease, etc.).

Ethics committee

The study protocol was approved by the Poznan University of Medical Sciences Bioethics Commission (approval No. 504/16 granted on May 5, 2016). The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in a prior approval by the institution's human research committee. All patients gave written informed consent.

Evaluation of *Heliobacter pylori* infection

A noninvasive method was used to assess the presence of HP in patients. A rapid, qualitative, one-step test for the detection of the HP antigen in stool samples was performed (Helicobacter Antigen Test; Hydrex Diagnostics, Warszawa, Poland) (Ref HXHPAg10, Ag20, Ag25). The principle of the test is based on the immunochromatographic method (monoclonal antibodies directed against HP on the test strip). The mean sensitivity and specificity of the test is 94.5%. The presence of the HP antigen in feces is confirmed by the formation of a colloidal gold antibody complex of HP antigen–antibody conjugate.

Anthropometric parameters and survey data

Anthropometric indicators (height, body weight, body mass index (BMI), waist and hip circumference (WHR index)) were analyzed. The daily dose of insulin was evaluated. The estimated glucose disposal rate (eGDR) was calculated using published formulae.[6] The visceral adiposity index (VAI) was evaluated based on a previously published gender-specific formula.[7]

Total body fat was assessed with electrical bioimpedance using a Tanita BC-418 MA (Tanita Polska, Poznań,

Poland) analyzer. As a result of those measurements, abdominal visceral body fat (AViscBF) content was presented on a scale from 1% to 59% (resolution: 0.5%) and percentile content of trunk body fat on a 5–75% scale (resolution: 0.1%). The device is CE certified and complies with European Union Directive MDD93/42EEC for medical appliances. Total body fat and visceral adipose tissue were estimated using electrical bioimpedance according to the WHO age- and gender-adjusted criteria.[8] The clinical presentation of the investigated group is shown in Table 1.

Evaluation of chronic complications

The presence and severity of chronic complications were evaluated. Retinopathy was diagnosed when at least 1 microaneurysm was found in both eyes and was classified according to the recommendations of the Polish Diabetes Association. Diabetic kidney disease was recognized by the assessment of renal function (creatinine serum concentration, calculation of glomerular filtration rate (GFR) following the pattern of Modification of Diet in Renal Disease (MDRD; the assessment of excretion of albumin in the urine)). Diabetic kidney disease was defined as albuminuria or evident proteinuria. Albuminuria was defined according to the recommendations of the Polish Diabetes Association.[9] Peripheral neuropathy was diagnosed based on 2 out of 5 abnormal parameters such as the sensation of touch, vibration, temperature, pain, or knee or ankle reflex removal. Touch sensation was assessed using Semmes–Weinstein 10 g pressure monofilament (Medische Vakhandel, Veendam, the Netherlands), vibration sensation was assessed with Tunning Fork C 128 Hz/C 64 Hz according to Ryder–Seiffer function diagnostics 2 (Suzhou Yongtaite Dianzikejiyouxiangongsi, Suzhou, China), temperature sensation was evaluated with a metal and plastic tip roller (Tiptherm; GIMA Italy, Gessate, Italy), and by examining the ankle jerk reflex. Autonomous neuropathy was diagnosed based on heart rate variability test (rest assessment, respiratory test, Valsalva test, orthostatic test) with the use of ProSciCard III® apparatus (2010; CPS GmbH, Rohrdorf, Germany).

Quantitative assessment of skin autofluorescence

The content of AGEs was evaluated using the AGE Reader device (type 214D00102; DiagnOptics, Groningen, the Netherlands). The device emitted ultraviolet light with a wavelength of 300–420 nm and was used to illuminate 1 cm^2 of skin on the inner forearm, about 10 cm away from the elbow joint. A built-in spectrometer registered light in the range of 300–600 nm (autofluorescence (AF)). The AF score was calculated automatically (the ratio of the amount of light emitted by the skin to the amount of light emitted by the device).

Table 1. Clinical characteristics of the study group (n = 103, median, 1st and 3rd quartile)

Variable	Value
Sex (female/male), n (%)	53 (51)/50 (49)
Age [years]	34 (30–42)
Diabetes duration [years]	17 (12–23)
Diabetic retinopathy, n (%)	39 (37.9)
Autonomic neuropathy, n (%)	21 (20.4)
Peripheral neuropathy, n (%)	32 (31.1)
Diabetic kidney disease, n (%)	10 (9.7)
IFI [years]	12.5 (8–18.5)
SBP [mm Hg]	120 (116–133)
DBP [mm Hg]	74 (70–80)
WC [cm]	93.5 (84–103)
HC [cm]	105.5 (100–111.5)
WHR, n	0.9 (0.8–0.9)
BMI [kg/m^2]	25.8 (22.9–29.3)
TBF [kg]	19 (13.5–26.5)
VBF, n	5 (3–8)
DDI [j/kg/d]	0.5 (0.4–0.6)
HbA1c [%]	8.2 (7.1–9)
AST [U/L]	19 (16–24)
ALT [U/L]	19 (14–26)
Creatinine [µmol/L]	79.6 (70.7–88.4)
eGFR [mL/min/1.73 m^2]	87.6 (76.5–90)
hsCRP [mg/dL]	1.5 (0.8–3.1)
T-Ch [mmol/L]	48 (41.6–53.8)
TAG [mmol/L]	1.1 (0.83–1.5)
HDL-C [mmol/L]	1.6 (1.3–2)
LDL-C [mmol/L]	2.6 (2.1–3.1)
non-HDL-C [mmol/L]	3.1 (2.6–3.6)
25-OH vitamin D [mg/dL]	19 (13–29)
VAI-K, n	1.8 (1.4–2.9)
VAI-M, n	2.5 (1.6–4.1)
eGDR [mg/kg/min]	8.2 (6–9.4)
AGEs, SAF	2.2 (1.9–2.7)

AGEs – advanced glycation end products; ALT – alanine aminotransferase; AST – aspartate aminotransferase; BMI – body mass index; DBP – diastolic blood pressure; DDI – daily dose of insulin; eGFR – estimated glomerular filtration rate; eGDR – estimated glucose disposal rate; HbA1c – glycated hemoglobin A1c; HC – hip circumference; HDL-C – high-density lipoprotein cholesterol; hsCRP – high-sensitivity C-reactive protein; IFI – intensive insulin therapy; LDL-C – low-density lipoprotein cholesterol; SBP – systolic blood pressure; SAF – skin autofluorescence; TBF – total body fat; T-Ch – total cholesterol; WC – waist circumference; WHR – waist-to-hip ratio; VAI – visceral adiposity index; VBF – visceral body fat; TAG – triglicerydes.

Advanced products of protein glycation can be assessed in serum; however, due to their high heterogeneity and instability, as well as physiological fluctuations in their concentrations, this test is not particularly reproducible.[10] Moreover, the cost of serum AGE determination is high.

Skin autofluorescence (SAF) is a simple and noninvasive technique that has been validated based on the gold standard, the assessment of AGE in skin biopsy.[11] The SAF directly correlates with both fluorescent (pentosidine) and nonfluorescent (Nε-carboxymethyllysine (CML) and Nεcarboxy-ethyllysine (CEL)) advanced glycation products in skin biopsy.[12] In a study by Koetsier et al., SAF reference values were established for healthy Caucasians.[13] The SAF increased linearly with age at approx. 0.023 units (AU) per year for those aged up to 70 years. Tobacco smoking was associated with an absolute increase in SAF of 0.16 AU, and in this case, age was not additive. Gender had no effect on SAF in non-smokers, while in the smoking group, women had 0.2 AU higher skin AF than men, with no further age-related increase.

Laboratory tests

Basic laboratory parameters were also evaluated: the value of glycated hemoglobin A1c (HbA1c) was measured with turbidimetric and immunoinhibition methods using the cobas® 6000 device (norm: 4.8%–6.5%; Roche Diagnostics, Basel, Switzerland); lipid metabolism parameters (total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) fractions, and triglycerides concentration in serum) using the enzymatic method and C-reactive protein (CRP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine using standard methods. The calculation of estimated glomerular filtration rate (eGFR) was based on MDRD study equation (norm: 90–120 mL/min/1.73 m^2). The concentration of vitamin 25(OH)D_3 was determined with radioimmunoassay.

Statistical analyses

The statistical analysis was performed using Statistica PL v. 13.3 (StatSoft Polska Sp. z o.o., Kraków, Poland), MedCalc v. 20.115 (MedCalc Software Ltd., Ostend, Belgium) and R software v. 4.1.1 (R Foundation for Statistical Computing, Vienna, Austria). The compliance of the interval data distribution with the normal distribution was assessed using the Kolmogorov–Smirnov test. Normal distribution was not observed in most of the data. In the analysis, a statistical method for nonparametric variables, the Mann–Whitney U test was used. The results are presented as numbers and percentages, as well as medians and interquartile range (IQR). The Fisher's test was applied to analyze the categorical data. In order to assess the influence of HPI on the onset of long-term complications of the disease, the logistic regression model with no automatic predictor selection was applied. Observations complied with the model's assumptions: 1) no extreme outliers (>3 IQR), after removing 2 such values; and 2) no multicollinearity among explanatory variables (variance inflation factor (VIF) of 0.1 for each of the numerical predictors). A value of $p < 0.05$ was considered statistically significant.

Results

In the study group, 30% of patients were found positive for HPI. The HP-positive and HP-negative groups did not differ in age, sex, duration of diabetes, metabolic control, and inflammatory response markers, as well as regarding fat content, BMI and lipid profile. The studied groups varied in the amount of AGEs in the skin (Table 2), and in the levels of vitamin D. Vitamin D serum concentrations were significantly higher in patients without HPI. However, no differences in fat content, BMI and lipid profile were noted. Differences between HP-positive and HP-negative groups are shown in Table 2. In the logistic regression analysis, lower vitamin D levels were associated with the presence of HPI (odds ratio (OR): 0.89; 95% confidence interval (95% CI): 0.81–0.96, p = 0.005), and the higher SAF was associated with the presence of HP (OR: 5.43; 95% CI: 1.88–18.55, p = 0.003). Age, gender, smoking status, BMI, arterial hypertension, HbA1c value, and LDL cholesterol (LDL-C) concentration were not related to the presence of HPI (Table 3).

Discussion

It has been previously demonstrated that an increased prevalence of HPI occurs in patients with autoimmune diseases, such as hypothyroidism and DMT1. The co-occurrence of HP and DMT1 is also associated with a higher level of islet cell antibodies (ICA). The intensification of the autoaggression process is associated with worse diabetes control and increases the risk of chronic complications, such as retinopathy and neurological disorders.[9]

The results of studies evaluating the influence of HPI on metabolic control and its relation to the occurrence of chronic complications in diabetic patients are inconclusive.[14,15] The meta-analysis by Dai et al. found significantly higher glycated hemoglobin values in patients with DMT1 and HPI.[15] However, this relationship was not confirmed in patients with DMT2. A study by Demir et al. showed that HPI was associated with the development of neuropathy. However, no such relationship was found when analyzing glycemic control, occurrence of retinopathy or diabetic kidney disease.[16] Moreover, patients with DMT1 may show higher levels of HP colonization because of delayed gastric emptying and the occurrence of gastroparesis, as the result of autonomic neuropathy.[17] Chung et al. demonstrated that the incidence of microalbuminuria was significantly higher in patients with DMT2 and coexisting HPI.[18] Similarly, in a study by Zizzi et al., it was found that HPI is associated with a higher incidence of proteinuria in patients with DMT2.[19] The relationship between HPI and diabetic nephropathy has been shown in a study by Bajaj et al.[20] The HPI also aggravates the symptoms of diabetic gastroparesis.[21] The association between HPI and retinopathy has also been demonstrated in the study by Agrawal

Table 2. Statistical differences between HP(–) and HP(+) groups (the Mann–Whitney U test)

Variable	HP(–) n = 72 (70%)	HP(+) n = 31 (30%)	p-value
Sex, female/male, n (%)	34 (47)/38 (53)	19 (61)/12 (39)	0.191*
Age [years]	35 (31–40)	34 (29–43)	0.661
Diabetes duration [years]	18 (12–23)	17 (12–23)	0.749
Diabetic retinopathy, n (%)	30 (41.7)	9 (29)	0.276*
Autonomic neuropathy, n (%)	15 (20.8)	6 (19.4)	0.071*
Peripheral neuropathy, n (%)	21 (29.2)	11 (35.5)	0.076*
Diabetic kidney disease, n (%)	8 (11.1)	2 (6.5)	0.179*
SBP [mm Hg]	125 (120–135)	120 (110–130)	0.165
DBP [mm Hg]	75 (70–80)	71 (70–80)	0.952
WC [cm]	94 (84–101)	93 (84–103)	0.687
HC [cm]	104 (99–111)	109 (103–113)	0.105
WHR, n	0.9 (0.8–0.9)	0.9 (0.8–0.9)	0.789
BMI [kg/m^2]	25.3 (22.5–29.3)	27.5 (24–30.3)	0.256
TBF [kg]	18.8 (11.8–26.7)	19 (16–25.3)	0.558
VBF, n	5 (3–8)	5 (3–8)	0.966
DDI [j/kg/d]	0.5 (0.4–0.6)	0 (0.3–0.5)	0.784
HbA1c [%]	8.2 (7.1–8.9)	7.9 (7.1–9.3)	0.868
AST [U/L]	19 (15–25)	19 (17–23)	0.899
ALT [U/L]	20 (14–26.5)	17 (13–24)	0.214
Creatinine [µmol/L]	70.7 (70.7–88.4)	79.6 (61.9–79.6)	0.263
eGFR [mL/min/1.73 m^2]	88.6 (76.7–90)	86.4 (75.5–90)	0.419
hsCRP [mg/dL]	1.4 (0.8–3.1)	1.9 (0.7–3)	0.524
T-Ch [mmol/L]	49.1 (42.7–54.8)	45.7 (41.1–51.2)	0.149
TAG [mmol/L]	1.1 (0.83–1.5)	1.1 (0.8–1.4)	0.956
HDL-C [mmol/L]	1.6 (1.3–1.9)	1.7 (1.4–2.1)	0.228
LDL-C [mmol/L]	2.7 (2.3–3.2)	2.3 (1.9–2.8)	0.051
non-HDL-C [mmol/L]	3.2 (2.6–3.7)	2.8 (2.5–3.1)	0.058
25-OH vitamin D [mg/dL]	21 (14.5–31)	16 (12–21)	0.024
VAI-K, n	1.7 (1.4–2.3)	2 (1.2–3.2)	0.681
VAI-M, n	2.6 (1.5–4.1)	2.4 (2–4.2)	0.891
eGDR [mg/kg/min]	8.2 (6.1–9.3)	8.3 (5.9–9.8)	0.574
AGEs, SAF	2.1 (1.8–2.5)	2.6 (2.3–3.1)	<0.001

Data are presented as median, 1st and 3rd quartile. Because not all of the assumptions to interpret the Mann–Whitney U test results in terms of medians were met, p-values shown in the table do not refer to the comparison between medians, but ordering between variables. * categorical variables (shown as numbers and percentages) were compared using the Fisher's exact test. AGEs – advanced glycation end products; ALT – alanine aminotransferase; AST – aspartate aminotransferase; BMI – body mass index; DBP – diastolic blood pressure; DDI – daily insulin dose; eGFR – estimated glomerular filtration rate; eGDR – estimated glucose disposal rate; HbA1c – glycated hemoglobin A1c; HC – hip circumference; HDL-C – high-density lipoprotein cholesterol; HP – *Helicobacter pylori*; hsCRP – high-sensitivity C-reactive protein; LDL-C – low-density lipoprotein cholesterol; SBP – systolic blood pressure; SAF – skin autofluorescence; TBF – total body fat; T-Ch – total cholesterol; WC – waist circumference; WHR – waist-to-hip ratio; VAI – visceral adiposity index; VBF – visceral body fat; TAG – triglicerydes.

et al.[22] However, in our study group, no statistically significant differences in disease complications were observed in patients with and without HPI (Mann–Whitney test, $p > 0.05$). This may be due to the relatively small number of individuals presenting with complications in the study group (24.7% on average) and the short or completely unknown duration of HPI. Indeed, a prolonged inflammatory process enhances the development of chronic complications. The HPI causes a local inflammatory process that may not reflect a long-term systemic response. However, chronic inflammation is a well-known risk factor for diabetic chronic complications. The HPI could cause a long-lasting, low-intensity inflammation that affects cell metabolism. In the study by Noach et al. that used a multivariable logistic regression model, HPI was not associated with elevated CRP levels. However, tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1β levels were significantly higher in HP-positive patients. This may be

Table 3. Multivariate logistic regression for *H. pylori* infection including age, gender, DM duration, HbA1c, BMI, LDL-C, arterial hypertension, smoking, and AGEs

Variables	OR and 95% CI	p-value
Gender = W	1.44 (0.33–6.72)	0.631
BMI1	0.82 (0.17–3.76)	0.798
BMI2	1.5 (0.31–7.41)	0.611
Age	1.04 (0.96–1.14)	0.316
AGEs	5.43 (1.88–18.55)	0.003
Vitamin D	0.89 (0.81–0.96)	0.005
HbA1c	1.18 (0.73–1.9)	0.498
DM duration	0.94 (0.84–1.03)	0.179
LDL-C	0.99 (0.97–1.01)	0.320
HA = 1	0.68 (0.14–2.93)	0.610
Smoking = 1	0.22 (0.02–1.31)	0.126

OR – odds ratio; 95% CI – 95% confidence interval; Hosmer–Lemeshow = 8.8081; p = 0.358746; Nagelkerke R^2 = 0.388; AGEs – advanced glycation end products; BMI1 – body mass index overweight; BMI2 – body mass index obesity; HbA1c – glycated hemoglobin A1c; LDL-C – low-density lipoprotein cholesterol; SAF – skin autofluorescence. DM – diabetes mellitis; HA – hypertension. W – women.

of particular importance in patients with newly diagnosed DMT1 enrolled into this study, who present with higher numbers of IL-1β-expressing monocytes and a reduced number of myeloid dendritic cells (mDCs) expressing IL-6, when compared to nondiabetic individuals.[23] There were no elevated inflammatory response markers in the study group, but the value of CRP was higher in the HP-positive group. However, this difference was not statistically significant. In addition, elevated IL-1 levels may contribute to increased insulin resistance and play a central role in driving tissue inflammation during metabolic stress in patients diagnosed with diabetes.[24]

The phenomenon of insulin resistance is associated not only with DMT2. A growing body of evidence shows that insulin resistance may play an important role in the development of complications in DMT1. Reduced sensitivity of peripheral tissues to insulin limits the possibility of achieving good metabolic control of carbohydrate, lipid and blood pressure. An increase in insulin resistance due to HPI is another factor that may affect the control of disease progression in diabetes.[25,26] Local gastritis, induced by the bacterium, can also affect the concentration of hormones such as gastrin and somatostatin, as well as leptin and ghrelin, which are important factors regulating the content and hormonal function of adipose tissue in the body.[27,28] In the present study, no differences were observed in insulin-resistant markers (VAI, eGDR) between both groups of patients (HP-positive and HP-negative). The HPI may also influence lipid profiles. A significant increase in LDL-C and a decrease in HDL cholesterol (HDL-C) concentrations were observed in patients without diabetes and in patients with DMT2.[29,30] However, no studies evaluating these parameters in patients with DMT1 and concomitant HPI have been published. No such connection was found in the group of patients studied by our team.

In a study by Meerwaldt et al., in diabetic patients, 70% of SAF was above 95% CI of the mean value in the control group.[12] In diabetic patients, SAF directly correlated with age, duration of diabetes and the value of HbA1c.[12,31] Samborski et al. indicate that SAF was significantly higher in DMT1 patients than in the controls without diabetes.[32] Moreover, there was a significant positive association between SAF and the age of patients. A significant positive relationship was found in diabetic subjects between SAF, the duration of diabetes and HbA1c. The SAF measurement reflects glycemic control over a longer period than that reflected by HbA1c levels.[33] It has also been demonstrated that SAF is a better predictor of the development of chronic complications and mortality from diabetes over time (5–10 years) than HbA1c.[34–36] Moreover, AGE values (>2.0 AU for over 5 years) are a significant marker of the development of vascular complications of micro- and macroangiopathy. An increase in SAF value is a risk factor for cardiovascular disease and death.[36]

Our results indicate that HPI is associated with AGEs in patients with DMT1. This is the first study assessing the relationship between HPI and the presence of AGEs in the skin using AF. In previous studies, the local receptor for advanced glycation end products (RAGE) concentrations in gastric epithelial cells was assessed. It has been found that RAGE expression increased in HP-infected cells compared to uninfected cells.[37]

The HP produces many virulence factors such as vacuolating cytotoxin A (VacA), which induces programmed cell necrosis, causes HMGB1 release and enhances the pro-inflammatory response.[38] However, the mechanisms of the activation of HMGB1 expression and RAGE by HPI to promote inflammation in gastric epithelial cells are not fully understood.[39]

Furthermore, a high rate of positive RAGE expression was observed in the gastric biopsies.

In addition, a significantly higher percentage of RAGE expression is found in biopsies with dysplasia or carcinoma in situ.[40] Many studies show an association between HPI and metabolic syndrome, Barrett's esophagus, esophageal adenocarcinoma, gastric and duodenal ulcers, and gastric and lower gastrointestinal oncogenesis.[41]

The role of RAGE in the process of HP adhesion to the epithelial cells of the stomach is also worth emphasizing and confirms the belief that HP is undoubtedly related to the content of final products of protein glycosylation in the body. In the study by Rojas et al., the role of the receptor for RAGE on the adhesion of HP to gastric epithelial cells was defined. Bacilli of HP bind with immobilized RAGE. It means that RAGE represents a new factor in the pathogenesis of HPI.[42] There is no evidence in the literature showing that the eradication of HP reduces AGEs; this would be an important follow-up study.

The significantly lower vitamin D levels in HP-positive patients are also important. As shown in our study, patients with lower vitamin D levels and DMT1 are more likely to be HP-positive. Vitamin D deficiency can have a negative impact on metabolic status and glycemic control in DMT1, although the supplementation of vitamin D improves these outcomes.[43] On the other hand, low vitamin D levels are associated with an increased risk of HPI.[44] Moreover, many data show that vitamin D deficiency may result in failure to eradicate HPI on treatment.[44,45] In a study performed by Yildirim et al., out of 220 patients with HPI, 22.7% were found to be unresponsive to eradication treatment. In the group where eradication failed, mean vitamin D levels were significantly lower when compared to the successful treatment group.[45] El Shahawy et al. demonstrated a negative influence of vitamin D deficiency on the results of eradication therapy.[46] Finally, a recent meta-analysis confirmed these findings and additionally showed that 25-OH vitamin D supplementation improved eradication outcomes.[47]

Population studies show that the efficacy of HPI eradication in patients with DMT2 is also lower than in those without diabetes. In patients with DMT1, the infection is at risk of recurrence.[48] This is an important observation since, in studies carried out in patients with DMT2, a statistically significant improvement in the metabolic control of diabetes was observed after effective treatment of the infection.[49]

Considering all the data mentioned above and the results of our study, we strongly believe that HPI eradication should be implemented as a potential adjunct therapy, maximizing the effectiveness of classical treatment of DMT1 in HP-infected patients. This is still a matter of debate whether vitamin D should be routinely included in therapeutic protocols in HPI. Appropriate clinical trials should help us to answer this question in the near future. Nevertheless, a growing body of evidence suggests that DMT1 patients would especially benefit from supplementation of vitamin D during and after treatment for HPI.

Limitations

The limitations of the study were a relatively small research group, lack of control group and restrictions related to the AGE measurement technique in the skin with the AGE Reader device.

Conclusions

An increased accumulation of AGEs in the skin of patients with DMT1 with coexisting HPI may be a reason to consider eradication therapy in this group of patients.

In the group of DMT1 patients, lower vitamin D levels were observed and associated with HPI.

Further studies on larger groups of DMT1 patients should be considered to assess the connection between AGE and HPI. Further studies are also necessary to indicate whether HP eradication reduced the accumulation of protein glycation end products in the skin.

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LncRNA SNHG12 promotes proliferation and migration of hepatic progenitor cells via the Wnt/β-catenin pathway

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Abstract

Background. Hepatic progenitor cells (HPCs) play an important role in the treatment of chronic liver disease.

Objectives. To investigate the effect and mechanism of long noncoding RNAs/small nucleolar RNA host gene 12 (*lncRNA SNHG12*) on the proliferation and migration of the HPC cell line WB-F344.

Materials and methods. Hepatic progenitor cells were divided into a no-treatment group (sham), empty vector transfection of plasmid pcDNA3.1 (NC vector), pcDNA3.1-SNHG12 (SNHG12), negative short hairpin RNA (sh-NC), SNHG12 shRNA (sh-SNHG12), and pcDNA3.1-SNHG12+salinomycin intervention (SNHG12+salinomycin) groups. Cell proliferation, cell cycle and migration ability, as well as albumin (ALB), alpha-fetoprotein (AFP), β-catenin, cyclin D1, and c-Myc protein expression in each group were determined using Cell Counting Kit-8 (CCK-8), 5-ethynyl-2'-deoxyuridine (EdU), flow cytometry, transwell migration assays, enzyme-linked immunosorbent assay (ELISA), and western blot.

Results. The overexpression of *lncRNA SNHG12* significantly upregulated proliferation, migration and cell cycle progression of WB-F344 cells. Furthermore, the overexpression of *lncRNA SNHG12* increased the level of ALB, and the protein expression of β-catenin, cyclin D1 and c-Myc in the cell line, while decreasing the level of AFP. Conversely, the knockdown of *lncRNA SNHG12* displayed the opposite effects. The inhibition of the Wnt/β-catenin signaling pathway with salinomycin significantly downregulated the β-catenin, cyclin D1 and c-Myc protein expression in WB-F344 cells.

Conclusions. The *lncRNA SNHG12* promotes the proliferation and migration of WB-F344 cells via activating the Wnt/β-catenin pathway.

Key words: proliferation, migration, Wnt/β-catenin, WB-F344, *lncRNA SNHG12*

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Background

With its complex structure and variety of biological functions, the liver is one of the most important organs in vertebrates.[1] Specifically, its biological functions include synthesis and decomposition of liver glycogen, synthesis and transportation of lipids, biosynthesis of hormones and proteins, as well as drug metabolism and the decomposition of harmful components.[2] Furthermore, there is a close link between the liver and other tissues, including skeletal muscle, adipose tissue, brain, and other viscera.[3] Hence, liver injury or functional impairment may be life-threatening. To this end, organ transplantation seems to be the only option for the most critical liver diseases. However, the lack of liver donors restricts the treatment of many patients who need liver transplants.[4,5] Therefore, hepatic progenitor cells (HPCs) have become a relevant research avenue because of their potential to replace liver transplant operations. Hepatic progenitor cells, also known as small hepatocyte or biliary epithelioid cells, are a heterogeneous cell type with bidirectional differentiation potential.[6] Studies have shown that HPCs play a vital role in the development of liver regeneration and liver cancer, which can be applied in the treatment of chronic liver diseases.[7,8] Najimi et al. transplanted human-derived HPCs into severe combined immunodeficient mice through the splenic vein. They found that HPCs could proliferate and differentiate into hepatocytes after transplantation, indicating that they could be used as a replacement or adjuvant therapy for the treatment of liver diseases.[9] Moreover, HPCs can be obtained from the patients themselves and the population expanded in vitro, thereby preventing the immune rejection caused by allogeneic transplantation. Therefore, HPC transplantation is expected to be another treatment option alongside liver tissue transplantation.[9] However, transplantation of HPCs is more complex due to their heterogeneity, the diversity of liver diseases and individual differences. It is necessary to further understand the regulation of HPC proliferation and differentiation before HPC transplantation can be applied as the gold standard treatment for liver diseases.

Long noncoding RNAs (lncRNAs) longer than 200 nucleotides (nt) are RNAs with noncoding functions. The lncRNAs are the most numerous, complex and the longest class of noncoding RNAs, and many of their functions remain to be revealed.[10] Previous studies have reported that lncRNAs are not only involved in most cellular activities, including cell growth, apoptosis, differentiation, inflammatory response, and angiogenesis, but are also directly linked to the development of a diverse set of diseases.[11–13] Recently, it has been pointed out that lncRNAs play an important role in the proliferation of hepatocytes and liver regeneration. Ruan et al. reported that lncRNA-Dreh could assist in the proliferation and migration of the HPC line WB-F344 during liver regeneration.[14] Furthermore, Zhang et al. found that the downregulation of lncRNA metastasis-associated lung adenocarcinoma transcript 1 (*lncRNA-MALAT1*) enhanced the ligands of the transforming growth factor-β (TGF-β)/small mother against decapentaplegic signaling responses, and then induced the differentiation of pluripotent stem cells into hepatocytes.[15] Deemed an oncogene, long noncoding RNAs/small nucleolar RNA host gene 12 (*lncRNA SNHG12*) was able to promote the development of a variety of tumors.[16] Ruan et al. found that *lncRNA SNHG12* accelerates the proliferation and migration of human osteosarcoma cells by boosting vascular actin gene expression.[17] A study by Wang et al. revealed that the overexpression of *lncRNA SNHG12* was capable of promoting cell proliferation and migration, as well as inhibiting apoptosis in triple-negative breast cancer.[18] Lan et al. showed that *lncRNA SNHG12* contributed to the development of hepatocellular carcinoma (HCC) by targeting microRNA-199a-5p.[19] Additionally, that study reported that *lncRNA SNHG12* was upregulated after 2/3 of hepatectomy in mice, and the overexpression of *lncRNA SNHG12* activated the Wnt/β-catenin signaling pathway to promote liver regeneration.[20] Those studies revealed the importance of *lncRNA SNHG12* in the proliferation of hepatocytes and liver regeneration. However, it is still unclear whether *lncRNA SNHG12* has the same function in HPCs. Therefore, this study aims to explore the effect of *lncRNA SNHG12* on the proliferation of HPCs. When *lncRNA SNHG12* is overexpressed or knocked down in the HPC cell line WB-F344 in vitro, the changes in cell function such as cell proliferation, migration and cell cycle progression can be observed.

Objectives

This study aims to explore the effect of *lncRNA SNHG12* on the proliferation of HPCs, which may provide new ideas for improving HPC transplantation.

Materials and methods

Cell culture

The WB-F344 cells at the 3rd passage were purchased from the American Type Culture Collection (ATCC) cell bank (Manassas, USA). All cells used as test samples were within 15 passages. The WB-F344 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 Nutrient Mixture (DMEM/F12; Gibco, Waltham, USA) containing 10% fetal bovine serum (FBS; Gibco) and 100 U/L penicillin/streptomycin (Solarbio, Beijing, China) at 37°C in an incubator with 5% CO_2.

Cell transfection and grouping

When reaching the logarithmic growth phase, the cells were digested using 0.25% trypsin (Solarbio). Then, the cells were resuspended in culture medium and seeded into

a 6-well plate at 4×10^4 cells/well. Empty vector pcDNA3.1 (NC vector), pcDNA3.1-SNHG12, negative short hairpin RNA (sh-NC), and SNHG12 shRNA (sh-SNHG12) were constructed and synthesized by RIBOBIO (Guangzhou, China). Next, the WB-F344 cells were transfected with the above fragments when they reached 70% confluence using the Lipofectamine™ 2000 kit (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. The WB-F344 cells were grouped as follows: no treatment (sham group), NC vector (NC vector group), pcDNA3.1-SNHG12 (SNHG12 group), sh-NC (sh-NC group), sh-SNHG12 (sh-SNHG12 group), pcDNA3.1-SNHG12 and 10 μM salinomycin (MedChemExpress, Shanghai, China) (SNHG12+salinomycin group).

Real-time quantitative polymerase chain reaction

Total RNA was extracted from cells using the TRIzol reagent (Thermo Fisher Scientific). Then, RNA was reverse-transcribed into cDNA using a random primer reverse transcription kit (Thermo Fisher Scientific). The real-time quantitative polymerase chain reaction (qPCR) assays were performed using the SYBR GREEN qPCR kit (Takara, Shiga, Japan) according to the manufacturer's instructions, with cDNA as the template and *lncRNA SNHG12* primers (Table 1). The β-actin was adopted as an internal control, and 6 replicates were used in the experiment. The experimental data obtained through qPCR were analyzed using the $2^{-\Delta\Delta Ct}$ method to calculate the relative expression of the target genes.

Table 1. Primers for quantification

RNA	Sequences (5′ to 3′)
lncRNA SNHG12	F: 5′-AGTGTACTGGGAGGAGG
	R: 5′-ATAAGTCCGTGCGTCC
β-actin	F: 5′-GCATGGGTCAGAAGGATTCCT
	R: 5′-TCGTCCCAGTTGGTGACGAT

Cell Counting Kit-8 test

The WB-F344 cells were seeded into 96-well plates at 1×10^3 cells/well. After 24 h, the cells were transfected with *lncRNA SNHG12* or treated with reagents. After serum starvation for 1 night, the medium was replaced and cells were cultured for 24 h, 48 h and 72 h. Cell Counting Kit-8 (CCK-8) cell dye solution (10 μL) (Beyotime Biotechnology Co., Ltd., Shanghai, China) was added to each well and plates were placed in an incubator for 1.5 h. The optical density (OD) at 450 nm was measured with a microplate reader (VL0000D2; Thermo Fisher Scientific). The proliferation rate of the cells was calculated from the absorbance values.

5-ethynyl-2′-deoxyuridine analysis

The treated WB-F344 cells were seeded in a 24-well plate. Then, the cells were stained using a 5-ethynyl-2′-deoxyuridine (EdU) staining kit (Thermo Fisher Scientific) and mounted with neutral resin. Fields (n = 6–10) were randomly selected for observation of cells under a fluorescence microscope (model FM-600; Shanghai Pudan Optical Instruments Co., Ltd., Shanghai, China). The number of positively stained cells (red) in each field was recorded. The EdU labeling rate (%) was calculated as the number of positive cells/total cell count × 100%.

Flow cytometry detection

The cells were cultured for 24 h and digested with trypsin after transfection. The cells were centrifuged at 800 rpm for 5 min at 4°C and washed twice with ice-cold sterile phosphate-buffered saline (PBS; Solarbio). Then, the cells were resuspended in 1 mL of ice-cold methanol with a volume fraction of 70% and fixed overnight at 4°C. After centrifugation (800 rpm, 4°C, 5 min), the cells were washed twice with ice-cold sterile PBS. Then, 500 μL PBS was adopted to resuspend cells, and propidium iodide (PI; 50 μg/mL) was added. Finally, the cells were incubated at room temperature for 15 min in the dark, and the cell cycle was analyzed with flow cytometry.

Transwell assay

After the transfection for 24 h, 2×10^4 cells were added in the upper chamber of a transwell, and 700 μL of medium containing 20% FBS was added to the lower chamber. The inserts were taken out after 12–24 h of culture at 37°C and 5% CO_2. Next, the inserts were washed 3 times with PBS, fixed with 1% glutaraldehyde for 30 min, and then washed again with PBS. Subsequently, the inserts were dried, and the cells were incubated with 0.1% crystal violet for 12 h. Then, the cells were washed with PBS, dried and observed with optical microscopy to identify cells that penetrated to the reverse side of the transwell inserts. Then, 6–10 fields were randomly selected to observe and record the number of positive cells in each field. Eventually, 3 fields were chosen to be photographed and analyzed.

Albumin and alpha-fetoprotein level detection

After 48 h of transfection, the cells were digested with trypsin and seeded in culture plates. Subsequently, the cells were cultured with minimal medium in the absence of FBS. After 24 h, the supernatant was collected and centrifuged at 800 rpm for 5 min at 4°C. The levels of albumin (ALB) and alpha-fetoprotein (AFP) were detected using an ALB assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and AFP assay kit (Nanjing

Jiancheng Bioengineering Institute) in accordance with the manufacturer's instructions.

Western blot assay

The total proteins were extracted from the treated cells using radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, St. Louis, USA). The concentration of total proteins was determined using a bicinchoninic acid (BCA) kit (Thermo Fisher Scientific), and 25 μg of proteins were boiled and denatured, then combined with ×1 loading buffer. The proteins were separated using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes (MilliporeSigma, Bedford, USA) using wettability conversion method. The membranes were blocked with 5% nonfat dry milk, and incubated with the primary antibody (Abcam, Waltham, USA) overnight at 4°C. Then, the membranes were washed 3 times, and the secondary antibody (Abcam) was added for 1 h. After washing 3 times, enhanced chemiluminescence (ECL) reagent (Sigma-Aldrich) was added to the membranes. A gel imaging system was utilized to collect images. Any visible protein bands were analyzed using ImageJ software (National Institutes of Health, Bethesda, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control to calculate the relative protein expression.

Statistical analyses

The statistical analysis was performed using IBM SPSS v. 22.0 software (IBM Corp., Armonk, USA) and GraphPad Prism 9 (GraphPad Software, San Diego, USA) for mapping. All data are presented as mean with 95% confidence interval (95% CI). All experiments were repeated independently 3 times. Data that passed normality (Shapiro–Wilk test) and homogeneity of variance tests (Brown–Forsythe test) (shown in Table 2–8) were compared using one-way analysis of variance (ANOVA) followed by the Tukey's post hoc test in order to analyze the differences among multiple groups. A value of $p < 0.05$ indicated a significant difference.

Results

Overexpression of *lncRNA SNHG12* promoted the proliferation of WB-F344 cells

To explore the effect of *lncRNA SNHG12* on the proliferation of WB-F344 cells, we analyzed cell proliferation in each group using CCK-8 and EdU assays. Furthermore, qPCR showed that compared with the NC vector group, *lncRNA SNHG12* expression was significantly increased in the SNHG12 group ($p < 0.05$; Table 2). Compared with the sh-NC group, the expression of *lncRNA SNHG12* in the sh-SNHG12 group was significantly decreased ($p < 0.05$; Fig. 1A, Table 2). These results revealed that *lncRNA SNHG12* was successfully knocked down or overexpressed through transfection. Furthermore, the proliferation rate of WB-F344 cells in the SNHG12 group was higher than in the NC vector group ($p < 0.05$; Table 3), while the knockdown of *lncRNA SNHG12* reduced the cell proliferation rate ($p < 0.05$; Fig. 1B, Table 3). The EdU staining demonstrated that the overexpression of *lncRNA SNHG12* increased, while the knockdown of *lncRNA SNHG12* decreased the percentage of EdU-positive cells ($p < 0.05$; Fig. 1C,D, Table 4). It suggested that the *lncRNA SNHG12* expression level was positively correlated with the proliferation of WB-F334 cells.

Overexpression of *lncRNA SNHG12* promoted the migration of WB-F344 cells

The results of the transwell assay showed that the migration ability of WB-F344 cells in the SNHG12 group was significantly increased compared with the NC vector group. When compared with the sh-NC group, the sh-SNHG12 group presented a notable decrease in the migratory ability of WB-F344 cells (Fig. 2). This suggests that the overexpression of *lncRNA SNHG12* promoted the migration of WB-F344 cells.

Table 2. Results of homogeneity of variance test, normality test and analysis of variance (ANOVA) for data presented in Fig. 1A

Group	Mean	95% CI for the mean change		Shapiro–Wilk normality test		Brown–Forsythe test
		upper	lower	W value	p-value	
Sham	1.055	1.359	0.751	0.9302	0.4892	p = 0.8634 conclusion: data have equal variance
NC vector	1.061	1.140	0.982	0.8024	0.1201	
SNHG12	1.569	1.837	1.302	0.8293	0.1866	
Sh-NC	1.050	1.340	0.760	0.9787	0.7204	
Sh-SNHG12	0.493	0.700	0.286	0.7904	0.0918	

ANOVA: $F_{contrast} = 45.20$; degrees of freedom (df) = 14; $p < 0.0001$. Tukey's test: p-value (NC vector compared to SNHG12) = 0.0006; p-value (sh-NC compared to sh-SNHG12) = 0.0003. 95% CI – 95% confidence interval; sham – no-treatment group; NC vector – empty vector transfection of plasmid pcDNA3.1 group; SNHG12 – pcDNA3.1-SNHG12 group; sh-NC – negative shRNA group; sh-SNHG12 – SNHG12 shRNA group.

Table 3. Results of homogeneity of variance test, normality test and analysis of variance (ANOVA) for data presented in Fig. 1B

Group	Mean	95% CI for the mean change		Shapiro–Wilk normality test		Brown–Forsythe test
		upper	lower	W value	p-value	
Sham	36.837	45.022	28.651	0.9999	0.9816	p = 0.8531 conclusion: data have equal variance
NC vector	35.363	42.671	28.056	0.7873	0.0844	
SNHG12	47.253	59.815	34.692	0.7744	0.0548	
Sh-NC	36.650	48.942	24.358	0.9986	0.9298	
Sh-SNHG12	25.473	28.794	22.153	0.8263	0.1789	

ANOVA: $F_{contrast}$ = 12.53; degrees of freedom (df) = 14; p = 0.0007. Tukey's test: p-value (NC vector compared to SNHG12) = 0.0209; p-value (sh-NC compared to sh-SNHG12) = 0.0298. 95% CI – 95% confidence interval; sham – no-treatment group; NC vector – empty vector transfection of plasmid pcDNA3.1 group; SNHG12 – pcDNA3.1-SNHG12 group; sh-NC – negative shRNA group; sh-SNHG12 – SNHG12 shRNA group.

Table 4. Results of homogeneity of variance test, normality test and analysis of variance (ANOVA) for data presented in Fig. 1D

Group	Mean	95% CI for the mean change		Shapiro–Wilk normality test		Brown–Forsythe test
		upper	lower	W value	p-value	
Sham	35.083	42.327	27.840	0.9517	0.5768	p = 0.9258 conclusion: data have equal variance
NC vector	31.412	36.206	26.618	0.9999	0.9857	
SNHG12	50.209	57.076	43.343	0.9930	0.8401	
Sh-NC	30.700	35.699	25.702	0.8035	0.1228	
Sh-SNHG12	14.177	25.266	3.088	0.8410	0.2165	

ANOVA: $F_{contrast}$ = 56.48; degrees of freedom (df) = 14; p < 0.0001. Tukey's test: p-value (NC vector compared to SNHG12) = 0.0001; p-value (sh-NC compared to sh-SNHG12) = 0.0003. 95% CI – 95% confidence interval; sham – no-treatment group; NC vector – empty vector transfection of plasmid pcDNA3.1 group; SNHG12 – pcDNA3.1-SNHG12 group; sh-NC – negative shRNA group; sh-SNHG12 – SNHG12 shRNA group.

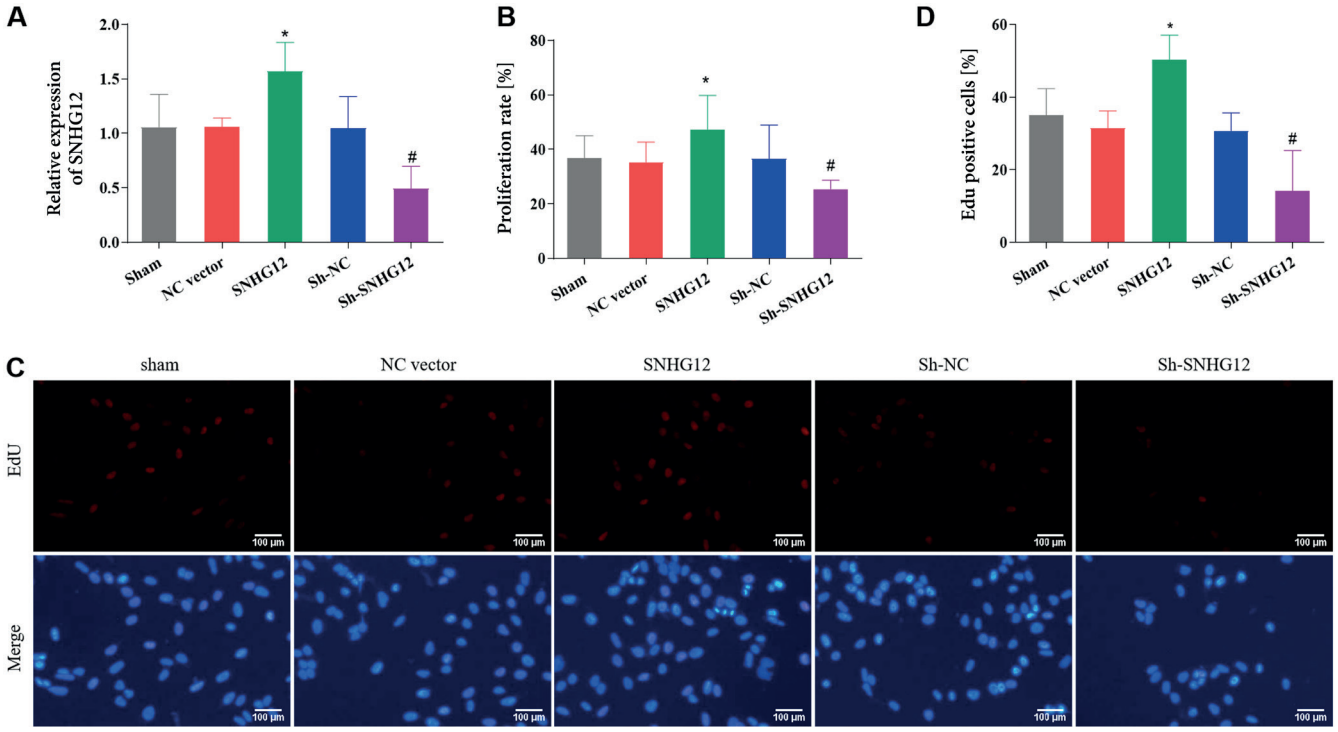

Fig. 1. The overexpression of long noncoding RNAs/small nucleolar RNA host gene 12 (*lncRNA SNHG12*) promoted the proliferation of WB-F344 cells. A. The expression level of *lncRNA SNHG12* in WB-F344 cells of each group was detected using real-time quantitative polymerase chain reaction (qPCR) (n = 3); B. The proliferation rate of WB-F344 cells was measured using Cell Counting Kit-8 (CCK-8); C,D. The number of 5-ethynyl-2′-deoxyuridine (EdU)-positive cells in each group was detected using EdU assay (n = 3); * p < 0.05 compared to the NC vector group; # p < 0.05 compared to the sh-NC group

sham – no-treatment group; NC vector – empty vector transfection of plasmid pcDNA3.1 group; SNHG12 – pcDNA3.1-SNHG12 group; sh-NC – negative shRNA group; sh-SNHG12 – SNHG12 shRNA group.

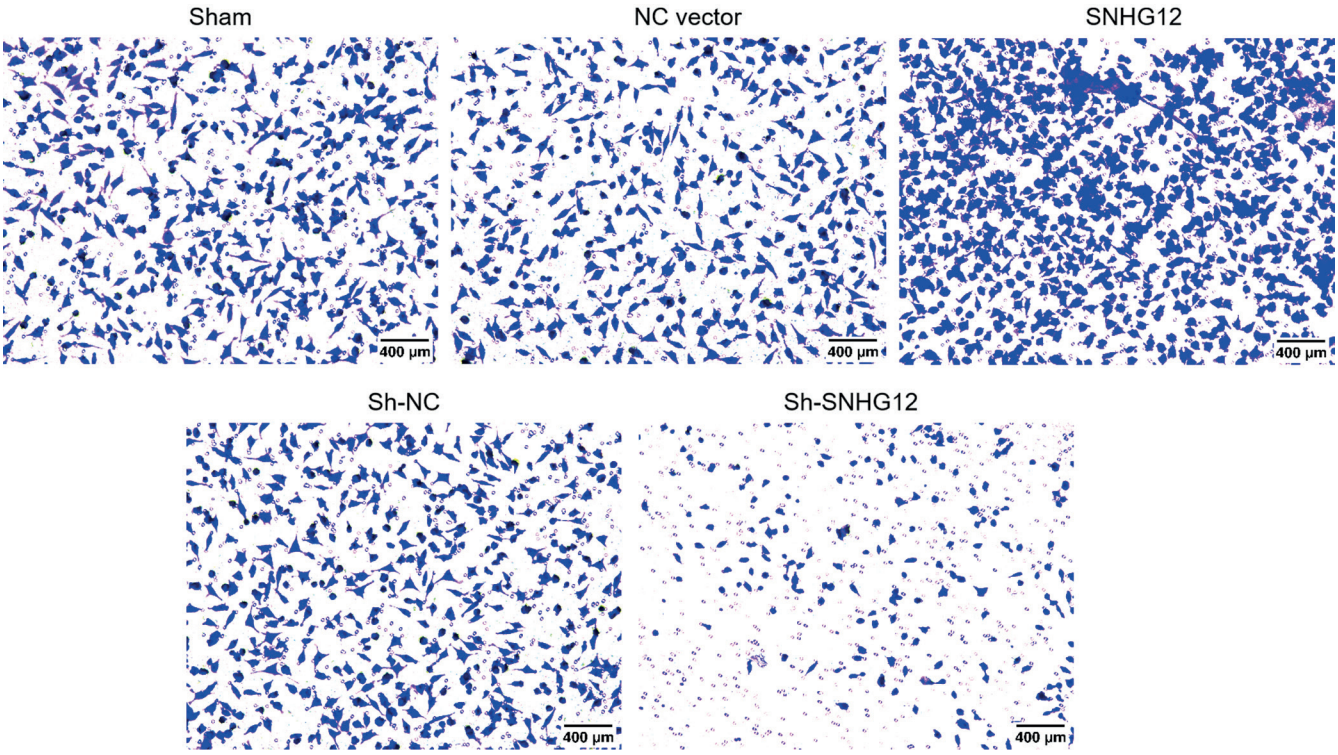

Fig. 2. The overexpression of long noncoding RNAs/small nucleolar RNA host gene 12 (*lncRNA SNHG12*) promoted the migration of WB-F344 cells. Images above show the results of WB-F344 cell migration as measured using transwell cell migration assay under specified conditions. Each test was performed 3 times under the same conditions

sham – no-treatment group; NC vector – empty vector transfection of plasmid pcDNA3.1 group; SNHG12 – pcDNA3.1-SNHG12 group; sh-NC – negative shRNA group; sh-SNHG12 – SNHG12 shRNA group.

Overexpression of *lncRNA SNHG12* expedited cell cycle progression of WB-F344 cells

Results of the cell cycle analysis revealed that when compared with the NC vector group, the number of cells in G0/G1 phase was significantly lower, while the number of cells in S phase was significantly higher in the SNHG12 group. In comparison with the sh-NC group, the sh-SNHG12 group showed an increase in the proportion of cells in G0/G1 phase and a significant decrease in the proportion of S phase cells (Fig. 3), suggesting that *lncRNA SNHG12* promoted S phase progression of WB-F334 cells.

Overexpression of *lncRNA SNHG12* promoted the differentiation of WB-F344 cells into hepatocytes

The ALB and AFP kits were used to detect the effect of intervention with *lncRNA SNHG12* expression on ALB and AFP synthesis in HPCs (Fig. 4A,B). Compared with the NC vector group, ALB levels were increased and AFP levels were significantly decreased in the SNHG12 group. When

Table 5. The results of homogeneity of variance test, normality test and analysis of variance (ANOVA) for data presented in Fig. 4A

Group	Mean	95% CI for the mean change		Shapiro–Wilk normality test		Brown–Forsythe test
		upper	lower	W value	p-value	
Sham	31.393	36.233	26.554	0.8115	0.1423	p = 0.9662 conclusion: data have equal variance
NC vector	32.357	36.176	28.537	0.9774	0.7120	
SNHG12	37.357	39.679	35.034	1.0000	0.9941	
Sh-NC	31.340	35.737	26.943	0.9999	0.9813	
Sh-SNHG12	27.343	30.538	24.149	0.8492	0.2382	

ANOVA: $F_{contrast}$ = 16.28; degrees of freedom (df) = 14; p = 0.0002. Tukey's test: p-value (NC vector compared to SNHG12) = 0.0172; p-value (sh-NC compared to sh-SNHG12) = 0.0591. 95% CI – 95% confidence interval; sham – no-treatment group; NC vector – empty vector transfection of plasmid pcDNA3.1 group; SNHG12 – pcDNA3.1-SNHG12 group; sh-NC – negative shRNA group; sh-SNHG12 – SNHG12 shRNA group.

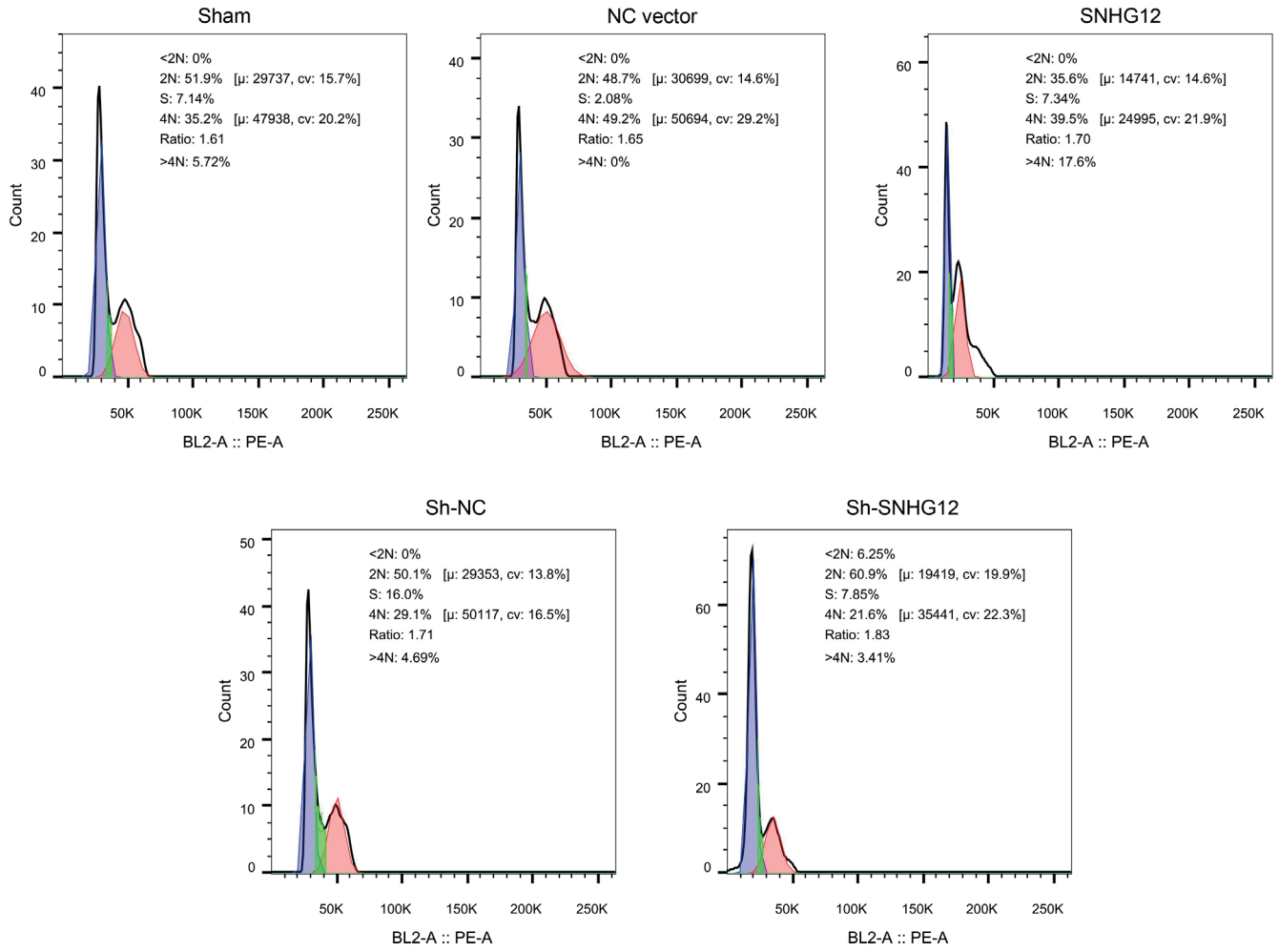

Fig. 3. The overexpression of long noncoding RNAs/small nucleolar RNA host gene 12 (*lncRNA SNHG12*) expedited cell cycle progression of WB-F344 cells. The distribution of *lncRNA SNHG12* in each cycle of WB-F344 cells under the specified conditions detected using flow cytometry

sham – no-treatment group; NC vector – empty vector transfection of plasmid pcDNA3.1 group; SNHG12 – pcDNA3.1-SNHG12 group; sh-NC – negative shRNA group; sh-SNHG12 – SNHG12 shRNA group.

compared with the sh-NC group, the sh-SNHG12 group displayed a lower ALB level and a higher AFP level (Fig. 4A,B; Table 5,6). These results revealed that WB-F344 cells showed an increase in ALB synthesis and a decrease in AFP synthesis after the overexpression of *lncRNA SNHG12*. The overexpression of *lncRNA SNHG12* promoted the differentiation of WB-F344 cells into hepatocytes.

Overexpression of *lncRNA SNHG12* activated the Wnt/β-catenin signaling pathway in WB-F344 cells

Studies have shown that the Wnt/β-catenin signaling pathway could mediate the regeneration of hepatocytes.[21] We hypothesized that *lncRNA SNHG12*

Table 6. Results of homogeneity of variance test, normality test and analysis of variance (ANOVA) for data presented in Fig. 4B

Group	Mean	95% CI for the mean change		Shapiro–Wilk normality test		Brown–Forsythe test
		upper	lower	W value	p-value	
Sham	14.847	23.560	6.133	0.9928	0.8379	p = 0.3839 conclusion: data have equal variance
NC vector	14.383	17.540	11.227	0.9990	0.9392	
SNHG12	7.930	9.515	6.345	0.8555	0.2553	
Sh-NC	14.640	15.746	13.534	0.9542	0.5882	
Sh-SNHG12	20.433	25.675	15.191	0.8236	0.1722	

ANOVA: $F_{contrast}$ = 15.51; degrees of freedom (df) = 14; p = 0.0003. Tukey's test: p-value (NC vector compared to SNHG12) = 0.0153; p-value (sh-NC compared to sh-SNHG12) = 0.0290. 95% CI – 95% confidence interval; sham – no-treatment group; NC vector – empty vector transfection of plasmid pcDNA3.1 group; SNHG12 – pcDNA3.1-SNHG12 group; sh-NC – negative shRNA group; sh-SNHG12 – SNHG12 shRNA group.

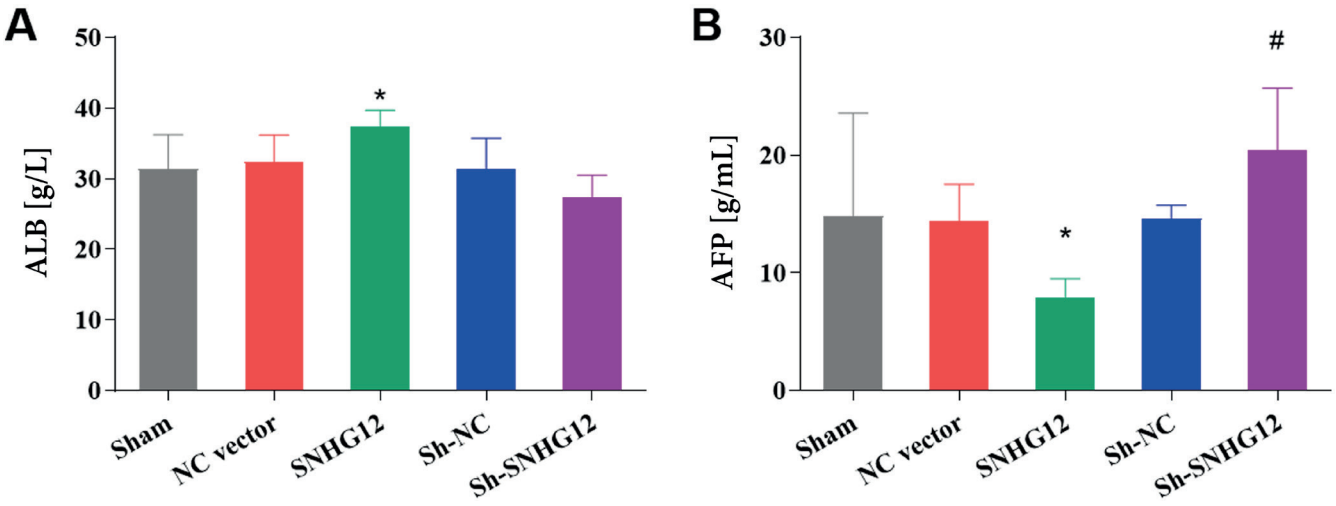

Fig. 4. The overexpression of long noncoding RNAs/small nucleolar RNA host gene 12 (*lncRNA SNHG12*) promoted the differentiation of WB-F344 cells into hepatocytes. A,B. The level of albumin (ALB) (A) and alpha-fetoprotein (AFP) (B) in WB-F344 cells of each group was measured (n = 3); * $p < 0.05$ compared to the NC vector group; # $p < 0.05$ compared to the sh-NC group

sham – no-treatment group; NC vector – empty vector transfection of plasmid pcDNA3.1 group; SNHG12 – pcDNA3.1-SNHG12 group; sh-NC – negative shRNA group; sh-SNHG12 – SNHG12 shRNA group.

stimulated the Wnt/β-catenin signaling pathway to participate in the proliferation, migration and differentiation of HPCs. To verify this, we used western blot to detect the expression of proteins related to the Wnt/β-catenin signaling pathway. The results showed that the expression of β-catenin, cyclin D1 and c-Myc protein significantly increased in the SNHG12 group compared with the NC vector group ($p < 0.05$; Table 7). When compared with the NC vector group, the expression of β-catenin, cyclin D1 and c-Myc protein was significantly decreased in the sh-SNHG12 group ($p < 0.05$; Fig. 5A, Table 7).

Finally, WB-F344 cells were treated with salinomycin (a specific inhibitor of the Wnt/β-catenin pathway). The western blot results indicated that the expression of β-catenin, cyclin D1 and c-Myc protein were significantly increased in the SNHG12 group compared with

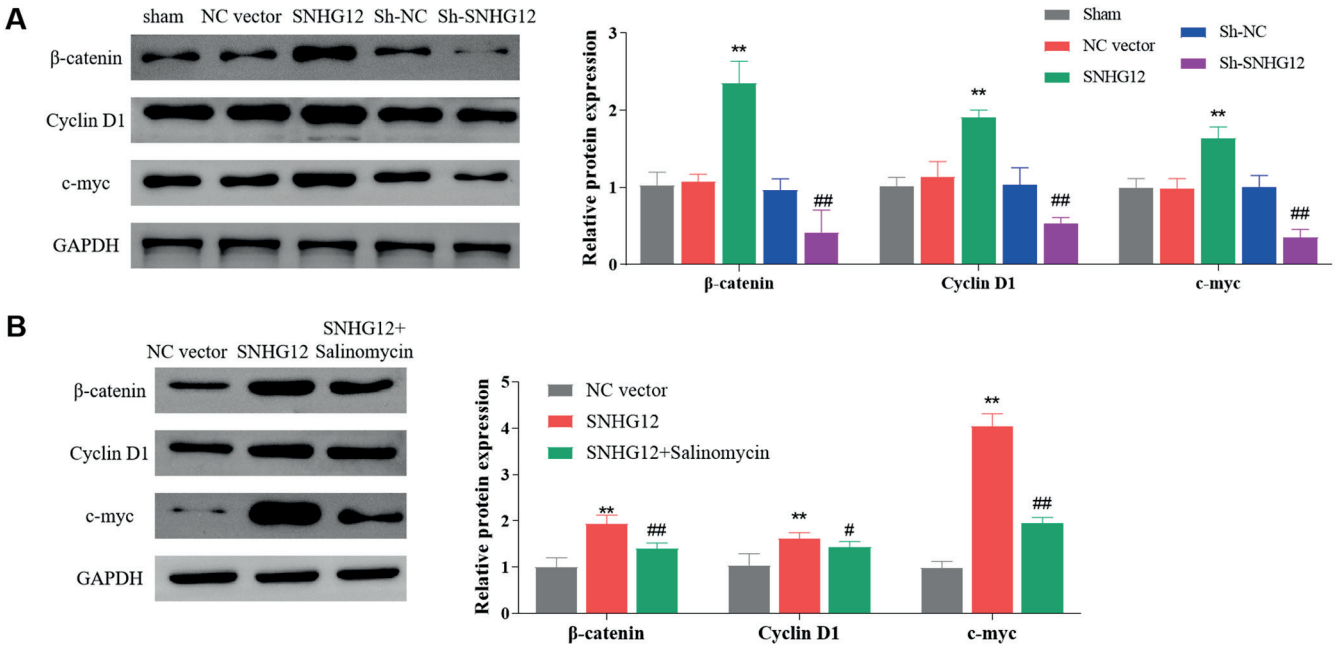

Fig. 5. The overexpression of long noncoding RNAs/small nucleolar RNA host gene 12 (*lncRNA SNHG12*) activated the Wnt/β-catenin signaling pathway in WB-F344 cells. A. The expression of Wnt/β-catenin signaling pathway-related proteins (β-catenin, cyclin D1 and c-Myc) in WB-F344 cells was detected using western blot (n = 3); ** $p < 0.01$ compared to the NC vector group; # $p < 0.05$ compared to the sh-NC group; B. The expression of β-catenin, cyclin D1 and c-Myc proteins in the WB-F344 cells that were treated with salinomycin was detected using western blot (n = 3); ** $p < 0.01$ compared to the NC vector group; $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ compared to the SNHG12 group

sham – no-treatment group; NC vector – empty vector transfection of plasmid pcDNA3.1 group; SNHG12 – pcDNA3.1-SNHG12 group; sh-NC – negative shRNA group; sh-SNHG12 – SNHG12 shRNA group; GAPDH – glyceraldehyde-3-phosphate dehydrogenase.

Table 7. Results of homogeneity of variance test, normality test and analysis of variance (ANOVA) for data presented in Fig. 5A

Protein	Group	Mean	95% CI for the mean change		Shapiro–Wilk normality test		Brown–Forsythe test	ANOVA			Tukey's test	
			upper	lower	W value	p-value		$F_{contrast}$	df	p-value	p-value (NC vector compared to SNHG12)	p-value (sh-NC compared to sh-SNHG12)
β-catenin	sham	1.028	1.198	0.859	0.9892	0.8009	p = 0.7109 conclusion: data have equal variance	209.3	14	<0.0001	<0.0001	0.0001
	NC vector	1.072	1.172	0.971	0.9825	0.7469						
	SNHG12	2.350	2.633	2.066	0.9998	0.9732						
	sh-NC	0.963	1.112	0.815	0.9333	0.5012						
	sh-SNHG12	0.419	0.707	0.130	0.9678	0.6553						
Cyclin D1	sham	1.014	1.129	0.899	0.9619	0.6250	p = 0.7485 conclusion: data have equal variance	192.6	14	<0.0001	<0.0001	<0.0001
	NC vector	1.135	1.335	0.935	1.0000	0.9921						
	SNHG12	1.908	2.003	1.812	0.9994	0.9540						
	sh-NC	1.033	1.256	0.809	0.8925	0.3621						
	sh-SNHG12	0.537	0.611	0.464	0.9273	0.4786						
c-Myc	sham	0.999	1.113	0.886	0.9112	0.4219	p = 0.9934 conclusion: data have equal variance	234.9	14	<0.0001	<0.0001	<0.0001
	NC vector	0.989	1.114	0.864	0.9867	0.7794						
	SNHG12	1.639	1.784	1.494	0.9861	0.7744						
	sh-NC	1.010	1.155	0.865	0.8970	0.3759						
	sh-SNHG12	0.355	0.458	0.251	0.9995	0.9573						

95% CI – 95% confidence interval; df – degrees of freedom; NC vector – empty vector transfection of plasmid pcDNA3.1 group; SNHG12 – pcDNA3.1-SNHG12 group; sh-NC – negative shRNA group; sh-SNHG12 – SNHG12 shRNA group.

Table 8. Results of homogeneity of variance test, normality test and analysis of variance (ANOVA) for data presented in Fig. 5B

Protein	Group	Mean	95% CI for the mean change		Shapiro–Wilk normality test		Brown–Forsythe test	ANOVA			Tukey's test		
			upper	lower	W value	p-value		$F_{contrast}$	df	p-value	p-value (NC vector compared to SNHG12)	p-value (NC vector compared to SNHG12+salinomycin)	p-value (NC vector compared to SNHG12+salinomycin)
β-catenin	NC vector	1.004	1.202	0.806	0.9504	0.5711	p = 0.8104 conclusion: data have equal variance	136.8	8	<0.0001	<0.0001	0.0010	0.0002
	SNHG12	1.933	2.123	1.744	0.9820	0.7433							
	SNHG12+salinomycin	1.404	1.519	1.290	0.9654	0.6428							
Cyclin D1	NC vector	1.038	1.291	0.785	0.8754	0.3110	p = 0.7417 conclusion: data have equal variance	50.94	8	0.0002	0.0002	0.0012	0.0492
	SNHG12	1.615	1.747	1.483	0.9565	0.5987							
	SNHG12+salinomycin	1.435	1.552	1.318	0.9679	0.6559							
c-Myc	NC vector	0.977	1.123	0.832	0.9994	0.9552	p = 0.7808 conclusion: data have equal variance	1228	8	<0.0001	<0.0001	<0.0001	<0.0001
	SNHG12	4.039	4.312	3.766	0.8288	0.1853							
	SNHG12+salinomycin	1.949	2.072	1.827	0.8724	0.3024							

95% CI – 95% confidence interval; df – degrees of freedom; NC vector – empty vector transfection of plasmid pcDNA3.1 group; SNHG12 – pcDNA3.1-SNHG12 group.

the NC vector group. The expression level of β-catenin, cyclin D1 and c-Myc protein were further notably decreased in the SNHG12+salinomycin group compared with the SNHG12 group ($p < 0.05$; Fig. 5B, Table 8). Hence, it was suggested that *lncRNA SNHG12* was able to promote the proliferation of hepatocytes through activation of the Wnt/β-catenin pathway.

Discussion

Liver transplantation is the only effective therapy for critical liver diseases. However, because of the global shortage of donor organs, many patients do not have access to this therapy. Hepatic progenitor cell therapy is theorized to be a remarkable alternative to liver transplantation.[22] However, HPC has presented unsatisfactory therapeutic effects when applied alone. Therefore, it is necessary to find some auxiliary factors to improve the efficacy of HPC transplantation. Studies have highlighted that the proliferative ability of HPC is positively correlated with the severity of liver injury. There are many factors that affect the activation, proliferation and differentiation of HPCs. Some cytokines or inflammatory factors that can regulate a variety of biological behaviors of HPCs have been found, including tumor necrosis factor alpha (TNF-α)-like weak inducer of apoptosis, hepatocyte growth factor, epidermal growth factor, and interleukin 6 (IL-6).[23,24]

In addition to their profound effect on liver transplantation, lncRNAs have been found to play an important role in a variety of biological processes. Xie et al. discovered that long noncoding small nucleolar RNA host gene 7 could bind and inhibit *miR-29b* expression in hepatic stellate cells and affect DNA-methyltransferase 3A expression. In that case, the activation, autophagy and proliferation of hepatic stellate cells could be impacted.[25] Liao et al. highlighted that the lncRNA G protein-coupled receptor 137b-pseudogene (*lncRNA Gpr137b-ps*) could affect the activation of C-X-C motif chemokine ligand 14 (*CXCL14*) by targeting and regulating the expression of *miR-200a-3p*, and thereby activating hepatic stellate cells.[26] Zhang et al. reported that the upregulation of liver fibrosis-associated lncRNA1 expression in the liver promoted the transcription of TGF-β, some mothers against decapentaplegic homolog 2 which transmits the activation signal from TGF-β receptors at the plasma membrane to transcriptional regulators in the nucleus, mothers against decapentaplegic homolog 3, and neurogenic locus notch homolog protein 2 and 3-induced TGFβ and notch pathway activation, as well as inhibited hepatic stellate cell activation.[27] However, there are few relevant studies on lncRNAs in HPCs. In our study, we found that *lncRNA SNHG12* possessed several functions, including facilitating the proliferation, migration and cell cycle progression of WB-F344. Moreover, *lncRNA SNHG12* was effective in upregulating ALB and downregulating AFP expression in cells. Alpha-fetoprotein is a glycoprotein derived from embryonic endodermal cells. Despite the high level in fetal serum, AFP content will gradually decrease to adult levels after birth. Therefore, AFP is often applied as a marker of HPC.[28] Albumin, on the other hand, is often considered a marker of mature hepatocytes.[29] It can thus be seen that *lncRNA SNHG12* is conducive to the proliferation, migration, cell cycle progression, and differentiation of HPCs.

The Wnt/β-catenin not only is a signaling pathway for stem cell differentiation, but also plays a role in cell survival, proliferation, embryonic patterning, organogenesis, differentiation, cell migration, and polarity.[21] Moreover, Wnt/β-catenin signaling regulates many biological processes in the development, homeostasis, metabolism, regeneration, and carcinogenesis of the liver.[30] It has been shown that the downregulation of Wnt/β-catenin signaling activity can assist the differentiation of umbilical cord stem cells to hepatocytes.[31] In a previous study, lncRNAs have been found to regulate the activation of the Wnt/β-catenin signaling pathway. Song et al. claimed that lncRNA AWPPH, promoted the proliferation and inhibited the apoptosis of non-small cell lung cancer cells by activating the Wnt/β-catenin signaling pathway.[32] Liu et al. concluded that the upregulation of lncRNA nuclear-enriched abundant transcript 1 could promote colon cancer progression by sponging *miR-486-5p* and activating nuclear receptor subfamily 4 group A member 1/Wnt/β-catenin pathway.[33] While Zhao et al. summarized that *lncRNA SMAD5-AS1*, as a competitive endogenous RNA, could increase adenomatous polyposis coli (APC) expression by stimulating miR-135b-5p and inhibit the proliferation of diffuse large B-cell lymphoma by the upregulation of the Wnt/β-catenin pathway.[34] The above studies indicate that lncRNA could regulate Wnt/β-catenin signaling though a variety of methods, and produce various results. In our study, *lncRNA SNHG12* was shown to be able to activate Wnt/β-catenin signaling. Several lncRNAs have been determined to be involved in Wnt/β-catenin signaling in order to elevate the proliferation of hepatocytes, including lncRNA liver regeneration 1, lncRNA transcription factor 7 and lncRNA lnc-β-Catm, which is highly expressed in human HCC tumors and liver cancer stem cells.[35,36] Therefore, it can be suggested that *lncRNA SNHG12* exerts its biological function by activating the Wnt/β-catenin signaling pathway.

Limitations

There are limitations to this study. The lncRNAs have multiple targets and the way *lncRNA SNHG12* regulates Wnt/β-catenin signaling needs further exploration. In addition, the involvement of *lncRNA SNHG12* in the regulation of hepatocyte proliferation was only confirmed through in vitro cellular experiments; therefore, in vivo animal experiments need to be performed. Finally, because of the very small sample sizes used in the ANOVA, it is more difficult to reliably verify the test assumptions, and our results need to be considered with caution.

Conclusions

In summary, the upregulation of *lncRNA SNHG12* expression in WB-F344 cells activated the Wnt/β-catenin signaling pathway, and promoted cell proliferation, migration and cell cycle progression. However, lncRNAs have been shown to have multiple targets. Further studies are required to thoroughly elucidate the mechanism of action of *lncRNA SNHG12* in order to provide a solid basis for clinical application.

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Molecular features as promising biomarkers in ovarian cancer

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Abstract

Ovarian cancer (OC) is a global challenge for modern medicine, ranking 7th for incidence and the 8th most common cause of mortality from cancers in women. Ovarian cancer has a poor prognosis, characterized by high morbidity and mortality, with detection occurring more frequently in advanced stages. Further issues lie within the heterogeneous nature of this pathology, as well as in its ability to develop multidrug resistance. Therefore, there is a burgeoning need to introduce effective screening for the general population, especially in high-risk groups such as individuals with a family history of cancer. Achieving this would be greatly assisted by identifying new biomarkers in order to, in turn, develop targeted therapies for patients. Advances in molecular biology techniques that enable cancer genetic characterization offer hope for personalized medicine. This article reviews the current findings on the biology of OC at the molecular level. Such knowledge may prove to be crucial and constitute a starting point for the development of new options for the early detection, prevention and treatment of OC.

Key words: ovarian cancer, molecular markers, early detection, genetic testing, ovarian cancer screening

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Introduction: Ovarian cancer

Ovarian cancer (OC) is a global issue. In 2018, 295,414 new cases of OC were reported, alongside 184,799 deaths. In Europe, the number of diagnosed women was 67,771, with a mortality rate of 44,576 (The World Ovarian Cancer Coalition Atlas 2020; World Health Organization (WHO) and GLOBOCAN data).[1] A range of risk factors may contribute to OC, including increasing age, obesity, family history, and genetic mutations.

Most cases of OC occur in women aged 50–79 years.[1,2] This may be related to hormonal and reproductive cycle changes – for example, early menarche, estrogen replacement therapy used for over 5 years, late menopause, late pregnancy, endometriosis, and polycystic ovary syndrome.[2,3] A high-fat diet and obesity also contribute to an increased risk of OC.[1] This may be in part due to the function of adipose tissue – a metabolically active organ that produces pro-inflammatory factors and regulates cell division.[4] Obesity may also result in hormonal changes such as a higher level of endogenous androgens.[5] It is postulated that obesity increases the risk of developing endometrial cancer of the ovary due to the increased level of estrogens involved in this cancer pathogenesis.[6,7]

Family history is linked to the likelihood of ovarian cancer; women with 2 first-degree relatives who have had breast cancer (with 1 diagnosed before the age of 50) have a higher risk of OC. A higher risk of OC in patients may also be seen if they have: 1) 3 or more first- or second-degree relatives with breast cancer; 2) first- and second-degree relatives with a combination of breast cancer and OC; 3) 1 first-degree relative with bilateral breast cancer; 4) 2 or more first- or second-degree relatives with OC; 5) 1 first- or second-degree relative with a combination of breast cancer and OC; and/or 6) a family history of male breast cancer.[2,3,4,8]

Specific genetic mutations are also related to an increased risk of developing OC. For example, women who carry the *BRCA1* or *BRCA2* mutation are at a higher risk of OC, and this prevalence may be seen especially in the Ashkenazi Jewish population, where 2% carry mutations in the *BRCA1* (185delAG and 5382insC) and *BRCA2* (6174delT) genes.[2,9] Mutations in the DNA repair genes *hMSH1*, *hMSH2*, *hPMS1*, and *hPMS2* are seen in those with Lynch syndrome (related to hereditary colon cancer), which may also predispose individuals to OC.

Epithelial OC (EOC) is responsible for the overwhelming majority of OCs (85–90%).[10] Epithelial OC is a heterogeneous disease, consisting of several histological subtypes with distinct biological features (Fig. 1). With the advancement of molecular biology techniques, emerging evidence suggests that different subtypes have different genetic characteristics which will result in changes in classification and, moreover, advances in treatment.[11] Currently, EOCs are classified into 5 main groups, according to the clinicopathological features of the disease: clear cell OC (CCOC), endometrioid OC (EOVC), low-grade serous OC (LGSOC), mucinous OC (MOC), and high-grade serous OC (HGSOC).[12,13] In clinical practice, EOCs are currently divided into 2 groups: HGSOC and non-serous high-grade OC (non-HGSOC) which contains CCOC, EOVC, LGSOC, and MOC.[10]

Genetic and molecular changes in HGSOC

High-grade serous OC is the most common type of OC, accounting for approx. 70% of cases, and in many instances diagnosed at more advanced stages (III–IV).[14,15] There are currently several drugs with molecular targets for the treatment of HGSOC (e.g., antivascular endothelial growth factor (VEGF) antibodies or poly (adenosine diphosphate (ADP)-ribose) polymerase inhibitors (PARPi)). Unfortunately, due to the lack of specific symptoms and late diagnosis, mortality is still high. New, more effective diagnostic molecular markers may be useful in combating this disease.[16,17]

DNA sequence

High-grade serous OC is characterized by acquired or inherited mutations in various DNA repair pathways.[18] Data from The Cancer Genome Atlas (TCGA) revealed that a mutation in the *TP53* gene occurs in 96% of HGSOC tumors, both in primary and metastatic sites.[19] The p53 transcription factor encoded by this gene activates further genes responsible for DNA repair, the cell cycle and apoptosis after irreversible DNA damage.[18] The *TP53* mutations appear to play a significant role in tumor initiation, with most presenting as missense mutations located predominantly in exons 4–8. However, nonsense mutations and frameshifts also occur.[19,20] The *TP53* mutations can lead to loss of function (LOF), thus depriving the cell of anticancer protection. Subsequent mechanism is the dominant negative effect, which either masks the wild-type allele function by the mutant allele or gains the function (gain of function – GOF), allowing the cell to promote tumorigenesis. Godwin et al. and Saretzki et al. revealed that the loss of heterozygosity (LOH) at the *TP53* locus is characteristic of OC. The *TP53* mutant tumors feature poor differentiation, increased invasiveness and high metastatic potential, leading to an aggressive phenotype.[acc.20]

The *BRCA1* and *BRCA2* are referred to as tumor suppressor genes (TSGs),[21] as they form multiprotein complexes which are involved in the regulation of transcription of DNA synthesis and recognition and correction of particular DNA damage double-strand breaks (DSBs). Moreover, these genes play an important role in controlling cell growth and maintaining genome integrity.[6] The *BRCA1/2* inactivation causes cancer cells to be devoid of DNA damage repair by homologous recombination (HR). Germline

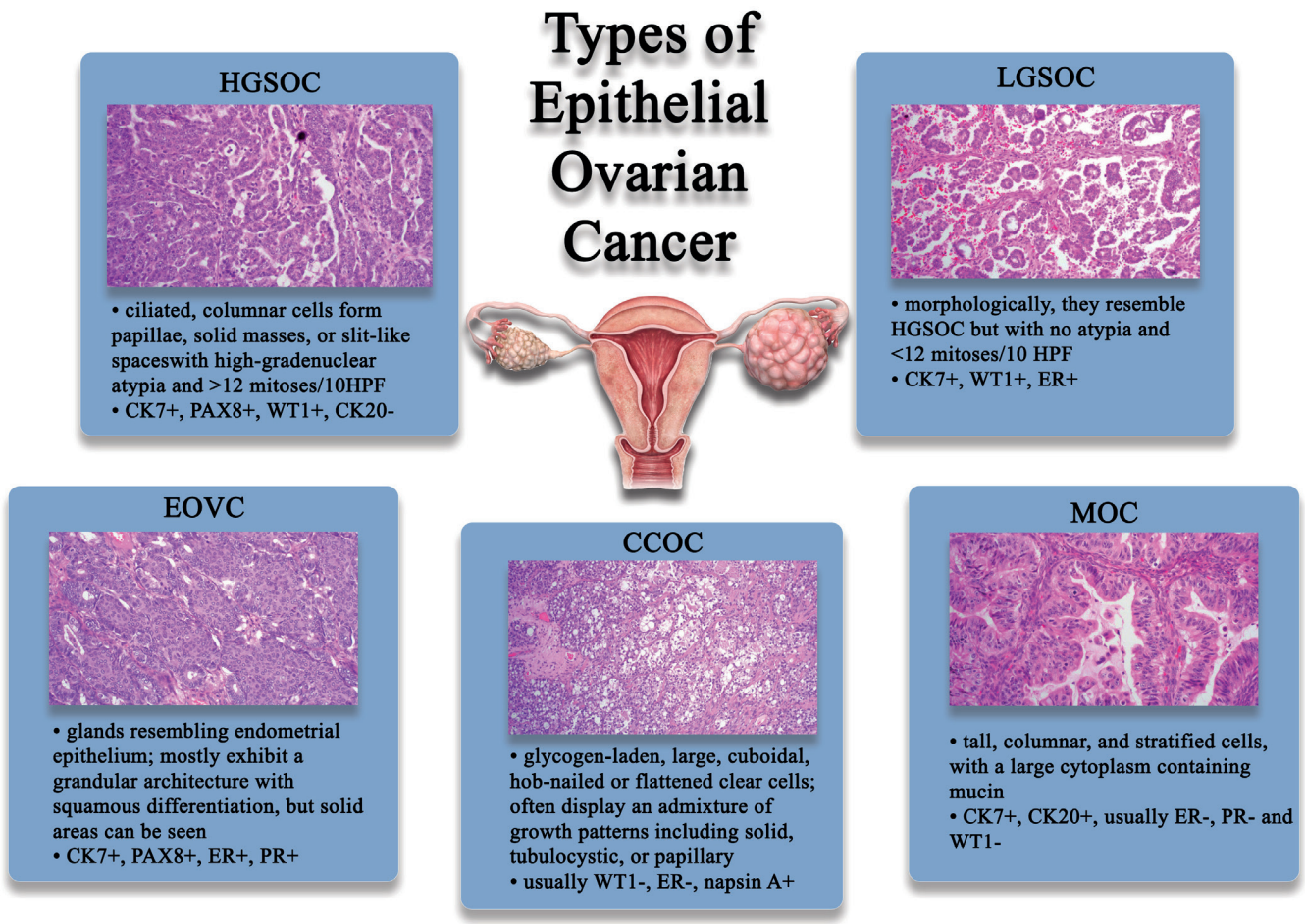

Fig. 1. Characteristics of histopathological types of ovarian cancer (Images courtesy by Department of Pathology, Johns Hopkins Hospital (Baltimore, USA))
OC – ovarian cancer; HGSOC – high-grade serous OC; EOVC – endometrioid OC; CCOC – clear cell OC; LGSOC – low-grade serous OC; MOC – mucinous OC.

mutations in the *BRCA1* and *BRCA2* genes are responsible for the majority of hereditary ovarian tumors.[15] However, many HGSOCs occur spontaneously, with altered BRCA functioning caused by somatic mutations in *BRCA1/2*, or as a result of methylation.[22] Both germline and somatic *BRCA1* mutations accompanied by a LOH suggest that the loss of functionality of this TSG plays a key role in the development of HGSOC.[23]

Based on TCGA data for HGSOC, mutations in *NF1* and *CDK12* are also frequently observed, as supported by research from Dugo et al., who found additional mutations in *RB1*, along with *CSMD1*, *NOTCH4* and *TMEM132D*.[24,25] Other mutations in HGSOC present in the *EMSY*, *RAD51*, *ATM*, *ATR*, *BARD1*, *BRIP1*, *PALB2*, *RB1*, *NF1*, and *CDKN2A* genes, potentially resulting in a homologous recombination deficiency (HRD).[18]

Structural and copy number changes

A characteristic feature of HGSOC tumors is the loss of genome integrity, which leads to extreme genome instability.[26] The HGSOCs show a relatively high number of somatic copy number changes (copy number variation (CNV)) and structural changes, with >100 repeated amplifications and deletions identified.[27] Genomic instability is manifested by common structural and numerical aberrations in chromosomes 3, 8, 11, 17, and 21.[28]

One of the most common CNVs in OC is the amplification of the 19q12 locus, where *CCNE1* resides. The *CCNE1* encodes cyclin E1, which is amplified in many solid tumors, as well as in about 20% of cases with HGSOC.[17] The overexpression of cyclin E1 increases the rate of passage of cells through the restriction point of the G1/S phase, leading to genomic instability, as an abnormal expression of cyclin E1 triggers unplanned DNA replication, centrosome amplification and chromosome instability.[17,29] The *CCNE1* amplification and *BRCA* mutations are mutually exclusive in HGSOC.[17] Copy number amplification of cyclin E1 has only been reported in wild-type *BRCA1/2* tumors and is associated with early primary treatment failure and reduced survival in patients suffering from OC. Etemadmoghadam et al. showed that *CCNE1*-amplified ovarian tumors require the presence of a functional BRCA1 protein and may respond to bortezomib, a proteasome inhibitor.[acc.30] Cases of *CCNE1* amplification are distinct from those with the *BRCA* mutation, which suggests that

there may be 2 different pathways driving the pathogenesis of HGSOC with heterogeneity among patients.[31]

According to TCGA data, the most common focal amplifications, apart from *CCNE1*, concerned *MYC* and *MECOM*, each with strong amplification in more than 20% of the analyzed cases.[32] Zeng et al. focused on the amplification of the *MYC* oncogene at the 8q24 locus, and proved that *MYC* copy number is significantly correlated with the level of expression, and OC is characterized by the highest frequency of *MYC* amplification compared to many other cancers.[33] Data suggest that MYC triggers the selective amplification of gene expression in order to promote cell growth and proliferation as the protein coordinates nutrient sourcing for the production of ATP and, as a result, DNA replication along with cell division occur and cells increase in mass.[34] The *MECOM* is located at the 3q26.2 locus and is essential in early cell development and differentiation (including neurogenesis and craniofacial development), as well as in the regulation of the transforming growth factor β (TGF-β) signaling pathway (allowing for hematopoietic proliferation, differentiation and/or cell death).[24,35] The *MECOM* somatic mutations are characteristic of leukemias, while the germline variants in RECQL4 occur in osteosarcoma and skin cancer.

Ballabio et al. identified 2 focal and minimal common regions (FMCRs) of amplification in the cytoband 3q26.2 (193 kb α region) and 8q24.3 (495 kb β region). The *MECOM* gene, located in the α region, is associated with favorable prognosis and relapse-free time in OC.[24] The researchers reported that the most frequent was the amplification of the cytoband 8q24 (48%), and the deletion concerned fragments: 5q, 6q, 8p, 13q, 16q, and 18q (from 40% to 48%), as well as 1p, 4p and 4q (approx. 30%).[24] Comparatively, Dugo et al. showed that in more than 80% of the HGSOC patients tested, the amplification of the 3q26.2, 17q11.2 and 19p13.3 region, as well as the 4q34.3 deletion was present.[25] Another locus, 6q24.2e26, was found to exhibit specific copy number loss, where a reduction in the expression level of 76% of the genes in the lost region was significantly associated with better survival. In a data-driven high-throughput study by Kamieniak et al., regional loss of 6q15eq27 was defined among the 8 copy number losses that significantly influenced the chemoreactive candidate pathways in EOC.[36] Amplification of genes such as *EMSY*, *FANC* family, *RAD51C*, and *PTEN* is able to disrupt the HR pathway in HGSOC tumors.[37]

Gene expression

Changes to gene expression in HGSOC may reveal underlying mechanisms in which the pathology persists. Differences in gene expression for human HGSOC and normal ovarian surface epithelium (OSE) samples were assessed by Si et al., and the researchers identified 103 differentially expressed genes (DEGs). These included 28 upregulated genes that appear to stimulate cell division and proliferation, and 75 downregulated genes that assist in a variety of processes such as the metabolism of retinol, tyrosine and drug/cytochrome P450 pathways, as well as Wnt signaling. Many studies suggest that these metabolic disorders play an important role in carcinogenesis. Ten genes that may play an important role in the pathogenesis of HGSOC were identified, with 8 being upregulated (*EPCAM*, *ZWINT*, *BUB1B*, *NEK2*, *DLGAP5*, *MELK*, *CEP55*, *CKS2*) and 2 downregulated (*ALDH1A1*, *KDR*). These genes may be useful in the early diagnosis of patients with HGSOC. Additionally, survival analyses showed that the expression levels of *MELK*, *CEP55* and *KDR* were significantly correlated with the overall survival (OS) rates of HGSOC patients. The overexpression of *MELK* was associated with the prolongation of OS, while increased levels of *CEP55* and *KDR* expression in cancerous tissues were significantly associated with the shortening of OS. Moreover, these findings suggest that *MELK*, *CEP55* and *KDR* are potential predictors of patient prognosis. Therefore, the genes listed above may be viable biomarkers to facilitate early diagnosis and treatment, and predict the prognosis of patients with HGSOC.[38]

Millstein et al. reported an association between OS and expression of 276 genes, and identified the 5 most significant genes (*TAP1*, *CXCL9*, *FBN1*, *PTGER3*, and *ZFHX4*). The *TAP1* codes a protein involved in the antigen presentation pathway and had the highest prognostic value in this study. This gene exhibited a reduced expression in metastatic HGSOC and was positively associated with OS as well as tumor regression in response to treatment. The CXCL9, a chemokine that mediates the recruitment of T cells into solid tumors, is a strong prognostic factor in HGSOC and a characteristic feature of the immunoreactive molecular subtype of HGSOC. High expression of intratumor *CXCL9* was associated with longer OS and more intensive lymphocyte infiltration. The FBN1 is an extracellular matrix protein, recognized as a biomarker associated with an early relapse in OC patients initially sensitive to chemotherapy and strongly correlated with desmoplasia in HGSOC. The expression of the prostaglandin E2 PTGER3 receptor in ovarian tumor cells is associated with relapse-free survival. The ZFHX4 was newly identified in that study as significant in HGSOC.[39]

Li et al. sought to underpin molecular features distinguishing HGSOC from serous borderline tumors (SBTs) and revealed significant differences in the expression of 11 genes. High-grade serous OC was characterized by: 1) lower expression of *SLC7A2*, *PIFO*, *AFF2*, *HES2*, *BBS12*, *RPL12*, *RPL7A*, *RPS15*, and *RPS12*; and 2) higher expression of *MAFB* and *CRABP2*. Currently, *AFF2*, *MAFB*, *HES2*, *RPL12*, *RPL7A*, *RPS12*, and *RPS15* are believed to be involved in pre-transcriptional and post-transcriptional regulatory processes. The expression levels of the transcriptome between stage II and III of HGSOC were also compared. The researchers identified 17 DEGs: *TSPAN1*, *ANK1*, *SERPINE2*, *SYNPO*, *PDGFC*, *NQO1*, *CA12*, *PPP1R14C*,

TMEM30B, *PGK1*, *DNAJB9*, *CLIC1*, *ECE1*, *ENPP4*, *ZDHHC7*, *GCLC*, and *CHST15*. Stage III was characterized by downregulation of all the genes mentioned. The PDGFC, SERPINE2, TSPAN1, ANK1, and SYNPO are involved in cell migration and cytoskeleton organization.[40]

Heterogeneity

The clonal composition of individual clinical samples and the coexistence of larger and smaller subclones were revealed through deep sequencing of epithelial tumors.[41] Next-generation sequencing (NGS) reveals significant genomic heterogeneity of breast, pancreatic, kidney, and HGSOC tumors.[17,41]

Changes in the BRCA and CCNE1 pathways represent 2 different genotypes displaying unrepeatable DNA repair susceptibilities.[17] The use of PARPi in women who have *BRCA1/2* mutations is an example of a shift towards precision medicine in HGSOC.[42] Therapeutic approaches for tumors with *CCNE1* amplification are being developed and will likely utilize their dependence on HR and replication fork protection routes.[17] Four partially overlapping transcriptional subtypes of HGSOC have been identified through the profiling of mRNA expression in HGSOC tumors: 1) C1, mesenchymal; 2) C2, immunoreactive; 3) C4, differentiated; and 4) C5, proliferative. The C1 and C5 subtypes showed the least favorable OS, whereas the C2 subtype exhibited better survival outcomes. Single-cell technologies have allowed for the exploration of intratumor heterogeneity (ITH), and multi-omics profiling has assisted in better understanding of the molecular changes underlying HGSOC. Molecular heterogeneity within the HGSOC tumor is demonstrated both spatially and temporally, and in the case of extensive intraperitoneal dissemination, multi-region molecular profiling of primary tumors and metastases may be useful in identifying the biology underlying HGSOC progression.[42]

The complex relationship between cancer cells and the tumor microenvironment (TME) also contributes to the success of cancer treatment (Fig. 2). In HGSOC,

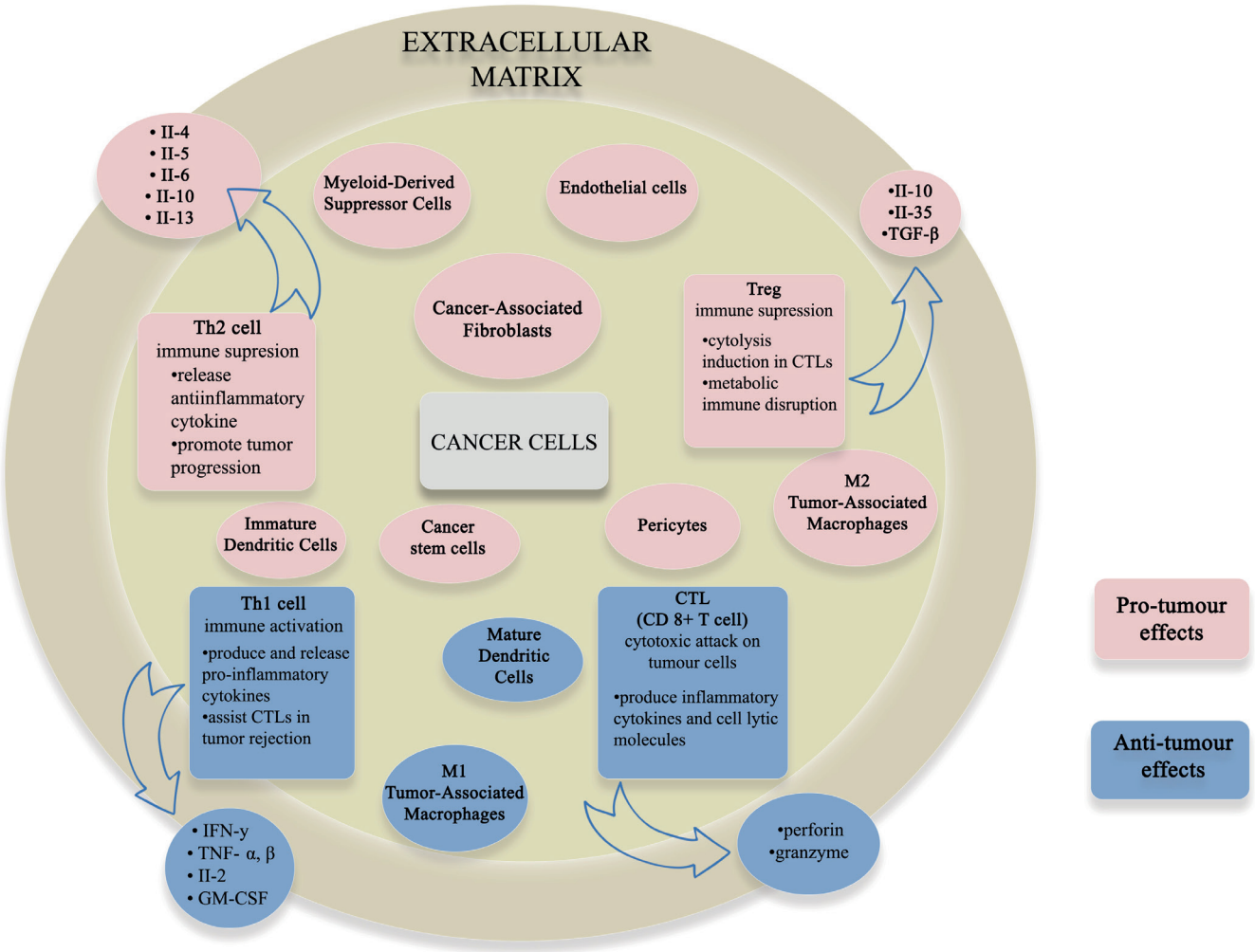

Fig. 2. Simplified characterization of tumor microenvironment (TME)

IL – interleukin; CTL – cytotoxic T lymphocyte (also called $CD8^+$ T cell); Th cell – T helper cell; Treg – regulatory T cell; M1 – macrophage type 1; M2 – macrophage type 2; TNF – tumor necrosis factor; IFN-γ – interferon gamma; TGF – transforming growth factor; GM-CSF – granulocyte-macrophage colony-stimulating factor.

a relatively low burden of point somatic mutations, high levels of aneuploidy and large changes in copy number are associated with low immunogenicity. The T-cell infiltration ($CD3^+/CD8^+$) has been shown to play a key role in the prediction of HGSOC survival in primary disease. Research to determine the interaction between mutational ITH and T-cell interactions, as well as the potential impact of chemotherapy on T-cell infiltration in HGSOC is ongoing.[43]

Jiménez-Sánchez et al. found that the heterogeneity of the transcriptomic pathway is mainly due to the presence or absence of immune and stromal cells. The degree of variability in the patient's immune signature was comparable to that of the metastatic HGSOC case study, in which different tumor immune microenvironments could be linked to certain clinical results. In this study, all patients had at least 1 tumor with low immune infiltration, which suggests that HGSOC is characterized by distinct microenvironmental niches that underpin both primary and acquired resistance to therapies. Integration of these data revealed, in the case of samples with excluded immune cells but high cellularity, that target genes are overexpressed in samples as well as mutations in the negative Wnt signaling regulators. As Wnt (and MYC) signaling exerts immunosuppressive effects, this could have the capacity to mask immune activation induced by cytotoxic chemotherapy treatment.[43]

Despite a high response rate to initial treatment, HGSOC is characterized by a high relapse rate likely due to its heterogeneous and adaptive nature described above. Data on significant heterogeneity in solid tumors may be crucial in the development of personalized medicine, which will contribute to increased quality of life and an extended lifespan of patients.[41,42] A more recent discovery of the chemotherapy-induced expansion of natural killer (NK) cells may also provide a translational pathway for novel treatment strategies connecting chemotherapy with immunotherapy.[43]

Molecular changes in acquired therapy resistance in HGSOC

Acquired resistance to chemotherapy is responsible for the majority of deaths in HGSOC.[44] The inborn genetic instability of a neoplastic cell allows for fast adaptation to changes in the local molecular microenvironment. This provides the cancer with an opportunity to use many innate and acquired resistance mechanisms to overcome both chemotherapy and targeted therapies. For HGSOC, acquired resistance research has focused on mechanisms such as drug efflux and increased DNA damage repair ability.[45]

Currently, platinum-based chemotherapy is the primary form of pharmacological treatment in all cases of advanced EOC. Carboplatin in combination with paclitaxel is the standard treatment, regardless of the histotype.[46] The PARPi are used in approx. 50% of patients with HGSOC; however, platinum resistance mechanisms often result in PARPi resistance. The BRCA1/2-deficient HGSOCs are platinum- and PARPi-responsive, yet chemoresistance may well develop through a restored function of the HR repair pathway. Most often, this is achieved through the acquisition of secondary somatic mutations in *BRCA1/2* carriers of the germline mutations that reverse mutations to restore the open reading frame (and subsequent BRCA1/2 function), as well as decreased *BRCA1* promoter methylation on relapse, resulting in increased *BRCA1* expression. Reverse mutations of *RAD51C*, *RAD51D* and other genes involved in the HR pathway have been reported in women who progressed after the administration of PARPi. The resistance to paclitaxel, a one-component second-line treatment in women with platinum-resistant HGSOC, arises through alterations in the activity or expression of these apoptosis-controlling proteins. In HGSOC, increased expression of antiapoptotic proteins such as Bcl-2, Bcl-XL, Mcl-1, and survivin (BIRC5) is associated with shorter progression-free survival and resistance to taxanes and platinum in preclinical studies. In addition, the downregulation of pro-apoptotic factors such as Bax and caspases is associated with acquired chemoresistance. One of the best-studied mechanisms underlying multi-drug resistance is the hyperactivity of drug efflux through membrane-bound transporters where cancer cells can lower the intracellular concentration of drugs due to ATP binding cassette (ABC) transporters.[44] In the case of OC chemoresistance, the ABCB protein subfamily, with the multi-drug resistance protein 1 (MDR1, other names: P-glycoprotein (P-gp) and ABCB1) has been most intensively studied.[45] The overexpression of the ATP-dependent P-gp efflux pump causes resistance to taxanes (paclitaxel and docetaxel) by allowing the expulsion of these substrates from cells.[44]

Epigenetic and microRNA dysregulation in HGSOC

Epigenetic modifications are mechanisms that affect gene expression without altering the DNA sequence. These modifications alter the packaging of DNA on the histones, which in turn regulates the functioning of the genome. Errant epigenetic changes may lead to pathological over- or underexpression of genes, resulting in various diseases, including cancer. The propensity for alteration makes them an additional site for cancer cell chemoresistance. The most widely known histone modifications include: 1) methylation; 2) acetylation; and 3) phosphorylation. Modifications to cell function may also be driven by microRNAs (miRNAs) – molecules that participate in the regulation of processes such as the cell cycle, cell differentiation, proliferation, apoptosis, and metabolism. The dysregulation of miRNA levels occurs in many types of cancer.[47] Unfortunately, there are sparse data describing

the role of the noncoding genome in the development of EOC, suggesting a gap in the literature in the area of understanding and developing novel drugs based on targeting epigenetic modifications.[48]

The DNA methylation plays a key role in the epigenetic regulation of gene expression and takes place mainly on cytosine followed by guanine in CpG dinucleotides. The CpG islands are often located in the regulatory region of genes. Hypermethylation of cytosines located on the CpG islands in the genes promoter region results in decreased gene expression, unlike hypomethylation, which results in increased gene expression. Abnormal methylation patterns are common in cancer and can usually be characterized by global hypomethylation and hypermethylation of TSGs.[48] Significant differences in methylation were found, both between the histological subtypes of OC and between the tumor tissue and the normal tissue.[46] The CpG island hypermethylation in OC is frequently observed in TSGs such as *BRCA1*, *p16*, *MLH1*, *RASSF1*, and *DARK*, suggesting reduced expression and, in turn, inability to repress tumor growth.[49]

Although HGSOC exhibits some hypermethylation of genes, it appears more hypomethylated than EOVC or CCOC. For example, *HNF1B* is overexpressed in CCOC while it is methylated in about 50% of HGSOC cases. The HNF1 homeobox B (HNF1B) plays a key role in the epithelial–mesenchymal transition (EMT), in which cells lose cell adhesion and polarization, acquiring an invasive phenotype. The altered expression of HNF1B has been associated with an increased cancer risk, and decreased expression of HNF1B is involved in cancer development.[50] A variant of *HNF1B* was identified as a HGSOC susceptibility locus, and Ross-Adams et al. reported that the susceptibility allele was associated with the methylation of the *HNF1B* promoter. Unmethylated *HNF1B* likely acts as an oncogene in CCOC, but, when hypermethylated, it acts as a tumor suppressor in the more aggressive histotype of HGSOC.[acc.46] Sanchez-Vega et al. compared DNA methylation between HGSOC and EOVC and normal tubal tissue. Twelve CpG loci differed in methylation for both HGSOC and EOVC compared to normal tissue, and of these, 11 had reduced methylation in tumors in comparison with normal tissue.[acc.46] Millstein et al. demonstrated that *TAP1* hypomethylation was associated with a reduction in the time to relapse.[39]

Histone acetylation has been explored thoroughly in OC.[48] As the vast majority of cases in TCGA are HGSOC, further studies are needed to ensure whether these findings can be applied to other histotypes.[46] Chapman-Rothe et al. identified gene sets associated with the H3K27me3 (active) and H3K4me3 (repressive) tags at the transcription initiation sites in HGSOC, and investigated their association with epigenetic silencing and malignant progression.[51] Researchers examined sets of histone-labeled genes in 8 benign ovarian lesions and 499 HGSOCs. A lower gene expression for H3K27me3 and bivalent gene sets have been demonstrated in neoplastic tissue.[46,51]

The miRNAs are post-transcriptional regulators of gene expression which reduce the expression of their target mRNAs by inhibiting translation or promoting degradation. This allows for the regulation of key biological processes such as development, differentiation, apoptosis, and proliferation.[28] The miRNAs have also been shown to be involved in cancer tumorigenesis and metastasis.[52] Dong et al. revealed that the overexpression of *miR-182* in HGSOC was shown to confer strong oncogenic properties by targeting BRCA1 and MTSS1. The overexpression of *miR-145* was found in vitro to inhibit proliferation, migration and invasion of OC cells, and in vivo to inhibit tumor growth and metastasis. The miRNA-145 was also found to directly target metadherin, an oncogene strongly overexpressed in breast cancer and OC.[52] The members of the *miR-106* family are involved in stem cell self-renewal and are highly induced during the early stages of cell reprogramming. In many solid cancers, miR-106a is known to act in tumor-initiating cells and regulate tumor differentiation through the retinoblastoma (Rb) pathway, the cell cycle and FoxO signaling. Liu et al. found significantly increased expression of *miR-106a* and its family members in the early and late stages of HGSOC. The tumor suppressor gene *RBL2*, which is mostly downregulated in HGSOC, is a specific target for miR-106a. These findings suggest that miR-106a may specifically inhibit protein expression of a member of the Rb family, and the overexpression of *miR-106a* results in rapid tumor growth and poor differentiation.[53]

As previously mentioned, an initiating event of neoplastic metastases in epithelial tumors is EMT. This manifests through increased gene expression and protein levels that are preferentially present in mesenchymal cells. Sun et al. integrated mRNA and miRNA data and subsequently identified a mesenchymal subtype associated with poor survival in HGSOC patients. They revealed a network consisting of 8 major miRNAs and 214 mRNAs. Of these, miR-101, miR-200c, miR-141, and miR-506 are EMT regulators but the role of the remaining 4 miRNAs (miR-25, miR-29c, miR-182, and miR-128) is less clear, with potential involvement in cell migration, invasion and metastasis.[47]

Genetic and molecular changes in non-HGSOC

Tumors other than HGSOC are often only defined as unclassified, atypical, non-serous, or indistinguishable between EOVC and CCOC. Moreover, non-HGSOC subtypes often show a poor response to chemotherapy; thus, the search for representative molecular features is essential for the development of new targeted therapies.[54]

EOVC

Endometrioid OC accounts for 10–20% of all EOC cases. Compared to HGSOC, this cancer occurs in younger women (mean age of 56) and is often associated with endometriosis and synchronous endometrial carcinoma.[55,56] The EOVC is characterized by abnormal PI3K signaling and mutations in *CTNNB1*, which is the major effector of the Wnt pathway.[57] Mutations in *PTEN* and microsatellite instability are also common. Both the histological and molecular profiles of EOVC appear to be more similar to endometrioid endometrial carcinoma (EEC) than to HGSOC.[55] Endometrioid carcinomas of the ovary and endometrium have been reported to contain mutations in *PTEN*, *PIK3CA*, *ARID1A*, *PPP2R1A*, and *CTNNB1* (β-catenin), but the frequency varies between these types of tumors. McConechy et al. showed that *PTEN* is more often mutated in EEC than in EOVC, but *CTNNB1* is more frequently mutated in EOVC than in EEC.[58]

Pierson et al. sequenced the entire exome of 26 EOVC samples, and their findings suggest that *PTEN*, *CTNNB1*, *PIK3CA*, *KMT2D*, *KMT2B*, *PIK3R1*, *ARID1A*, and *TP53* are significantly mutated in EOVC, which also occurred with a similar incidence in uterine corpus endometrial carcinoma (UCEC). Hypermutation due to mismatch repair deficiency (MMRD), as well as *POLE* mutation, were also observed in EOVC, again with a frequency comparable to UCEC. Common features of EOVC with HGSOC include 1) *TP53* mutations, 2) mutations leading to HRD and 3) widespread CNV.[55] The new EEC molecular classification suggests EOVC be grouped based on the following characteristics: *POLE* mutant, abnormal MMR, abnormal p53, and non-special type (NST, individuals without *POLE* mutation, without abnormal MMR or p53 protein expression). Such classification may have an important prognostic value and enable the selection of the most effective therapy.[59]

CCOC

Clear cell OC accounts for approx. 10% of EOC in Europe and is more common in younger women.[54] It is considered a high-grade neoplasm.[59] The most common mutations of CCOC are *ARID1A*, *PIK3CA*, *TP53*, and *KRAS*.[54] Endometriosis is associated with 33–37% cases of CCOC.[59] Clear cell tumors are characterized by a transcriptional profile that occurs in clear cell carcinoma of the ovary, endometrium and kidney.[54] Clear cell EOC, like renal clear cell tumors (clear cell renal cell carcinoma (ccRCC)), often have inactivating mutations in the chromatin remodeling factor SWI/SNF.[57,60] High similarity of CCOC to ccRCC indicates that the mTOR pathway and angiogenesis may be therapeutic targets. One of the molecular features of CCOC is the overexpression of hypoxia-inducible factor 1α (HIF-1α) and 2α (HIF-2α) with activation of this pathway.[57] One characteristic of CCOC is a lower incidence of *TP53* mutations than in other histological types. In addition, according to in vivo studies, the inactivation of *ARID1A* is not efficient in tumor initiation, and further mutations (such as in *PIK3CA)* are necessary for tumor evolution.[59]

The amplification of primary copy numbers in CCOC has been revealed in several regions. In 40% of women with CCOC, the amplification of 17q21-24 was present and associated with a worsened prognosis. The *PPM1D* oncogene and other genes on chromosome 17 were amplified in 10% of this population, with microRNA-21 overexpressed in 14% (resulting in loss of phosphatase tensin homolog (PTEN)). The amplification has also been demonstrated in the 8p, 20q, 17q, and 3p chromosomes that encode genes for CCOC-related proteins such as MYC, ZNF217, HNF1b, and PIK3CA, respectively. The *CCNE1* locus amplification occurred in 26% of patients with CCOC. The increase in the copy number resulted in increased protein expression and was associated with a worse prognosis.[60]

The MMRD occurs only in the histological types associated with endometriosis and is more common in EOVC (18%) than in CCOC (2%).[61] Defects in the MMR DNA repair genes cause microsatellite instability. Recent high-case studies have shown that 2.8% of CCOCs are characterized by the loss of at least 1 protein in the MMR pathway.[59] Rosen et al. observed a high level of microsatellite instability in the CCOC development, and a strong relationship between changes in the expression of *hMLH1* and *hMSH2* and microsatellite instability in this type of OC.[56]

MOC

Mucinous OC is an ovarian tumor of uncertain etiology that accounts for 3–5% of all epithelial OCs. As MOC is morphologically characterized by an epithelium with intestinal differentiation, it is difficult to determine whether the disease is primary OC or secondary metastatic mucinous adenocarcinoma.[62]

Primary mucinous tumors are classified as benign, borderline or malignant, depending on their histopathological features.[56] Genetic analysis of primary MOCs confirms a progressive model of carcinogenesis in which benign cystadenoma develops a *KRAS* or *CDKN2A* mutation, resulting in progression to borderline tumors, with the probability of both events and additional CNV, and then further to overt cancer that exhibits a greater frequency of KRAS and TP53 mutations and greater CNV than borderline tumor.[54] Mucinous ovarian tumors are characterized by a high frequency of *KRAS* mutations (46%). The *KRAS* mutations were found in histologically benign, borderline and malignant regions of the same tumor, suggesting this may be an early (or first) event in carcinogenesis of the ovarian mucosa.[56] Comparatively, *HER2* amplification or *TP53* mutation occurs later in malignant transformation as it is only observed in cancers.[63] The comparison of the incidence of lesions between mucinous borderline tumors (MBTs) and MOC showed that *TP53* mutations

occur in 10–18% of MBTs, and therefore contribute to the progression from MBT to MOC.[59]

Cheasley et al. indicated that TP53 mutations and copy number aberrations (CNAs), including the noteworthy 9p13 amplicon, are the key factors of cancer progression.[62] A high burden of CNA is associated with the progression of MBT to MOC and a worse prognosis in MOC.[59,62] Data from ovarian mucosal tumor sequencing were also compared with records from TCGA and other exome sequencing databases, and MOC revealed different genetic features from high-grade serous tumors of the ovary, endometrium, stomach, and colon, including mucinous colorectal carcinomas and appendicitis. Pancreatic adenocarcinoma was genetically the most similar to the MOC, with common features including the inactivation of *CDKN2A*, mutation of *KRAS* and *TP53*. Contrasting features in primary MOCs were *ERBB2* amplification and *RNF43* mutations, whilst pancreatic tumors exhibited frequent changes in *SMAD4*.[62]

The *TP53* mutation is often associated with HGSOC; however, ~25% of MOCs also possess this alteration. The *HER2* amplification and overexpression can be observed in 18% of MOC, and high microsatellite instability (MSI-H) may also be seen. Mutations in the *CTNNB1* or *APC* genes were detected, resulting in abnormal signaling in the Wtn pathway.[63] Other molecular changes identified in MOCs include *RNF43*, *BRAF*, *PIK3CA*, and *ARID1A* mutations (8–12%), as well as *ERBB2* amplification (26%). On the other hand, CNVs are key carcinogens associated with the increase in the staging and progression of metastases.[54] It has also been shown that the copy number changes most strongly enriched in grade 3 MOCs are gains of 1p and 19p, which affect many oncogenes (e.g., *JUN*, *JAK1*, *MYCL*, *BRD4)*.[59]

LGSOC

Low-grade serous OC accounts for approx. 3–5% of the EOC.[54,57] The LGSOC differs from HGSOC in: 1) age of onset (younger for LGSOC); 2) pathological features; 3) reduced aggressive features; and 4) longer OS.[54] However, LGSOCs, unlike HGSOCs, are usually resistant to platinum-based chemotherapy.[54,57] The HGSOC expression profiling revealed an increased expression of genes involved in chromosomal instability and cell proliferation, whilst LGSOC had less overall karyotype instability and a lower mutation rate.[57]

The LGSOC often contains activating mutations in genes involved in the MAPK signaling pathway, including *KRAS* (20–35%), *BRAF* (10–40%), *ERBB2* (5%), and *NRAS* (10%). Mutations in key genes in the MAPK pathway are mutually exclusive.[54] The RAS encodes the guanosine triphosphate (GTP) binding protein that is frequently activated in low-grade serous, mucinous and endometrioid OCs. The RAS pathway can also be activated by eliminating regulatory proteins such as Dab2. The Dab2 is known to be highly expressed in normal human tissues, especially in ovarian surface epithelial cells, whereas most OCs were found to lack or inhibit the expression of Dab2 mRNA and protein. Therefore, loss of *Dab2* expression may contribute to cell transformation or growth of neoplastic cells.[56] The *BRAF* and *KRAS* mutations lead to constitutive activation of MAPK/Erk signaling and its downstream pathway, and in turn to increased survival and proliferation of neoplastic cells. These data suggest that the inhibition of MAPK hyperactivation may be a therapeutic target in women with LGSOC who respond very poorly to conventional platinum-based therapy.[57]

Hunter et al. compared SBT with LGSOC. The mutation frequency in *KRAS*, *BRAF*, *HRAS*, or *ERBB2* was significantly higher in SBT than in LGSOC. In contrast, *NRAS* mutations were recorded in 26% of LGSOC, but not observed in SBT, which indicates a much greater oncogenic potential of this change. Similar results were obtained by Emmanuel et al., who identified a 9% frequency of *NRAS* mutations that were associated only with cancer, which suggests that it is an oncogenic factor in serous OC.[acc.64] The findings of Hunter et al. confirmed that *BRAF* mutations are less common in LGSOC. Scientists also linked these changes to an earlier stage of cancer, better patient results and a lower likelihood of relapse in both SBT and LGSOC.[65] The low frequency of the *BRAF* mutation in LGSOC challenges the notion that all SBTs and LGSOCs are on a continuum, suggesting that the mutation may be protective. The studies supporting this findings show more favorable results in patients whose tumors have *BRAF* mutations than in the case of *KRAS* or *BRAF* mutations and wild-type *KRAS*.[64] The CNAs are associated with the progression of SBT to LGSOC, but are less common than in HGSOC. Hunter et al. reported that the most significant CNAs in LGSOC was the loss of 9p and homozygous deletions of the *CDKN2A/2B* locus. They were able to identify markers of progression from SBT to LGSOC, as well as novel LGSOC stimulants. The *USP9X* and *EIF1AX* are associated with mTOR regulation, suggesting that mTOR inhibitors may be a crucial adjunct therapy in trials of targeted therapy with MEK and RAF inhibitors.[65] Mutations in the *TP53* gene are rare in LGSOC, with an incidence of less than 8% in studies, although the absence of *TP53* mutation is sometimes used as a criterion for inclusion in LGSOC.[54] Finally, when considering CNV, the most common in LGSOC revealed by Van Nieuwenhuysen were loss of 1p, 6q, 9p, 16p/q, 17p, 18p/q, 22q, and gain in 1q, 7p/q and 8q. The most common focal lesion was the loss of 1p36.33 (54.1%). Many human neoplasms are characterized by an alteration in this locus.[acc.59]

The data discussed above suggest that LGSOC develops gradually from SBT. The formation of SBT appears to be closely related to abnormalities in KRAS signaling, which occurs very early in tumorigenesis. The acquisition of an invasive character, or evolution in LGSOC, appears to be associated with the acquisition of CNVs such as 9p21.3 hemizygous/homozygous deletion and hemizygous deletion of 1p36, which occurs in the final stages of carcinogenesis.[66]

Conclusions

Ovarian cancer is a challenge of modern medicine. Such tumors are characterized by considerable heterogeneity and their genetic profiles show significant differences (Table 1). The varied molecular characteristics of the individual cases result in differences in the typical stage of presentation (the stage in which the disease gives clinical manifestation), prognosis and success of targeted therapy. Given the heterogeneity between cancers, the individual and the TME, future directions of therapy are based on the concept of personalized medicine. To reduce the mortality rate in OC, there is also a need for an effective strategy to detect the disease at an early stage, which will likely utilize NGS technology.

Target populations for screening may be divided into 2 groups based on average lifetime risk, with general (1.32%) and high-risk (10%) populations. There is a need to modify and standardize the guidelines for screening, as the high-risk population is currently identified through family history and genetic testing only for *BRCA1* and *BRCA2*. Additional SNPs should be included as these contribute to the polygenic risk of OC.[67]

The current standard of care for women at high risk of OC is risk-reducing salpingo-oophorectomy (RRSO). This procedure is recommended to be performed up to 40 years of age in carriers of the *BRCA1* mutation and up to 45 years of age in the case of carriers of the *BRCA2* mutation. In Europe and the UK, the RRSO is recommended for high-risk women without screening, while in the USA, the CA125 blood test and transvaginal ultrasound are performed before the surgery. The RRSO reduces the risk of OC and mortality; however, many factors may influence the choice of undergoing surgery. Oophorectomy may result in complications but also increase the risk of cardiovascular disease.[68] In their review, Nebgen et al. emphasize the role of the detection of circulating tumor DNA (ctDNA) in the blood, tests based on DNA methylation analysis and miRNA as potential biomarkers in detecting cancer at an early stage, as well as in complementing and improving screening strategies offered to patients before RRSO.[69]

For patients diagnosed with EOC, germline genetic testing is recommended, and in addition, pathogenic variants (PVs) of genes other than *BRCA1* and *BRCA2* should be included. After obtaining a negative germline PV result, a tumor tissue test to assess somatic mutations for *BRCA1* and *BRCA2* (at the minimum) should be performed.[70] It is reasonable to use multigene NGS in OC where there are somatic BRCA1/2 mutations associated with increased benefit for PARPi. As recommended by the European Society for Medical Oncology (ESMO), larger panels can only be used under specific contracts with payers who must consider the total cost of such a strategy. However, ESMO stresses the need for multi-gene sequencing by cancer clinical research centers and the development of new treatment strategies.[71]

To conclude, current research has revealed many changes at the molecular level in OC. However, further, large-scale investigations are required to assess the increased application in diagnostics. In turn, this would allow for both

Table 1. Main genetics and molecular differences between ovarian carcinomas subtypes

HGSOC	EOVC	CCOC	MOC	LGSOC
mutations in: *TP53*, *BRCA1/2*, *CDK12*, *NF1*, *RB1*, *CSMD1*, *NOTCH4*, *TMEM132D*	mutations in: *PTEN*, *PIK3CA*, *ARID1A*, *PPP2R1A*, *CTNNB1*, *KMT2D*, *KMT2B*, *PIK3R1*, *TP53*	mutations in: *ARID1A*, *PIK3CA*, *TP53*, and *KRAS*; inactivating mutations in the chromatin remodeling factor SWI/SNF	mutations in: *KRAS*, *CDKN2A*, *TP53*	– activating mutations of genes involved in the MAPK signaling pathway, including *KRAS*, *BRAF*, *ERBB2*, and *NRAS* – mutually exclusive – *TP53* mutations are rare
widespread CNV	widespread CNV	CNV: – 17q21-24 amplification, chromosome 17 – *PPM1D* oncogene – amplification of 8p – *MYC*, 20q – *ZNF217*, 17q – *HNF1b*, and 3p – *PIK3CA* (genes for proteins related to CCOC) – amplification at the *CCNE1* locus	CNV: – *HER2* and *ERBB2* amplification – 1p and 19p – impact on multiple oncogenes (*JUN*, *JAK1*, *MYCL*, *BRD4*)	CNV: – loss of 1p, 6q, 9p, 16p/q, 17p, 18p/q, and 22q – gain in 1q, 7p/q and 8q – loss of 1p36.33 – the most common focal change
mutations leading to HRD	mutations leading to HRD	microRNA-21 overexpression lead to loss of PTEN	mutations in the *CTNNB1* or *APC* gene lead to abnormal signaling in the Wtn pathway	less overall karyotype instability and lower mutation rate than HGSOC
very frequent structural and numerical aberrations in chromosomes 3, 8, 11, 17, and 21 lead to genomic instability	MMRD (EOVC – 25%) causes MSI-H	MMRD (CCOC – 2%) causes MSI-H	MSI-H	

OC – ovarian cancer; HGSOC – high-grade serous OC; EOVC – endometrioid OC; CCOC – clear cell OC; LGSOC – low-grade serous OC; MOC – mucinous OC; SWI/SNF – switch/sucrose nonfermentable; CNV – copy number variation; HRD – homologous recombination deficiency; MMRD – mismatch repair deficiency; MSI-H – high microsatellite instability; PTEN – phosphatase and tensin homolog.

the extension of the gene panels offered to patients with EOC or suspected family predisposition, as well as for tailoring therapeutic intervention.

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The beneficial role of simple inflammatory blood indices in pediatric cardiology

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Abstract

Simple whole blood analysis can effectively demonstrate complex changes in inflammatory responses to cardiovascular disorders in adults and enable the prediction of adverse outcomes or diminished survival. Such inflammatory activation has also been detected in the pediatric population. Blood analysis results are repeatable and readily available, which gives the method an advantage over others. Inflammatory phenomena such as a high leukocyte count and an increased neutrophil-to-lymphocyte ratio (NLR) are related to a poor prognosis of advanced heart defects and worse outcomes after pediatric cardiac surgery in the advanced stages of the disease. Surgery-associated inflammation exacerbates these diseases, and the inflammatory response may further complicate the postoperative period. Simple blood cell counts and indices may be beneficial for evaluating cardiac surgery outcomes and cardiovascular disorder prognosis in infants and children. This review summarizes current knowledge on inflammatory markers in pediatric cardiovascular diseases and surgery.

Key words: surgery, inflammation, congenital heart disease, neutrophil-to-lymphocyte ratio

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Introduction

Inflammation is a well-known causative factor in cardiovascular disorders in adults, and several studies have underlined the significant contribution of inflammation in the occurrence and progression of coronary artery disease.[1,2] Atherosclerotic plaque formation and enlargement are related to neutrophil and macrophage infiltration, pro-inflammatory cytokines and chemokines, lipid accumulation in the core, and thinning of the fibrous cap.[3,4] Moreover, inflammatory responses were reported in heart failure[5–7] and worsened with its advancement and complications, such as pulmonary hypertension.[8] Furthermore, the assessment of inflammatory activation demonstrated its value in the pediatric population.[9]

The assessment of cardiovascular disease prognosis and procedural outcomes highlighted several parameters and biomarkers.[10–15] Many of them, including interleukins, microribonucleic acids (microRNAs), tumor necrosis factor alpha (TNF-α), and platelet-leukocyte aggregates,[16,17] have high prognostic value. However, using such markers in clinical practice is not possible, as a profound analysis of inflammatory phenomena is challenging and not cost-effective.[18,19] Hence, simpler methods, such as whole blood analysis, were introduced and described as sufficient for demonstrating complex changes in the inflammatory response to cardiovascular disorders and predicting adverse outcomes or diminished survival. The results of whole blood analysis are readily available, and the method is repeatable, which gives it an advantage over other methods.

Objectives

The objective of this review was to summarize current knowledge on inflammatory markers in pediatric cardiovascular diseases. The contemporary studies concerning the assessment of inflammatory activation using simple blood count analysis were collected and analyzed.

Outline of the issue

The neutrophil-to-lymphocyte ratio (NLR) is the most commonly described biomarker that represents the relationship between neutrophils and lymphocytes in the blood. Other indices include the monocyte-to-lymphocyte ratio (MLR) and platelet-to-lymphocyte ratio (PLR). More complex indices, such as the systemic inflammatory index (SII), systemic inflammatory response index (SIRI) and aggregate index of systemic inflammation (AISI), combine even more morphological elements of blood.

Leukocytes are the primary cellular mediators of inflammation, and changes in their subpopulation counts may reflect the immune response to several inflammation-associated phenomena. Neutrophils are a marker of ongoing nonspecific inflammation, while lymphocytes signify the immune regulatory response.[20] At the same time, lymphocyte count indicates physiological stress but is inversely proportional to inflammation.[21] The NLR combines 2 leukocyte subtypes and may have high predictive value in several cardiovascular and noncardiac disorders.

Platelets are involved in inflammatory processes and secrete thromboxane, chemokines, proinflammatory cytokines, and growth factors, which play roles in vascular inflammation and thrombosis.[21] Mean platelet volume (MPV) is a marker of platelet activation associated with active rheumatic arthritis, bowel disease[20] and revascularization processes.[22]

Inflammatory markers in cardiac disorders

Hematological count and index derangements, functioning as predictors of clinical outcomes, are presented in Table 1.

Cardiac arrhythmias

Cardiac arrhythmias usually occur in children as paroxysmal supraventricular tachycardia (SVT).[23] There are 2 peaks of its incidence – the 1st in infancy and the 2nd between 8 and 12 years of age.[24] Some hypotheses explain the etiology of SVT with an inflammatory state as its trigger. The fact that viral infections in children, especially those promoting myocarditis, are commonly related to the prevalence of arrhythmias supports these hypotheses.[25] Furthermore, Aydin et al. emphasised that patients with SVT have higher NLR values.[26]

Frequent premature ventricular beats may be associated with chronic myocardial injury that is related to many inflammatory response factors.[27] Therefore, an early prediction of a higher risk of myocardial damage is crucial. Among the available laboratory tests, troponin and creatine kinase are established markers of myocardial injury in acute and chronic heart failure. Recently, leukocyte count and NLR correlated positively with troponin and perioperative damage.[27] Preoperative neutrophil count has been proposed as a predictive factor for postoperative atrioventricular block in pediatric cardiac surgery.[28]

Kawasaki disease

Kawasaki disease is an acute febrile disorder characterized by systemic inflammation and vasculitis.[29,30] Characteristic hallmarks of the disease include coronary artery lesions (CALs), coronary dilatation, aneurysm, stenosis, myocardial infarction, and valvular lesions. Chang et al. underlined the critical role of simple markers in preventing CAL development by identifying at-risk children.[29] They proposed 4 independent risk factors for predicting CALs,

Table 1. Hematological count and index derangements as predictors of clinical outcomes

Parameters	Outcome	Reference
Neutrophilia	longer hospital stay need for postoperative mechanical circulatory support	65
Lymphopenia	longer hospital stay longer mechanical ventilation time postoperative nitric oxide use increased mortality postoperative sepsis increased susceptibility to infections postoperative effusion and edema heart failure	43, 44, 64, 65
Thrombocytopenia	longer hospital stay postoperative sepsis	64
NLR	longer mechanical ventilation time increased length of ICU stay increased hospital stay increased mortality higher vasoactive–inotrope score low cardiac output syndrome pleural effusion, chylothorax Kawasaki disease – coronary artery lesions IVIG resistance in Kawasaki disease Kawasaki disease diagnostics in uncertain cases necrotizing enterocolitis in PDA children arterial hypertension heart failure acute kidney disease pulmonary hypertension neonatal sepsis exclusion progression in pulmonary hypertension related to congenital heart defects cyanotic congenital heart disease Fontan patients, PLE coronary artery lesions in Kawasaki disease supraventricular tachyarrhythmia frequent ventricular premature beats lymphatic malformations and complications after Fontan surgery ECMO use	21, 27, 29, 30, 31, 32, 33, 41, 44, 45, 46, 55, 56, 57, 59, 60, 62, 63, 71
Platelet count	Kawasaki disease – coronary artery aneurysms acute rheumatic carditis arterial hypertension	20, 30, 41
PLR	Fontan patients neonatal sepsis ECMO use IVIG resistance in Kawasaki disease arterial hypertension	21, 41
MLR	necrotizing enterocolitis in PDA children severity of valvular involvement in acute rheumatic carditis	33, 35]

ECMO – extracorporeal membrane oxygenation; ICU – intensive care unit; IVIG – intravenous immunoglobulin; MLR – monocyte-to-lymphocyte ratio; NLR – neutrophil-to-lymphocyte ratio; PDA – patent ductus arteriosus; PLE – protein-losing enteropathy; PLR – platelet-to-lymphocyte ratio.

namely C-reactive protein (CRP) >103 mg/L, NLR > 3.5, male gender, and intravenous immunoglobulin (IVIG) resistance. High-dose IVIG effectively resolves inflammation and reduces the risk of CALs. However, 10% of patients are resistant to this therapy. Kanai et al. showed high NLR and PLR to be strong predictors of IVIG resistance.[31] Moreover, Smorczewska-Kiljan et al. showed that high platelet count is one of the most important predictors of coronary artery aneurysm occurrence.[30] In addition, Yan et al. proposed NLR and CRP as markers deserving special attention in patients suspected of Kawasaki disease who do not initially meet the diagnostic criteria.[32]

Necrotizing enterocolitis and congenital heart disease

Infants diagnosed with necrotizing enterocolitis with patent ductus arteriosus (PDA) and congenital heart defects had significantly higher NLR and MLR than children without cardiac abnormalities.[33] Higher NLR and MLR resulted in very intense local inflammation involving infiltration of the intestines by neutrophils and circulating monocytes. The monocytes differentiated into macrophages in situ in infants with impaired intestinal perfusion and systemic circulation caused by cardiac anomalies.

Acute rheumatic disease

Inflammation is fundamentally involved in the pathogenesis of acute rheumatic disease. The infiltration of several inflammatory cells into the myocardium and endocardium of valves is observed, including neutrophils, macrophages and subpopulations of lymphocytes. Neutrophils and macrophages influence atrial remodeling, and the actions of macrophages include the generation of oxygen free radicals.[34] The healing process results in fibrosis and changes in the vasculature and dimensions of atrial cells. Increased NLR, PLR and MLR, and decreased MPV are associated with the severity of valvular involvement in patients with acute rheumatic carditis (ARC).[20,35] In addition, NLR correlated with leukocyte count, erythrocyte sedimentation rate (ESR) and CRP.[20,35] In other studies, ESR, CRP and red blood cell distribution width (RDW) were higher in patients with ARC.[36–38] Moreover, an increase in platelet count is relevant and reflects the production of new reactive platelets through cytokine stimulation.[20] Interleukin 6 (IL-6) is a platelet effector, and its serum levels increase significantly in episodes of acute rheumatic fever.[39] In the presence of IL-6, inflammatory processes are activated and generate thrombogenicity.[40]

Arterial hypertension

Arterial hypertension is an increasing problem in children due to obesity, sedentary lifestyle and excessive

salt intake.[41] Subclinical inflammation may contribute to the pathogenesis of primary hypertension. Skrzypczyk et al. analyzed simple blood morphology and found higher NLR, PLR and platelet counts in hypertensive patients than in healthy subjects, and NLR correlated with arterial stiffness.[41] Based on the different diagnostic methods used in the study, the authors concluded that the intramural inflammatory process affects multiple arteries in primary hypertension in children.

Heart failure

Heart failure in children is a rare but serious complication of several cardiological disorders and may lead to death or the need for a heart transplant.[42] Several clinical, echocardiographic and laboratory parameters characterize its advance and severity. Although brain natriuretic peptide (BNP) and N-terminal BNP are commonly analyzed, simple blood morphology predicted worse survival or the need for a heart transplant.[43]

Lymphocytopenia is multifactorial and may reflect the degree of sympathetic activation.[43] Araújo et al. reported a worse prognosis and a higher risk of death or cardiac transplant in children with dilated cardiomyopathy and higher NLR (>5.2) and lymphopenia (<1000/μL) values.[44] Gursoy et al. correlated inflammatory markers, including NLR, with the progression of pulmonary hypertension related to congenital heart defects.[45] In addition, mean pulmonary artery pressure and NLR significantly increased during the postoperative period in patients with a pulmonary hypertensive crisis.

Surgery in cardiac disorders in children

The surgery itself is related to a certain degree of inflammatory response. However, it can be unpredictably exaggerated in some congenital heart diseases.

Cardiopulmonary bypass

Cardiopulmonary bypass (CPB) use is usually associated with a systemic inflammatory response, a nonspecific inflammatory syndrome that may be similar to infection and lead to the unnecessary use of broad-spectrum antibiotics. The contact of blood with the surface of the CPB circuit results in a cascade of pro-inflammatory cytokines, complement activation, blood coagulation, and an increase in leukocytes, platelets and vascular endothelial cells. The neutrophils are essential components of the systemic inflammatory response to tissue and reperfusion injury.[46] Furthermore, ischemia–reperfusion injury and endotoxemia due to hypothermic perfusion lead to endothelial injury and the release of reactive oxygen species. Neither leukocytosis, nor neutrophil count, nor CRP can discriminate between infection and a nonspecific inflammatory syndrome,[47] as these markers reflect the inflammatory process. Fortunately, the biomarker procalcitonin enables differentiation between bacterial infection and noninfectious systemic inflammatory responses[48,49] after surgery with CPB. Indeed, an increase in procalcitonin above a proposed cutoff value of 2 ng/mL should lead to the commencement of antibiotic therapy.[48] However, the cutoff points are different on consecutive days due to the evolution of procalcitonin over time, and increase rates differ between infected and noninfected patients. Indeed, Haponiuk et al. pointed out the importance of changes and trends in values in the early postoperative hours rather than concentrating on single values of inflammatory markers.[50] Deviation from the typical kinetics of leukocyte count, CRP and procalcitonin should pique the attention of physicians.

Manuel et al. observed that children with cyanotic congenital heart diseases exhibited higher preoperative NLR than acyanotic patients.[51] Therefore, the authors assumed that cyanosis was related to a higher degree of preoperative inflammation. Similarly, more sophisticated methods, including the analysis of interleukins, showed their higher levels in cyanotic children.[52] A lower perioperative anti-inflammatory cytokine balance may contribute to postoperative mortality.[53] There are still some gaps in the literature and questions, such as why some patients with the same disease have a higher NLR than others, which extends to other biomarkers of the same lineage. Manuel et al. recently proposed a probable mechanism to explain this increase and the association with unfavorable outcomes in pediatric cardiac surgery patients (Fig. 1).[54] Cyanotic patients are continuously exposed to myocardial hypoxia, promoting myocardial stress, which causes a permanent inflammatory response characterized by high oxidative stress, reactive oxygen species and the recruitment of neutrophils.[54] The exacerbation of these phenomena may negatively influence postoperative outcomes, especially with CPB use.[51,55] The described mechanism causes cellular apoptosis and tissue injury.[54] In turn, increased blood flow to the lungs induces pulmonary vascular stress, vascular remodeling and endothelial dysfunction, followed by pulmonary hypertension and preceded by chronic inflammatory processes in the pulmonary tissue of acyanotic patients. Similarly, surgery under CPB is associated with an exacerbated inflammatory response and a negative impact on surgery outcomes.

Moosmann et al. recommended calculating NLR and PLR for univentricular patients during the course of total cavopulmonary connection and follow-up.[56] In their study, NLR and PLR correlated with the degree of lymphatic malformations, which are associated with early complications after Fontan surgery and Fontan failure, and may also occur after Glenn surgery despite a lack of clinical manifestation. The authors suggested that patients with higher

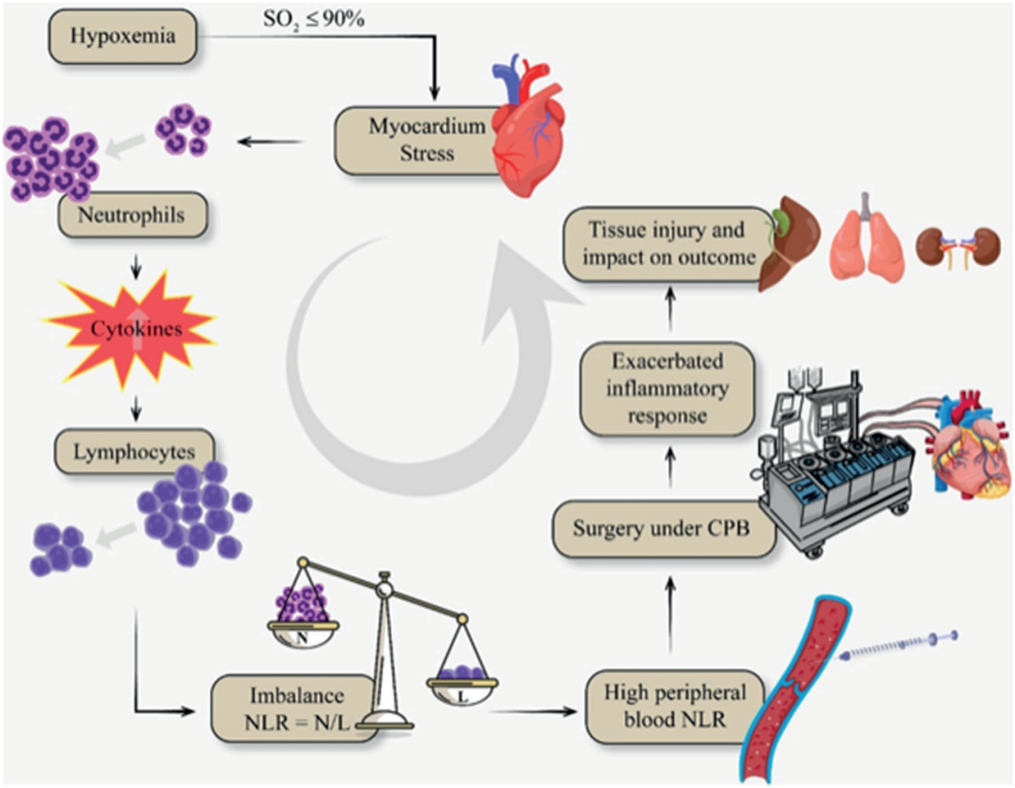

Fig. 1. Probable mechanism of high neutrophil-to-lymphocyte ratio (NLR) in cyanotic patients presenting with hypoxemia and myocardial stress linked with exaggerated inflammatory response and negative impact on postoperative outcomes. High pulmonary flow with pulmonary vascular stress seems to represent a comparable factor in acyanotic patients (see interpretation in the text). Adapted and modified, with permission, from Manuel V, Miana LA, Jatene MB. Neutrophil-lymphocyte ratio in congenital heart surgery: What is known and what is new? *World J Pediatr Congenit Heart Surg*. 2022;13(2):208–216. doi:10.1177/21501351211064143 [54]

CPB – cardiopulmonary bypass.

values require closer monitoring and evaluation for signs of Fontan complications, such as lymphatic malformations and protein-losing enteropathy (PLE). Lymphopenia in Fontan patients is associated with portal hypertension, PLE and lymphatic malformations. Lymphangiogenesis occurs during inflammation due to mediators released from inflammatory cells, including neutrophils. An increase in neutrophil and a decrease in lymphocyte count were described in Fontan patients.[56]

Postoperative complications

Studies performed in acyanotic patients with co-existing pulmonary hypertension demonstrated a significant prognostic value of NLR and SII on a higher vasoactive–inotrope score (VIS), prolonged mechanical ventilation time, extended time in the intensive care unit (ICU), and the length of hospital stay.[57–59] The aforementioned inflammatory markers have been introduced in the assessment of prognosis in pediatric cardiac surgery.[46,54,60] In a study by Savluk et al., NLR varied between children with failed and successful extubation (following prolonged intubation).[61] Moreover, a high preoperative NLR was associated with acute kidney injury after tetralogy of Fallot repair.[62] Therefore, NLR may be used to identify patients at risk of postoperative complications.

Elevated preoperative NLR was associated with higher mortality in hypoplastic left heart syndrome patients.[63] Cabrera et al. reported preoperative lymphopenia as a predictor of adverse outcomes such as longer postoperative length of stay, mechanical ventilation, postoperative nitric oxide use, and mortality.[64] Other authors made similar observations.[65,66] Perioperative complications associated with abnormal blood cell counts include an increased risk of perioperative mortality (lymphopenia), longer postoperative length of stay (lymphopenia, thrombocytopenia and neutrophilia), increased occurrence of postoperative sepsis (lymphopenia and thrombocytopenia), and the need for postoperative mechanical circulatory support (neutrophilia). A decreased absolute lymphocyte count secondary to acquired or inherited deficiency resulted in an increased susceptibility to infections.[64] The causes are multifactorial, with increased destruction or loss secondary to sequestration and decreased production. The authors explained their results by the presence of pulmonary lymphatic dysplasia and lymphangiectasia. These lesions may be associated with cardiac disorders with pulmonary venous obstruction, such as hypoplastic left heart syndrome or total anomalous pulmonary venous return.

Postoperative pleural effusion is a common complication after CPB during cardiac surgery.[67] Prolonged accumulation may lead to the deterioration of postoperative recovery, extended hospital stay and a higher mortality rate due to malnutrition.[68] Yakuwa et al. found no significant differences in baseline characteristics, while NLR change had prognostic value in predicting prolonged pleural effusion, including chylothorax.[68] Besides several factors such as increased right-sided hydrostatic pressure, decreased collagen osmolarity, slow bleeding, warfarin use, longer

CPB time, and postoperative infection, enhanced permeability due to systemic inflammation is an important etiological factor.[68] Gupta-Malhotra et al. showed a weak correlation between pleural fluid volume and IL-6, and in their further study,[69] they demonstrated an association between total duration and the amount of pleural effusion and troponin.[67] Bocsi et al. recommended the evaluation of preoperative neutrophil count and percentage, as well as a decreased percentage of lymphocytes, as suitable for identifying patients at risk of postoperative effusions and edema.[70] Crucially, NLR includes both blood elements in its calculation.

Extracorporeal membrane oxygenation

The use of extracorporeal membrane oxygenation (ECMO) in the postoperative period may be critical for pediatric patients with low output and pulmonary difficulties[21] to facilitate pulmonary or cardiac recovery. The therapeutic outcomes vary due to several factors, complications and comorbidities. Inflammation, infection and heart failure are common problems. Considering the interaction between blood flow and the foreign surfaces of ECMO, changes in the severity of inflammatory indices may show the degree of inflammation and provide information concerning prognosis. In a study by Arslanoğlu et al., NLR and PLR significantly increased compared to the preoperative period in patients who received ECMO, but its association with mortality was uncertain.[21] Iliopoulos et al. demonstrated a significant relationship between preoperative NLR and low cardiac output syndrome after cardiac surgery in children, particularly during the first 12 h.[71]

Limitations

The limitation of the evaluated inflammatory indices casts doubt over their normal ranges in children, especially if multiple pathological conditions and comorbidities exist.

Conclusions

The greatest advantages of using NLR and other indices are their accessibility in clinical practice and low evaluation costs. Several of the aforementioned reports highlighted their beneficial role in evaluating infants and children in terms of cardiac surgery outcomes and the prognosis of several cardiological disorders.

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Lead-related tricuspid regurgitation and ventricle dysfunction: Current management and future perspectives

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Abstract

The implantation of cardiac implantable electronic devices (CIEDs) may result in or worsen previously existing tricuspid regurgitation (TR). The prevelence of lead-related tricuspid regurgitation (LRTR) in patients with CIEDs is between 7.2% and 44.7% when the degree of worsening TR is not reported, or from 9.8% and 38% when it is diagnosed as worsening of TR severity by at least 2 grades after a CIED has been implanted. It has been suggested that a CIED lead positioned over or pinning a leaflet may be the main cause of TR in this patient population. The septal and posterior leaflets of the tricuspid valve have been reported to be the most affected by CIED leads. Severe LRTR is related to the development of heart failure (HF) or worsening of previously existing dysfunction; it is also associated with elevated mortality. However, there are no definitive predictors of LRTR development or standardized methods of treatment. Some studies have suggested that imaging-guided lead placement can reduce the occurrence of LRTR. This review summarizes current knowledge concerning the development, evaluation, consequences, and management of LRTR.

Key words: cardiac implantable electronic devices, lead-related tricuspid regurgitation, heart failure

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Introduction

The etiology of tricuspid regurgitation (TR) is predominantly functional (93% of cases) and includes left- and right-sided heart failure (HF), dysfunction of the aortic and mitral valves, pulmonary hypertension (PH), and arrhythmias. The incidence of isolated TR induced by atrial fibrillation is estimated to be approx. 8% of cases.[1] The prevalence of significant TR among patients with a cardiac implantable electronic device (CIED) has not been precisely determined and varies from 7.2% to 44.7%[2–23] or from 9.8% to 38%[4,12,18,21,23,24] when it is diagnosed as worsening of TR severity by at least 2 grades after a CIED has been implanted. A summary of current studies on TR occurrence after the implantation of CIED is presented in Table 1. Lead-related tricuspid regurgitation (LRTR) is associated with the development of HF and increased mortality,[5,6,21,25] yet different authors have different views about its clinical relevance. The differences between reports concerning TR and CIEDs include the number of patients, the type of investigated CIED, the method of TR assessment, and definitions of its significance.[9]

Imaging techniques for the assessment of lead position

Two-dimensional echocardiography

Conventional 2D transthoracic echocardiography (TTE) has limitations in its anatomical assessment of the tricuspid valve (TV) because only 2 leaflets can be visualized simultaneously on the atypical parasternal view. Furthermore, the posterior leaflet can only be seen on the right ventricular two-chamber view. Therefore, identifying a relationship between the leaflets and a CIED lead can be a challenging task.[3] The evaluation of LRTR severity is based on standard criteria of the right ventricle (RV) and TV assessment. It includes RV dimensions, fractional area change, tricuspid annular plane systolic excursion (TAPSE), right ventricular systolic pressure, proximal isovelocity surface area (PISA), vena contracta, effective regurgitant orifice, and regurgitation volume.[10,12,13,24,26,27] To avoid an underestimation of LRTR, it is crucial to record

Table 1. Summary of the recent reports

Author	Year	n	CIED	Assessment of TR severity	2D or 3D TTE	Frequency (%)	p-value
Paniagua et al.[2]	1998	374	PPM	onset of severe TR defined as III–IV	2D	7.2	<0.001
Seo et al.[3]	2008	87	PPM, ICD, CRT	onset of severe TR	2D and 3D	15.0	–
Kim et al.[14]	2008	248	PPM, ICD	worsening by at least 1 grade	2D	24.2	–
Webster et al.[17]	2008	123	PPM	worsening by at least 1 grade	2D	22.0	–
Klutstein et al.[18]	2009	410	PPM	worsening by at least 2 grades	2D	18.3	<0.001
Alizadeh et al.[19]	2011	125	PPM	moderate-to-severe TR	2D	31.6	<0.001
Addetia et al.[20]	2014	100	ICD, PPM, CRT	worsening by at least 1 grade	2D and 3D	36.0	–
Höke et al.[21]	2014	239	ICD, PPM	worsening to a grade ≥2	2D	38.0	–
Mediratta et al.[16]	2014	121	PPM, ICD, CRT	severe TR	2D and 3D	21.5	–
Fanari et al.[22]	2015	206	PPM, ICD	worsening by at least 1 grade	2D	44.7	<0.001
Lee et al.[23]	2015	382	PPM, ICD	worsening by at least 2 grades	2D	10.0	–
Arabi et al.[4]	2015	41	PPM, ICD, CRT	worsening by at least 2 grades	2D	17.1	–
Al-Bawardy et al.[5]	2015	1596	PPM, ICD	prevalence of grade 3 or 4+ TR	2D	35.0	–
Delling et al.[6]	2016	634	PPM	onset of STR defined as moderate-to-severe or ≥3+	2D	16.0	<0.001
Rydlewska et al.[7]	2017	100	ICD/CRT-D, PM, CRT-P	onset of severe TR	2D	28.0	–
Nakajima et al.[8]	2020	143	PPM, ICD, CRT	worsening of lead-related and lead non-related TR	2D and 3D	20.3	–
Seo et al.[9]	2020	373	PPM, ICD	worsening of lead-induced and non-lead-induced TR	2D	13.1	–
Seo et al.[24]	2020	429	PM	worsening by at least 2 grades	2D	9.8	–
Papageorgiou et al.[10]	2020	304	ICD/CRT-D, PM, CRT-P	onset of ≥moderate TR	2D	21.7	–
Lee et al.[11]	2021	1075	PPM	increased TR grade ≥2 degrees and TRPG >30 mm Hg	2D	18.4	–
Riesenhuber et al.[12]	2021	990	PPM	worsening by at least 2 grades	2D	25.6	–
Kanawati et al.[13]	2021	165	PPM, ICD	worsening by at least 1 grade	2D	27.0	–

CIED – cardiac implantable electronic device; CRT – cardiac resynchronization therapy; CRT-D – cardiac resynchronization therapy defibrillator; CRT-P – cardiac resynchronization therapy pacemaker; ICD – implantable cardiac defibrillator; PPM – permanent pacemaker; STR – severe tricuspid regurgitation; TTE – transthoracic echocardiography; TR – tricuspid regurgitation; TRPG – tricuspid regurgitation pressure gradient.

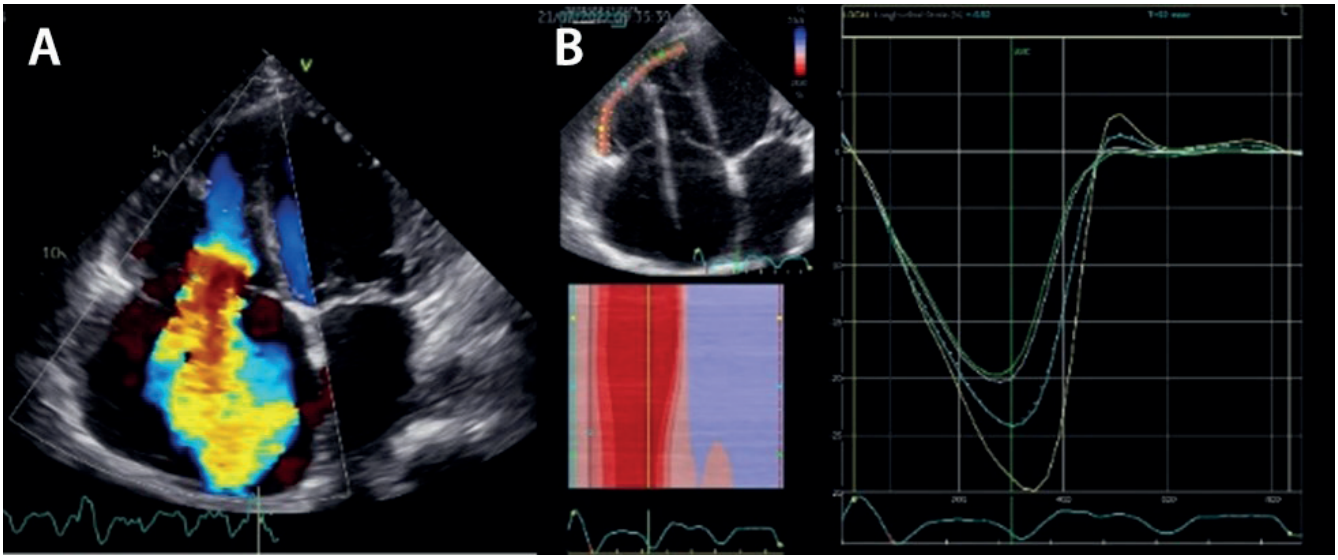

Fig. 1. A. Massive tricuspid regurgitation in a patient with an implantable cardioverter/defibrillator; B. Strain of a free wall of the right ventricle (–24%), indicating good systolic function despite dilatation of the right ventricle

regurgitant flow during inspiration. Massive TR in a patient with a CIED and calculated strain of the RV are shown in Fig. 1.

Three-dimensional echocardiography

Some limitations of conventional TTE can be overcome using 3D echocardiography.[28,29] It enables clinicians to make a complete assessment of the anatomy of the TV and subvalvular apparatus, as well as the geometry, volume and ejection fraction of the RV. The optimal view to enable visualization of all the TV leaflets and commissures is the en face view with the septal leaflet located at the 6 o'clock position.[28] In patients with CIEDs, 3D echocardiography makes it possible to identify the lead position and localize a leaflet prolapse, perforation or vegetations, in addition to the origin of the regurgitation jet. The 3D technique may be helpful in the evaluation of the TV orifice area for grading tricuspid stenosis.[28,30] It substantially improves the determination of the mechanism of valve dysfunction and is feasible in 74–94.2% of patients with CIEDs.[3,16,31]

Transesophageal echocardiography

Transesophageal echocardiography (TEE) should be considered a complementary technique of imaging in nonconclusive assessment of lead position. Moreover, TEE seems to be a more accurate method for evaluating TR severity. Lin et al. performed preoperative TTE in patients with LRTR undergoing cardiac surgery, and TR severity was underestimated in 37% of patients in subsequent intraoperative TEE.[32] In Fig. 2, TV imaging methods and lead position in TEE are presented. Figure 3 explains how to assess TR and lead position.

Chest radiography

Chest radiography is used to determine the dislocation of the leads and their position in relation to each other, as well as to check their continuity. Pang et al. in their study divided the RV region with 3 horizontal lines to assess the lead position in the posteroanterior (PA) view using fluoroscopy. Those regions are referred to as right ventricular outflow tract (RVOT) (superior region), middle RV and inferior region, with the last one divided into RV inflow and RV apex.[33] Yu et al. assessed the position of the leads on chest radiography in the PA view and found that nonapical RV lead position was not as often associated with TV interference as apical RV lead position.[34]

Techniques for implantation of CIEDs

The implantation of CIEDs is performed under fluoroscopic guidance. The tip of the lead is directed toward the interventricular septum (IVS) or RV apex. The 3 most commonly used techniques are[35]:

1. "Prolapsing" – the lead is put into the right atrium (RA) with an inner stylet 5–10 cm from the lead tip and pushed to create a limp loop hanging on the tricuspid annulus. Next, the lead is slightly retracted, and the stylet is slipped forward for prolapsing the lead to the RV. The tip of the lead may hook on the TV or trabeculation, with the remaining part of the lead hanging within the RV. In such situation, the stylet is pushed forward to free the lead tip, or the stylet and lead are slightly withdrawn together while still maintaining the loop shape of both of them in order to enable prolapsing of the lead to the RV in the next step. This operation should be performed very

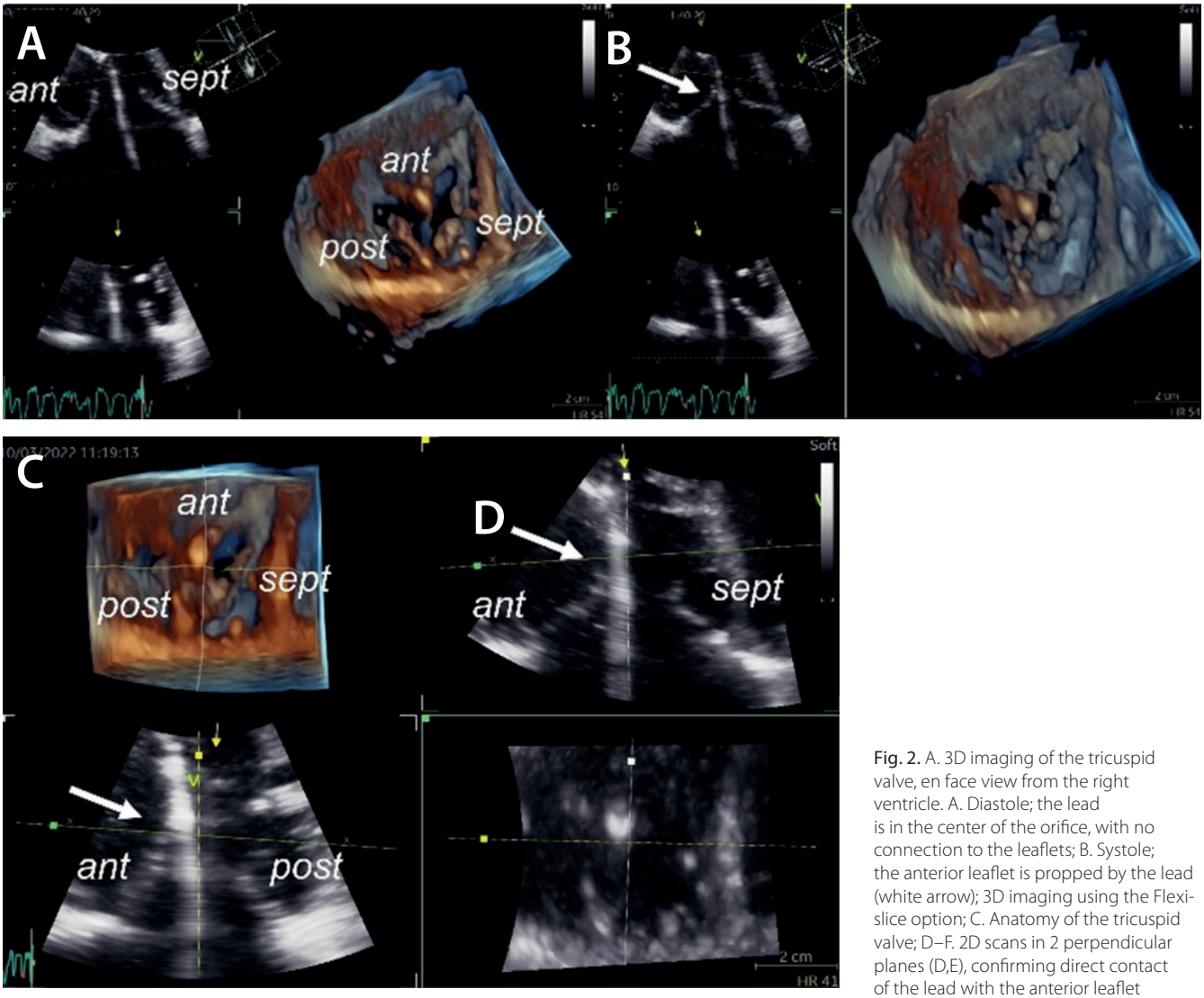

Fig. 2. A. 3D imaging of the tricuspid valve, en face view from the right ventricle. A. Diastole; the lead is in the center of the orifice, with no connection to the leaflets; B. Systole; the anterior leaflet is propped by the lead (white arrow); 3D imaging using the Flexi-slice option; C. Anatomy of the tricuspid valve; D–F. 2D scans in 2 perpendicular planes (D,E), confirming direct contact of the lead with the anterior leaflet

cautiously because overly vigorous movements can result in entanglement of the lead within the chordae tendineae or injury of the TV and subvalvular apparatus;

2. "Direct crossing" – used especially for leads with a larger diameter. The inner stylet is reshaped to gain less curvature, and the lead is advanced directly from the RA into the RV. As the adjustment of the tip location (by moving and rotating the lead body or ejecting and retracting the inner stylet) is performed after crossing the TV, it may lead to damage of the TV and subvalvular apparatus;

3. "Dropping down" – this technique is also used for thicker leads. The lead is inserted into the RV with the inner stylet maintained in the large curvature, which results in reaching the RV outflow tract by the lead tip directly after crossing the TV. If the tip is too high within the RVOT or the desired position is the IVS, the lead has to be withdrawn. If the final location of the lead tip is the RV apex, the stylet is replaced with a straight one and slipped forward through the lead with simultaneous withdrawal of the lead, which makes the tip "drop down" to the apex.

Mechanisms of lead-related tricuspid valve dysfunction and regurgitation

Many studies have indicated that CIEDs directly affect the function and structure of the TV.[3,6,8,16,20,22,30–32,36] The mechanisms of LRTR include the following:

1. Leaflet perforation[6,29,32];
2. Damage of the subvalvular apparatus; chordae tendineae entanglement or rupture and papillary muscle perforation[32,36,37];
3. Leaflet impingement by a lead or limitation of the movement of a leaflet caused by adherence to the lead[3,9,20,29,31,32,36];
4. Fibrosis involving the lead and the TV[32];
5. Device-related infections.

The technique of CIED implantation has an impact on the occurrence of LRTR. According to Trankle et al. and Chang et al., "prolapsing" reduces the risk of perforation and laceration of the TV leaflets and subvalvular

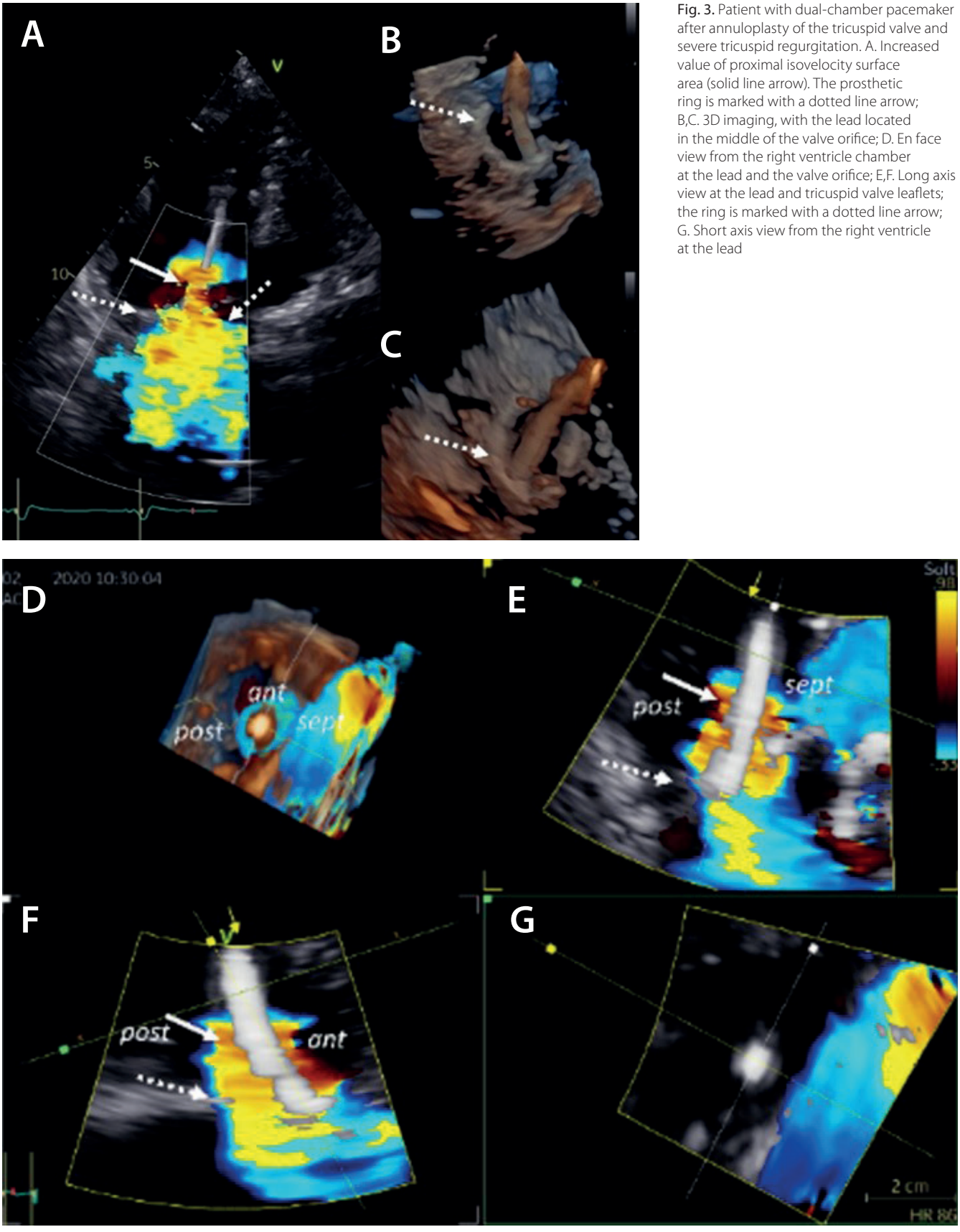

Fig. 3. Patient with dual-chamber pacemaker after annuloplasty of the tricuspid valve and severe tricuspid regurgitation. A. Increased value of proximal isovelocity surface area (solid line arrow). The prosthetic ring is marked with a dotted line arrow; B,C. 3D imaging, with the lead located in the middle of the valve orifice; D. En face view from the right ventricle chamber at the lead and the valve orifice; E,F. Long axis view at the lead and tricuspid valve leaflets; the ring is marked with a dotted line arrow; G. Short axis view from the right ventricle at the lead

apparatus. Rajappan, in turn, indicated that "direct crossing" results in a lower risk of damage to the TV apparatus and the development of TR.[30,35,38] The position of the lead tip is very important. Septal pacing is regarded as more physiological and less associated with the progression of HF.35 Alizadeh et al. found an association between

apical pacing and tricuspid and mitral valve regurgitation.[19] On the other hand, in a study by Cheng et al., a significant increase in proximal isovelocity surface area (PISA) radius was shown in patients with the lead tip in the IVS compared to those with the lead tip in the RV apex or the outflow tract.[31] Placement of the lead within the IVS more often affects the chordae tendineae and causes adherence of the lead to the septal or posterior leaflet, reducing their mobility and inducing TR. In some studies, placing the lead within the RVOT was the position least often related to TR worsening.[39,40]

Positioning the lead in the "center of the valve"[3,16,31,41] or in the anterior, especially posteroseptal commissure, results in only minimal restriction of the leaflet motion.[3,16,20,31,42] In that context, a promising method of visualization of the TV and optimization of the implantation of a cardiac device's leads is 3D TTE.[6,16] Gmeiner et al. carried out lead implantation guided with TEE, which resulted in no worsening of TR compared to the control group ($p < 0.001$); this makes TEE an alternative for procedures performed only with fluoroscopy.[43]

Polewczyk et al. indicated that loops formed due to an excess of atrial or ventricular leads falling into the TV orifice may cause irritation of the leaflets and their malcoaptation.[36] Many authors have suggested that it is not merely the presence of the lead between the leaflets or within the TV commissures but irritation or pinning of the leaflets by the lead that is the main cause of TV dysfunction.[3,8,16,20,31] The septal and posterior leaflets are reported to be the most often affected.[3,8,9,16,20,31,32,37] Henry et al. presented interesting cases of explanted hearts obtained from patients with CIEDs who had undergone cardiac transplantation. In their study, 40% of the cases showed device/lead interference with the TV. Three cases showed interference with the leaflets (the septal leaflet in 2 cases and the anterior leaflet in 1 case), while another 3 cases – interference with the subtricuspid apparatus.[37]

According to some authors, the type of CIED has an impact on the development of LRTR. In comparison to pacemakers, there is a greater risk of LRTR with implantable cardioverters/defibrillators (ICDs) and cardiac resynchronization therapy defibrillators (CRT-Ds)[10,14] due to the larger diameter of the defibrillation leads. Other authors did not find significant differences in TR occurrence between patients with pacemakers and those with other types of CIEDs.[4,5,13,16,21–23]

Many authors have investigated the relationship between TR development and the mode[22] or percentage of cardiac pacing.[5,11,23,24,38] In a study by Fanari et al., RV pacing dependence had no effect on the worsening of TR severity (39% for a high frequency of RV pacing compared to 45% for a low frequency of RV pacing; $p = 0.52$). This was similar for the mode of stimulation (43.2% in single-chamber ventricular pacing compared to 45.6% in dual-chamber stimulation; $p = 0.5$).[22]

Predictors of TV regurgitation development in patients with CIEDs

Apart from the technical aspects described above, other factors can affect the function of the TV after CIED implantation. The dilatation of the RV plays a significant role in the development of TV regurgitation after CIED implantation,[3,6,9,12,23,30] as does impairment of the systolic or diastolic function of the left ventricle (LV).[30,38,44] Other risk factors mentioned in reports include increased RA area,[20,23] elevated right atrial pressure[8,9] and elevated pulmonary systolic pressure.[6,23,45] Lee et al. found that an elevated tricuspid pressure gradient before implantation is an independent predictor of progressive LRTR.[11] Even more risk factors for the development of TR have been reported, including age over 73 years (73 years or older according to Lee et al., 76 years according to Delling et al., and 80 years or older according to Riesenhuber et al.[6,12,23]), female sex,[12] atrial fibrillation,[12,23] elevated heart rate,[6] history of mitral valve dysfunction,[6,12,23] and left atrial area enlargement.[12,23]

On the other hand, a study by Höke et al. found no significant differences in clinical, echocardiographic or device-related factors (age, sex, atrial fibrillation, history of mitral valve dysfunction, left atrial volume, type of device, or percentage of pacing) between patients with TR compared to those with no significant TR after CIED implantation.[21]

Consequences of TR

Many studies have shown that at least moderate TR is connected with increased mortality and more frequent and prolonged hospitalizations due to HF.[4–6,9,11,13,21,26,46,47] The same observations apply to LRTR, as patients with tricuspid dysfunction develop RV failure more often than patients without TR.[4,6,9,10,21,44] A summary of studies concerning the consequences of LRTR is presented in Table 2.

Management of LRTR

In recent years, some new treatment strategies for severe TR and LRTR have emerged. According to the current European Society of Cardiology (ESC) guidelines for the management of valvular heart disease,[48] surgery for secondary TR should be performed simultaneously with a left-sided valvular operation if the regurgitation is severe or considered when there is dilatation of the TV annulus in mild or moderate TR. Repair or replacement of the TV, independent of a left-sided operation, should also be considered in cases of severe TR that is causing symptoms or when there is dilatation of the RV in the absence of LV or RV failure and PH; this would promote reverse remodeling of the RV and improve its functional state.[49]

Table 2. All-cause mortality and hospitalizations for HF due to LRTR

Author	Year	n	Period of observation [years]	Endpoints	p-value
Höke et al.[21]	2014	239	1–1.5	all-cause mortality	0.047
				HF	0.019
Al-Bawardy et al.[5]	2015	1596	6	all-cause mortality	<0.05
Arabi et al.[4]	2015	41	1	RV HF	<0.001
Delling et al.[6]	2016	634	1	all-cause mortality	0.027
Seo et al.[9]	2020	373	1	hospitalization for HF	0.003
Papageorgiou et al.[10]	2020	304	11.6	all-cause mortality	0.01
Lee et al.[11]	2021	1075	4.9	hospitalization for HF	<0.001 in univariable analysis
				all-cause mortality	0.503
Kanawati et al.[13]	2021	165	2.5	hospitalization for HF	0.03
				all-cause mortality	0.09
Riesenhuber et al.[12]	2021	562	10	all-cause mortality	0.028

HF – heart failure; LRTR – lead-related tricuspid regurgitation; RV – right ventricle.

Among asymptomatic patients, an intervention should be considered when RV dilatation or declining function is observed.[48] Severe RV/LV dysfunction or severe PH are considered contraindications for surgery. When a CIED lead is present, the technique should be adapted to the patient's condition and the surgeon's experience.[50] In Table 3, we summarize the statements and recommendations from various guidelines on TR caused by CIEDs. In selecting the most appropriate and safe treatment strategy for patients with LRTR, the procedures described in the following sections should be considered.

Surgical annuloplasty or valve replacement

According to the 2021 ESC Guidelines for the management of valvular heart disease, when there is no severe TV degeneration or annulus dilatation, the repair of the valve is preferred over the replacement. Wong et al. demonstrated that repair in such cases is associated with better short- and long-term outcomes in both isolated and concomitant TV surgery compared with valve replacement,[51] because long-term anticoagulation is not needed and it allows for avoiding thrombosis and degeneration of the bioprosthetic valve.[52] In the case of LRTR requiring only an annuloplasty with an open ring (without additional procedures within the leaflets), the lead may be left in the previous position. However, to avoid further complications associated with the interference between the lead and parts of the repaired valve, the lead should be placed between the artificial and native ring or removed and implanted into the coronary sinus, epicardially[50] or in another alternative position.[53] There are also the options of implantation of a leadless pacemaker or subcutaneous ICD (sICD). In some cases, classic implantation of a new lead within the valve is acceptable. The same rules apply to biological prostheses. When a mechanical prosthesis must be implanted, the lead cannot be placed within the valve. The implantation of a leadless pacemaker is technically impossible secondary to valve surgery, but is acceptable during the procedure.[54]

Transcatheter tricuspid valve intervention

Transcatheter tricuspid valve intervention (TTVI) is an alternative treatment for patients with secondary severe symptomatic TR and contraindications for surgical intervention. It is not a standard procedure, as it still remains under evaluation and is only conducted in highly specialized centers.[48]

Leaflet approximation

Lurz et al. conducted transcatheter tricuspid valve repairs using the TriClip device and demonstrated that this device was safe and effective in patients with moderate or severe TR. They achieved a significant reversal of RV remodeling in terms of size and function. However, their study did not provide information about patients with CIEDs.[55]

Direct annuloplasty

Nickenig et al. presented results on the Cardioband system in patients with symptomatic and moderate to severe functional TR. This system was previously successfully applied in functional mitral regurgitation. The researchers reported a significant reduction in TR through a decrease in annular dimension, as well as a decrease in HF symptoms and improvement in quality of life. In their study, the presence of a CIED was one of the exclusion criteria.[56]

Table 3. Statements and recommendations of guidelines on the occurrence of TR caused by CIEDs

Guidelines	Statements	Recommendations
2021 ESC/EACTS Guidelines for the management of valvular heart disease[48]	"Cardiac implantable electronic device-lead implantation leads to progressive tricuspid regurgitation in 20–30% of the patients and predicts its progression over time." "The Heart Team with expertise in the treatment of tricuspid valve disease evaluates anatomical eligibility for transcatheter therapy including jet location, coaptation gap, leaflet tethering, potential interference with pacing lead."	"Annuloplasty with prosthetic rings is preferable to valve replacement which should only be considered when the tricuspid valve leaflets are tethered and the annulus severely dilated." "In presence of a cardiac implantable electronic device lead, the technique used should be adapted to the patient's condition and the surgeon's experience."
2021 ESC Guidelines on cardiac pacing and cardiac resynchronization therapy[50]	"CIED leads may interfere with tricuspid valve function intraoperatively by causing damage to the tricuspid valve leaflets or the subvalvular apparatus, or chronically after operation or lead extraction." "Moderate to severe tricuspid regurgitation is generally associated with excess mortality and occurs at a significantly higher rate in CIED patients." "Most studies attribute a greater harm with ICD leads and in the presence of multiple RV leads." "Methods for percutaneous tricuspid repair have recently gained major attention, but evidence in favour of such interventions in the context of lead-related tricuspid regurgitation is still limited."	"Indications for surgical valve repair or replacement in the context of CIED-induced tricuspid regurgitation follow current recommendations based on the presence of symptoms, severity of tricuspid regurgitation, and RV function."
ASE Guidelines and Standards. Recommendations for Noninvasive Evaluation of Native Valvular Regurgitation (2017)[73]	"Pacemaker leads can result in significant TR by interfering with closure of the TV but rarely cause a flail leaflet or a perforation of the leaflet."	none
2020 ACC/AHA Guideline for the Management of Patients With Valvular Heart Disease[74]	"In patients with severe symptomatic primary TR from either device leads or endomyocardial biopsy, TR develops rapidly, and surgery can be done before the onset of RV dysfunction." "Risk factors for persistence or progression of TR include tricuspid annulus dilatation, degree of RV dysfunction or remodelling, leaflet tethering height, pulmonary artery hypertension, AF, and intra annular RV pacemaker or implantable cardioverter-defibrillator leads." "There is renewed interest in earlier surgery for patients with severe isolated TR before the onset of severe RV dysfunction or end-organ damage." "There is growing interest in the development of catheter-based therapies for these patients with severe isolated TR."	"In patients with symptomatic severe primary TR, reduction or elimination of the regurgitant volume load by tricuspid valve surgery can alleviate systemic venous and hepatic congestion and decrease reliance on diuretics." "Patients with severe congestive hepatopathy may also benefit from surgery to prevent irreversible cirrhosis of the liver." "Tricuspid valve repair is preferable to replacement, but replacement may be necessary if there is marked dilatation of the annulus or intrinsic disease of the tricuspid leaflets."
Recommendations for the echocardiographic assessment of native valvular regurgitation: An executive summary from the European Association of Cardiovascular Imaging (2013)[75]	"TEE is of interest for the diagnosis of endocarditis, venous catheters and pacemakers lead infection, and visualization of traumatic rupture of the tricuspid valve."	none

AF – atrial fibrillation; CIED – cardiac implantable electronic device; ICD – implantable cardioverter defibrillator; RV – right ventricle; TEE – transesophageal echocardiography; TR – tricuspid regurgitation; TV – tricuspid valve; ESC – European Society of Cardiology; EACTS – European Association for Cardio-Thoracic Surgery; ASE – American Society of Echocardiography; ACC – American College of Cardiology; AHA – American Heart Association.

Valve replacement

Fam et al. reported that the use of the EVOQUE transcatheter tricuspid valve replacement (TTVR) system brought about significant clinical improvement among patients with severe TR and right-sided HF, with high effectiveness (92% of cases) and safety. In their study, 36% of patients had transvenous pacemakers, and TTVR was successful in all cases.[57]

Taramasso et al. compared medical treatment with TTVI and demonstrated that all-cause mortality and rehospitalizations at 1 year were lower among patients who received the intervention.[58] In the TriValve registry for the years 2015–2018, TTVI was conducted in 121 patients with CIEDs. Only 7 patients had isolated device-induced TR, in which the only mechanism for TR was interference between the lead and the TV components. The interventions in that registry included the following: edge-to-edge technique (MitraClip Abbott Vascular, Santa Clara, USA – 106 patients; 87%); PASCAL (Edwards Lifesciences, Irvine, USA – 1 patient; 0.8%), implantation of a coaptation device (FORMA; Edwards Lifesciences – 2 patients; 1.6%), annuloplasty (Cardioband; Edwards Lifesciences – 1 patient; 0.8%), transcatheter valve

implantation (CAVI; Edwards Lifesciences – 10 patients; 8%); and NaviGate (NaviGate Cardiac Structures, Lake Forest, USA – 1 patient; 0.8%). Procedural success was achieved in 78.6% of patients, with an in-hospital mortality rate of 3.7%. Symptomatic improvement was observed at 30 days in 65.0% of patients, and survival at 12 months was 73.6 ±5.0%.[59]

Transvenous lead extraction

A study by Polewczyk et al. showed an improvement in TV function after transvenous lead extraction (TLE) in 35.29% of patients. In that group, the survival rate after 5 years of follow-up was higher compared to patients without improvement after the procedure. One of the most common mechanisms of LRTR in their study was propping the leaflet upward or clamping it down using the lead (85.71%).[60] According to Glikson et al., TLE entails a high risk of TV avulsion with worsening TR.[50] Nazmul et al. reported some cases of TLE in patients with moderate or severe TR and stated that extraction did not result in a significant reduction in TR, particularly in patients with a dilated TV annulus.[61]

After TLE, the lead (including the defibrillation one) may be implanted in an alternative site within the RV or into the coronary sinus for LV pacing. Stimulation of the His bundle seems to be the most physiological technique and the least related to secondary TR evoked by pacing.[62] In some cases, epicardial leads are used.[53] Other solutions may involve a subcutaneous ICD or a leadless pacemaker; however, in the latter case, TR secondary to RV dysfunction associated with nonphysiological distribution of electric pulses or procedural complications can also occur.[63–65] A leadless pacemaker may also be used in combination with an sICD, or a lead can be implanted into the coronary sinus for biventricular pacing.[66] Some reports have shown good results with pericardial, extrapleural and substernal placement of defibrillator coils, separately or in conjunction with epicardial pacing leads, although their implantation requires employing surgical techniques.[53]

Medical therapy

According to the 2021 European Society of Cardiology/European Association for Cardio-Thoracic Surgery (ESC/EACTS) Guidelines for the management of valvular heart disease, conservative therapy of TR should be reserved only for patients with severe RV failure or PH. The treatment is based on diuretics and aldosterone antagonists as well as pharmacotherapy of arterial PH. In patients with atrial fibrillation, maintenance of sinus rhythm may be helpful in the prevention of dilatation of the TV annulus and progression of TR.[48]

The treatment strategy for severe TR is still not standardized, and more research is needed to establish such a strategy. Guidelines suggest that only severe RV/LV dysfunction or severe PH are considered contraindications for surgery.[48] Stocker et al. demonstrated that severe PH, defined as mean pulmonary arterial pressure (mPAP) >30 mm Hg and transpulmonary gradient >17 mm Hg, were associated with higher mortality after TTVR.[67] Taramasso et al. reported that systolic pulmonary artery pressure is the strongest parameter related to death and that patients must be treated with optimal medical therapy before the intervention, which allows for the best RV/pulmonary artery coupling in the peri-interventional period with the lowest possible RV afterload.[59] In addition, Kavsur et al. demonstrated in their study that pulmonary capillary wedge pressure (PCWP) is a predictive outcome parameter in TTVR patients. They reported that patients with a PCWP ≤ 16 mm Hg had a favorable outcome with lower mortality and morbidity. Furthermore, they suggested that right heart catheterization should be considered a routine diagnostic tool in the process of TTVR evaluation.[68] Regarding operative risk, Färber et al. suggested that the Model for End-Stage Liver Disease (MELD) score might be a tool to identify high-risk individuals among patients qualified for isolated TV surgery. In their study, classic surgical risk stratification scores of the Society of Thoracic Surgeons or the European System for Cardiac Operative Risk Evaluation (EuroSCORE II) failed to predict perioperative mortality in patients with severe liver dysfunction.[69] A proposal for the management of LRTR is presented in Fig. 4.

Discussion

Tricuspid regurgitation has stood on the sidelines of medical interest for many years. In comparison to atrial and mitral valvular diseases, fewer reports about indications and treatment results have been published. In the case of LRTR, the quantity of information is even more limited.

Some authors have stated that there is a lack of evidence regarding the progression of TR after CIED implantation.[17,70,71] The development of TR in patients with HF and ICDs or CRT-Ds is also questionable. Valve dysfunction may be either a result of mechanical impairment caused by a CIED[4,21] or an effect of the progression of LV and RV failure. Some studies have shown a reduction in TR severity through an improvement in hemodynamic function and an increase in cardiac output after normalization of heart rhythm following CIED implantation.[12,70]

It also remains unclear whether the progression of RV remodeling is a cause or a consequence of significant TR. Höke et al. did not identify any significant differences in RV function (TAPSE and RV fractional- area change) in patients with severe LRTR compared to patients without severe LRTR, but larger RV diastolic area (17 ±6 mm^2 compared to 16 ±5 mm^2; $p = 0.009$), right atrial enlargement (39 ±10 mm compared to 36 ±8 mm; $p < 0.001$) and higher pulmonary arterial pressure (41 ±15 mm Hg compared

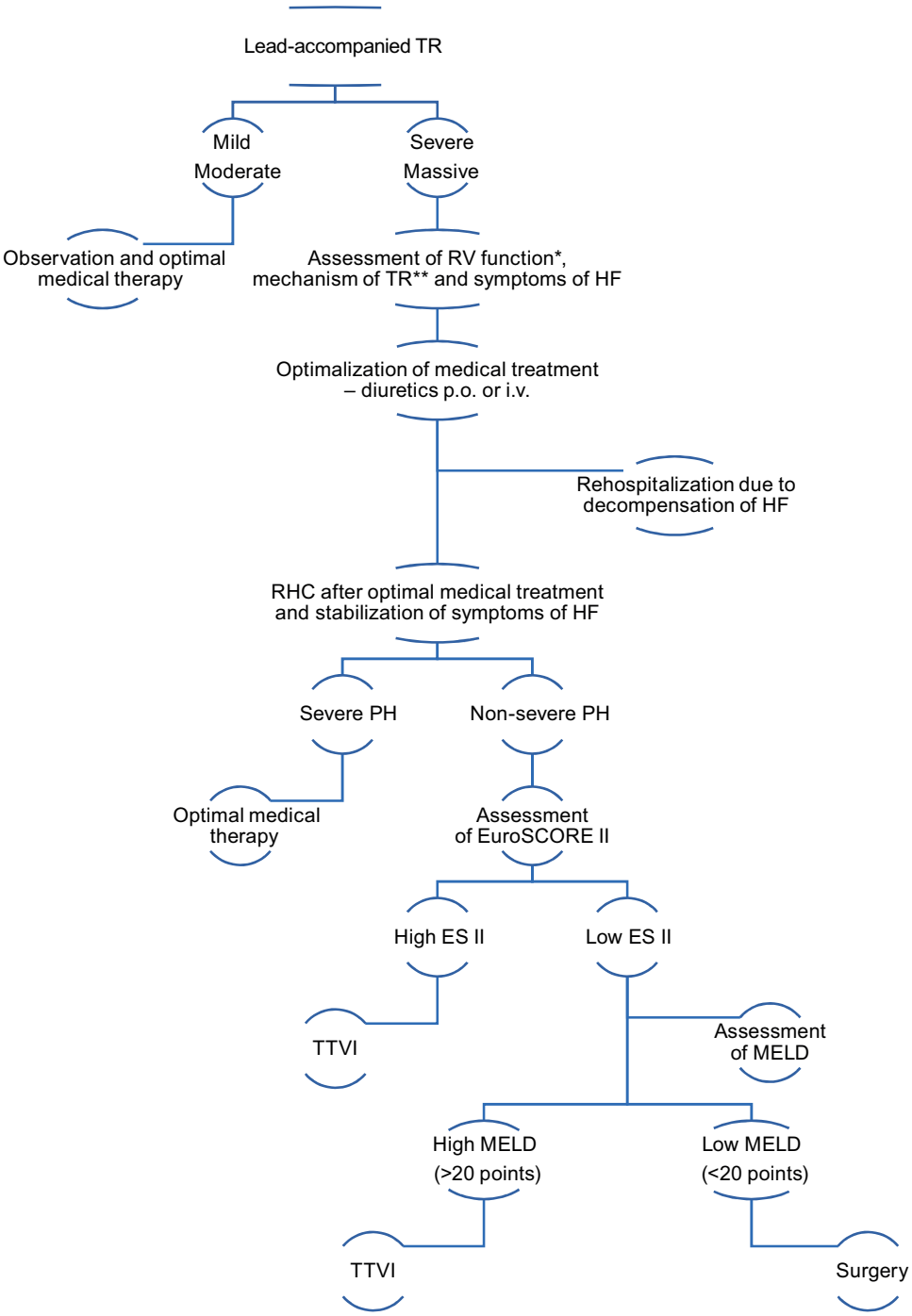

Fig. 4. Management of lead-related tricuspid regurgitation (TR)

ES II – European System for Cardiac Operative Risk Evaluation (EuroSCORE II); HF – heart failure; MELD – Model for End-Stage Liver Disease; PH – pulmonary hypertension; RHC – right heart catheterization; RV – right ventricle; TTVI – transcatheter tricuspid valve intervention; * RV function: tricuspid annular plane systolic excursion (TAPSE) [mm], FAC – fractional area change [%], RV strain [%]; ** mechanisms of TR: perforation, impingement, entanglement, fibrosis.

to 33 ±10 mm Hg; $p < 0.001$) were found in patients with significant LRTR after 1–1.5 years of follow-up. However, longer follow-up periods might lead to a decrease in RV function.[21] In addition, no significant differences were observed regarding changes in LV volume or systolic and diastolic function in patients with severe LRTR compare to patients without significant LRTR. Furthermore, the difference in the severity of mitral regurgitation was also similar in patients with compared to without significant LRTR.[21]

Seo et al. stated that LRTR might induce HF resistant to pharmacotherapy, as a result of continuous progression of TR with TV and RV remodeling.[9] Nakajima et al. demonstrated lower effectiveness of HF pharmacotherapy in patients with LRTR in comparison with TR not related to CIEDs, which suggests a real impact of CIED on TR development.[8] Papageorgiou et al. observed that new post-implant moderate or severe TR (hazard ratio (HR): 3.14 (95% confidence interval (95% CI): 1.29–7.63); $p = 0.01$) and RV impairment (HR: 2.82 (95% CI: 1.33–5.98); $p = 0.01$) were independent predictors of mortality.[10]

Confirmation of direct contact between the lead and tricuspid valvular apparatus may be challenging. The authors of this article propose that the term "LRTR" should be used in a situation when there is clear evidence of an interaction between the lead and a valve leaflet. The evidence of such an interaction are, among others:

1. Leaflet perforation[6,32];
2. Entanglement of the lead within the chordae tendineae[32,36];
3. Leaflet impingement by a lead or leaflet movement limitation caused by adherence to a lead[3,9,20,31,32,36];
4. Fibrosis involving the lead and TV apparatus.[32]

There are many questions related to the treatment of LRTR. Lead extraction is a risky procedure, especially if it is performed many years after primary implantation. In our opinion, the decision for TLE should be considered if the interaction between the electrode and the leaflet is confirmed using 3D TTE, because the presence of an electrode in the center of the tricuspid orifice without any evidence of contact with the leaflet suggests that a change in the position of the electrode after TLE will not affect the valve's function. The use of a leadless pacemaker or LV pacing with a lead inserted into the coronary sinus also does not prevent TR from developing.[72] The occurrence of LRTR should be avoided using available methods, such as His bundle pacing for the most physiological way of stimulation, or the use of TEE[43] to implant the ventricular lead intercommissurally or in the middle-of-the-annulus position[16] to minimize the interaction of the lead with TV components.

Although different authors differ in their opinions concerning the relevance of LRTR, it seems to be an important clinical problem, with an impact on right and left ventricular function and prognosis. Taking into account the low interest in TV diseases, further studies are required to formulate guidelines concerning LRTR prevention and to choose the time and method of treatment aimed at reducing the risk of complications and achieving optimal results.

Conclusions

Lead-related tricuspid regurgitation is common in patients with CIEDs. Risk factors for its development and impact on RV and LV function are difficult to predict and require further systematic clinical registries and observational studies. The preferred treatment methods for patients with LRTR have not yet been determined.

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Application and efficacy of transcutaneous electrical acupoint stimulation (TEAS) in clinical practice: A systematic review

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Abstract

Transcutaneous electrical acupoint stimulation (TEAS) is an emerging therapeutic approach that combines the effects of transcutaneous electrical nerve stimulation (TENS) with acupuncture point stimulation. Due to its noninvasive nature, it possesses relative advantages over traditional acupuncture and needle-based electrostimulation. Despite the large number of randomized clinical trials (RCTs) describing the effectiveness of TEAS in different applications, its role and mechanism are still not fully understood. The aim of this study was to systematically compare and summarize the latest studies examining a variety of TEAS applications in clinical practice. Databases, including Medline (PubMed), Cochrane Library and Google Scholar were searched without any time restrictions (as of March 2021). The analysis was performed according to the Cochrane Collaboration criteria. Out of 637 studies, only 22 RCTs were selected. Nine studies evaluated the impact of TEAS on nausea and vomiting (NV), showing beneficial effects compared to standard therapy. Eight RCTs examined the effectiveness of TEAS in pain management, reporting pain alleviation described using the visual analog scale (VAS) and lowering of total opioid doses. Improvement of postoperative recovery, in vitro fertilization and pregnancy outcomes, as well as display of cardioprotective properties were found to positively correlate with TEAS. As a noninvasive modality with advantages over classical acupuncture and needle-based electrostimulation, TEAS may be a valuable tool in clinical practice, particularly for pain and NV management. However, considering the methodological quality of the RCTs, rigorous large-scale clinical trials are required to evaluate the clinical utility of this method.

Key words: systematic review, randomized controlled trials, TEAS, clinical practice, transcutaneous electrical acupoint stimulation

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Introduction

Over the last 1,000 years, acupuncture has remained a crucial component of Traditional Chinese Medicine.[1] Originally, it was performed by applying specialized acupuncture needles into specific loci on the body, known as meridians. However, during the last several decades, the technique of acupuncture has evolved, and currently, a modified form called transcutaneous electrical acupoint stimulation (TEAS) has garnered widespread interest. The TEAS is a contemporary therapeutic method combining stimulation of acupuncture points and transcutaneous electrical nerve stimulation (TENS). Consequently, the essence of TEAS is the stimulation of sensory nerve endings along acupuncture meridians through the application of low-voltage electrical current. The modality exerts its biological effects through various molecular pathways, including the release of endogenous opioids.[2,3] Hence, the results of recent studies reveal a broad spectrum of both therapeutic and prophylactic applications of TEAS in clinical practice. The TEAS remains a widely used tool with a variety of uses, including recovery of gastrointestinal function, reduction of the occurrence of postoperative cognitive dysfunction, nausea and vomiting relief, enhancement of immune function, protection of organ function, acceleration of postoperative recovery, reduction of systolic blood pressure in patients with hypertension, and enhancement of patients' degree of overall comfort.[4] This technique has also been proven helpful in alleviating inflammation and cancer-related pain.[5] Moreover, TEAS has found a clinical application in treating various kinds of reproductive disorders, such as polycystic ovary syndrome (PCOS), pain induced by oocyte retrieval, diminished ovarian reserve, embryo transfer, and oligospermia.[6] Its promising potential is predominantly reported in pain prevention, obstetrics and anesthesiology.[7–9] Moreover, further analysis of the literature shows that TEAS may be utilized as a standalone therapy or as an adjuvant to established therapies, which could positively impact the quality of patient care. Furthermore, unlike traditional acupuncture, TEAS utilizes electrodes that are fixed to the skin through a patch, making the method noninvasive and, above all, safe for the patient.[2,10] To date, its usage has been reported in both children[11] and adolescents.[12,13] Furthermore, the therapy has the advantage of being easy to use and requires minimal training for physicians, technicians and patients. As a noninvasive acupuncture strategy, TEAS can even be performed at home by patients themselves without a prescription. Both patients and caregivers could participate in the clinical application of TEAS after a short but accurate training. However, despite many clinical applications, there are some limitations of TEAS. Firstly, there is a significant amount of conflicting data in the literature regarding the effectiveness of the technique.[14,15] Moreover, limited access to electrostimulators for patients has also been reported to be a challenge.[2] In addition, the lack of TEAS-trained health personnel is a significant issue that needs to be addressed.[16] Growing research on the application of TEAS in evidence-based practice emphasizes the need to systematize the current evidence. Therefore, we conducted a systematic review of randomized clinical trials (RCTs) evaluating the possible applications of TEAS in clinical practice and reviewed the benefits in the reported indications.

Objectives

The aim of this systematic review was to compare and summarize the findings of TEAS trials across various medical indications and provide an evaluation of its effectiveness in comparison to placebo or standard therapy.

Materials and methods

Databases

Medline (PubMed), Cochrane Library and Google Scholar databases were searched without any publication date restrictions (publication date: from inception to March 2021). Articles published after March 2021 were not included in this review due to either their publication date being beyond the custom time range or not meeting custom criteria. Although the search strategy had to be altered to meet limitations of each database, the following phrases were searched invariably: "transcutaneous electrical acupoint stimulation" OR "TEAS". The aforementioned terms were searched in titles and abstracts in order to initially qualify the research for a systematic review. Subsequently, the papers were thoroughly analyzed and assessed according to the Cochrane Collaboration criteria[17] and Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).

Criteria of inclusion and exclusion

For the purpose of evaluating the efficacy and efficiency of TEAS, we included only RCTs in which the TEAS treatment group received either standalone TEAS treatment or where it was an adjuvant to standard therapy and was compared to a) a control group (not receiving TEAS and/or receiving standard therapy) or b) a placebo group (receiving sham TEAS and/or standard therapy). Moreover, the participants had to be randomly assigned to the appropriate group, irrespective of age, sex, ethnicity, place of residence, underlying disease, or comorbidities. It is worth noting that not all of the analyzed RCTs contained information about each of these patient characteristics. Furthermore, this systematic review only considered research assessing transdermal nerve stimulation in points typical for acupuncture, regardless of length, voltage or acupoint chosen for TEAS therapy.

The following studies were excluded from the analysis: 1) not stating inclusion or exclusion criteria; 2) evaluating the effectiveness of traditional acupuncture, electroacupuncture (with the use of needles) or transdermal nerve stimulation in loci other than acupoints; 3) assessing the efficacy and effectiveness of TEAS without comparison to a control group; 4) applying any – even minimal – voltage in either the control or placebo group; 5) evaluating TEAS in places other than acupuncture points; 6) conducted on a pediatric population; and 7) conducted on animals.

Data extraction and assessment of the risk of bias

One author (RK) conducted an initial analysis followed by a detailed review of studies. The 2nd author (SA) verified the research included in the systematic review. Any discrepancies were critically discussed and revised. The primary analysis of RCTs comprised of assessing their 1) validity; 2) type; 3) means of randomization; 4) description of all medical procedures among participants (e.g., type of anesthesia, form and dosage of medication); 5) means of arranging experimental control and placebo groups; and 6) tools (e.g., scales, computer programs) used to extract and present the results. The next phase of analysis involved a detailed assessment of the test group, in particular 1) sample size; 2) acupuncture points applied with precise names and locations; 3) voltage (mA), frequency (Hz) of applied current and length of therapy (s or ms); and 4) exact moment of initiation of treatment (e.g., 30 min before the general anesthesia). At the same time, in the placebo/control group assessment, the following was examined: 1) sample size; 2) location of sham electrodes; and 3) type of intervention.

Results

A total of 22 RCTs (Fig. 1, Table 1) that met the inclusion criteria were selected from 637 studies. Almost all of the studies were conducted in Asia (China in particular). The included RCTs focused mainly on assessing the efficacy and effectiveness of TEAS in preventing nausea and vomiting (NV) and pain alleviation. However, some studies examined other indications for TEAS therapy. The RCTs were significantly diversified in terms of the sample size, although most of them had a small experimental TEAS and control/placebo groups. Among the analyzed RCTs, only a few were based on a sample of female participants only.

In terms of randomization, from a total of 22 analyzed studies, only a few were appropriately conducted. For instance, most of the excluded studies included participants randomized using incorrect baseline information, multiple randomizations being performed for the same participant or a lack of double-blinding, which might have impacted the observed outcomes.

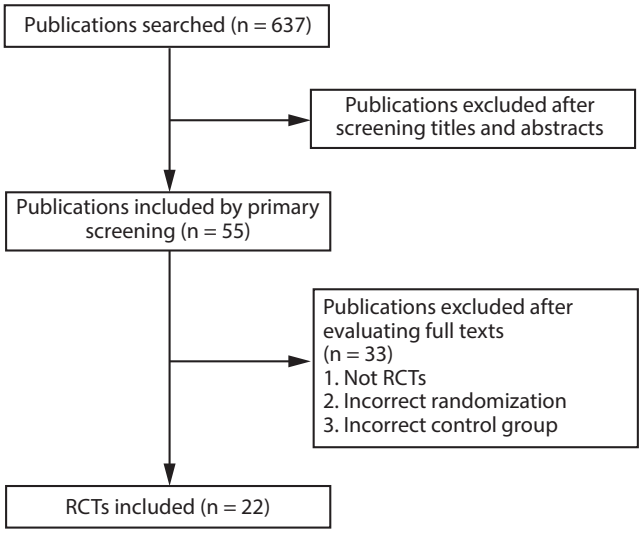

Fig. 1. Analysis of studies

RCTs – randomized controlled trials.

Effectiveness of TEAS in preventing NV

Yang et al. compared the effectiveness of TEAS with dexamethasone (Acu group), dexamethasone with tropisetron (Trp group), and dexamethasone only (Dxm group) in counteracting postoperative nausea and vomiting (PONV) 24 h after surgery. Compared to standalone glucocorticosteroid therapy, the Acu group presented a significantly lower risk of PONV ($p = 0.048$, odds ratio (OR) = 0.389, 95% confidence interval (95% CI): 0.170–0.891). Simultaneously, the authors did not show any difference in the reduction of PONV between the Acu group and Trp group ($p = 0.857$). The same study showed no difference between all of the groups in demand for anti-emetics (Acu group: 10%, 95% CI: 1–19%; Trp group: 8%, 95% CI: 0–15%; Dxm group: 14%, 95% CI: 4–24%).[18] Similarly, Liu et al. have shown the benefits of TEAS therapy in reducing PONV incidence 24 h after operation. Authors have noted 14/48 (30%) PONV in the TEAS group and 31/48 (65%) in a standard therapy group. The attained relation was statistically significant ($p < 0.05$). At the same time, the TEAS group received fewer anti-emetics than the control group ($p < 0.05$).[19] Zheng et al. also observed a reduction of PONV in the first 24 postoperative hours among patients receiving TEAS (5/30, 17%) in comparison to the control group (14/30, 47%). The correlation was statistically significant ($p < 0.05$).[20] Xu et al. reported a possible efficiency in PONV prevention on the day after surgery, comparing the TEAS group to the non-TEAS group (nausea: 33% compared to 58%, $p = 0.008$; vomiting: 22% compared to 41%, $p = 0.025$).[21] On the other hand, Ho et al. demonstrated that the occurrence of PONV was only slightly lower in the TEAS group compared to traditional therapy (9/25 (36%) compared to 11/25 (44%), respectively), and the relationship was not statistically significant.[22]

Table 1. Characteristics of clinical trials

Study	Publication year	Country	Study type	Acupuncture points	Characteristics of the study participants						Clinical indication
					intervention group				control/placebo/other group		
					n*	mA	Hz	duration	n*	description	
Xie et al.[23]	2017	China	single-blind RCT	Hegu (LI4) Neiguan (P6) Zusanli (ST36)	72 (active–acupuncture)	7–15	4	twice daily for 30 min for 6 days	70 (placebo–acupuncture)	electrodes applied in the same place as in the intervention group but with no stimulation	TEAS combined with palonosetron on chemotherapy-induced NV
Yang et al.[18]	2015	China	prospective double-blind RCT	Neiguan (P6)	50 (acupoint stimulation+ dexamethasone)	6–20	2	N/D	53 (control – tropisetron+ dexamethasone)	tropisetron+ dexamethasone	PONV in gynecological patients undergoing laparoscopic surgery
									50 (dexamethasone)	dexamethasone	
Xu et al.[21]	2012	China	prospective blind and randomized study	Neiguan (P6)	65 (TEAS)	2	2–100	30 min before the induction of anesthesia and lasted up to 24 h postoperatively	65 (sham TEAS/control)	electrodes applied in the same place as in the intervention group but with no stimulation	TEAS on PONV in patients after infratentorial craniotomy
Liu et al.[19]	2008	China	prospective double-blind randomized study	Neiguan (P6)	48 (treated)	0.5–4	2–100	at least 30 min but no longer than 60 min before the induction of anesthesia and continued until the end of surgery	48 (control)	electrodes applied in the same place as in the intervention group but with no stimulation	preventing PONV after laparoscopic cholecystectomy
Zheng et al.[20]	2008	China	RCT	Hegu (LI4) Neiguan (PC6)	30 (TEAS)	8–10	2/100	30 min before analgesia induction to 24 h after operation	30 (sham TEAS/control)	electrodes applied in the same place as in the intervention group but with no stimulation	TEAS on NV induced by patient-controlled intravenous analgesia with tramadol
Ho et al.[22]	1990	Taiwan	randomized clinical trial	Neiguan (P6)	25 (TENS)	ind.**	30	15 min	25 (control)	no treatment	postoperative vomiting
					25 (EAP)	ind.**	3	15 min	25 (drugs)	prochlorperazine 5 mg intravenously	
Zhan and Tian[30]	2020	China	single-blind RCT	Zusanli (ST36) Neiguan (PC6)	30 (TAP+TEAS)	ind.**	2–100	before leaving PACU and at 24 h after surgery	30 (control)	usual care group	TEAS to transverse abdominis plane block for postoperative analgesia in abdominal surgery
									30 (TAP)	transverse abdominis plane block	
Tu et al.[24]	2019	China	RCT	Shenyu (BL23) Yinlingquan (SP9)	60 (TEAS)	ind.** 5–10 for upper limbs 10–30 for lower limbs and trunk	2/100	before and 4 h, 8 h and 12 h postoperatively; re-implementing TEAS 3 times on the target acupoints (at 7 AM, 11 AM and 15 PM) on the next 2 days postoperatively	60 (control)	participants treated with tramadol hydrochloride 100 mg tablets for postoperative analgesia, twice daily (8 AM, 8PM), and electrodes applied in the same place as in the intervention group but with no stimulation	TEAS and postoperative analgesia after ureteroscopic lithotripsy
AminiSaman et al.[25]	2018	Iran	double-blind randomized clinical trial	Hegu (LI4) Zusanli (ST36)	25 (acu-TENS)	5–10	1–10	4 times in 24 h for 30 min	25 (placebo)	electrodes applied in the same place as in the intervention group but with no stimulation	TENS at the acupuncture points to relieve pain of patients under mechanical ventilation

Table 1. Characteristics of clinical trials – cont.

Study	Publication year	Country	Study type	Acupuncture points	Characteristics of the study participants						Clinical indication
					intervention group				control/placebo/other group		
					n*	mA	Hz	duration	n*	description	
Yeh et al.[26]	2018	Taiwan	randomized clinical trial	Chengshan (BL57) Erbai (EX–UE2)	39 (TEAS)	0.06–2.30	2/100	20 min; a total of 4 times: 1st time at the 4th hour after surgery, 2nd time at the 6th hour after surgery, 3rd time at 7 AM on the day after surgery, and 4th time at 11 AM on the day after surgery	41 (control)	electrodes applied in the same place as in the intervention group but with no stimulation	TEAS on post-hemorrhoidectomy-associated pain, anxiety and heart rate variability
Liu et al.[28]	2015	China	randomized blind controlled trial	Hegu (LI4) Waiguan (TE5) Jinmen (BL63) Taichong (LR3) Zusanli (ST36) Qiuxu (GB 40) Fengchi (GB20) Tianzhu (BL10) Cuanzhu (BL2) Yuyao (EX–HN4)	46 (TEAS)	4.89 ±2.15 6.79 ±3.51 7.04 ±3.35 5.61 ±2.13 ind.**	2/100	30 min before anesthesia induction, maintained throughout the operation and terminated at the end of surgery	46 (sham TEAS)	electrodes applied in the same place as in the intervention group but with no stimulation	intraoperative and postoperative anesthetic and analgesic effect of multipoint TEAS combined with sufentanil anesthesia in patients undergoing supratentorial craniotomy
Chen et al.[29]	2015	China	prospective triple-blind randomized placebo-controlled trial	Jiaji (EX–B2)	114 (TEAS)	optimal intensity was set to initiate visible slight twitching of the surrounding muscle	2/100	30 min	115 (sham TEAS)	electrodes applied in the same place as in the intervention group but with no stimulation	TEAS reduces abdominal pain after colonoscopy
Lan et al.[31]	2012	China UK	RCT	Neiguan (P6) Hegu (LI4) Zusanli (ST36) Fengshi (GB31)	30 (acu-TENS)	9–20	2/100	30 min before incision, and at several time points 2 days after surgery: at 2 h, 4 h, 20 h, and 44 h	30 (sham TEAS/control)	electrodes applied in the same place as in the intervention group but with no stimulation	TENS on acupoints reduces fentanyl requirement for postoperative pain relief after total hip arthroplasty in elderly patients
Wang et al.[27]	1997	USA	prospective single-blind randomized sham-controlled study	Hegu (LI4)	25 (low-TEAS)	4–5	2 and 100	36 min	26 (PCA only)	intravenous PCA alone with hydromorphone	effect of the intensity of TEAS on the postoperative analgesic requirement
					25 (high-TEAS)	9–12	2 and 100	29 min	25 (sham TEAS)	PCA with hydromorphone+sham TEAS (no electrical stimulation, but with functional indicator lights on)	
Yu et al.[36]	2020	China	prospective double-blind randomized placebo-controlled trial	Baihui (GV20) Yingtang (EX–HN3) Zusanli (ST36) Neiguan (PC6)	30 (TEAS)	12–15 ind.**	2/100	30 min before anesthesia	30 (control)	electrodes applied in the same place as in the intervention group but with no stimulation	TEAS on the quality of early recovery in patients undergoing gynecological laparoscopic surgery

Table 1. Characteristics of clinical trials – cont.

Study	Publication year	Country	Study type	Acupuncture points	Characteristics of the study participants						Clinical indication
					intervention group				control/placebo/other group		
					n*	mA	Hz	duration	n*	description	
Chi et al.[35]	2019	China	prospective randomized sham-controlled trial	Zusanli (ST36) Sanyinjiao (SP6) Neiguan (PC6) Quchi (LI11)	26 (TEAS)	ind.**	2/10	30 min before the epidural anesthesia and on postoperative day 1 and 2	26 (sham TEAS)	electrodes applied in the same place as in the intervention group but with no stimulation	TEAS for improving postoperative recovery, reducing stress and inflammatory responses in elderly patients undergoing knee surgery
Li et al.[32]	2019	China	single-center prospective exploratory randomized therapeutic clinical trial	Neiguan (PC6) Ximen (PC4)	61 (TEAS)	ind.**	4/20	30 min before anesthesia induction until the end of surgery	61 (control)	electrodes applied in the same place as in the intervention group but with no stimulation	cardioprotective effect of TEAS on perioperative elderly patients with coronary heart disease
Bai et al.[9]	2018	China	prospective randomized controlled clinical trial	Hegu (LI4) Neiguan (PC6) Lieque (LU7) Chize (LU5) Futu (LI18) Renying (ST9)	37 (TEAS)	6–15	2/10	30 min before anesthesia to 5 min before the end of surgery	38 (control)	electrodes applied in the same place as in the intervention group but with no stimulation	TEAS on the stress response during extubation after general anesthesia in elderly patients undergoing elective supratentorial craniotomy
Qu et al.[37]	2017	China	prospective randomized controlled study	Xuehai (SP10) Diji (SP8) Taichong (LR3) Zusanli (ST36) Zigong (EX–CA1) Guanyuan (RN4) Neiguan (PC6) Zhongwan (RN12)	108 (TEAS–2)	N/A	2	30 min respectively at 24 h before TVOR and 2 h before ET	109 (control)	routine procedure of IVF treatment and no TEAS was applied	TEAS improves the outcomes of IVF
					111 (TEAS–100)		100				
					114 (TEAS–2/100)		2/100				
Zheng et al.[38]	2015	China	randomized controlled study	Guanyuan (RN4) Zhongji (RN3) Sanyinjiao (SP6) Zigong (EX–CA1) Tianshu (ST25) Shenyu (BL23) Yaoyangguan (DU3) Mingmen (DU4)	56 (TEAS)	2–25	2	lasted for 30 min and was given once a day; after 3 courses, the treatment continued during the ovulation cycle until the day of egg retrieval	56 (FHP) 54 (AEC) 60 (control)	regardless of the displayed electric current, its output current is stable at 5 mA oral tablets of estradiol valerate and dydrogesterone	TEAS on ovarian reserve of patients with diminished ovarian reserve in in vitro fertilization and embryo transfer cycles
Jones and Ngai[33]	2014	Australia China	randomized placebo-controlled cross-over design	Neiguan (PC6)	10 (acu-TENS)	ind.**	2	45 min before exercise	10 (placebo)	similar to the acu-TENS protocol but without electrical output from the TENS unit	acu-TENS lowers blood lactate levels and enhances heart rate recovery after exercise
Ngai and Jones[34]	2013	China	double-blind randomized controlled cross-over study	Feishu (BL13)	9 (acu-TENS)	the highest tolerable intensity but short of pain	2	45 min	9 (placebo-TENS)	electrodes applied in the same place as in the intervention group but with no stimulation	skin impedance and heart rate variability with application of acu-TENS to BL13

RCT – randomized controlled trial; TEAS – transcutaneous electrical acupoint stimulation; TENS – transcutaneous electrical nerve stimulation; TAP – transverse abdominal plane block; IVF – in vitro fertilization; EAP – electroacupuncture; N/A – not applicable; N/D – no data; PONV – postoperative nausea and vomiting; PACU – post-anaesthesia care unit; TVOR – transvaginal oocyte retrieval; ET – embryo transfer; AEC – artificial endometrial cycle treatment; FHP – comforting false Han's placebo; PCA – patient-controlled analgesia; n* – the number of participants included in the appropriate group; ind.** – individually – the highest tolerable level that caused no discomfort for the participants.

Table 2. Characteristics of randomized clinical trials (RCTs) assessing the effectiveness of transcutaneous electrical acupoint stimulation (TEAS) in nausea and vomiting (NV) prophylaxis

Study	Publication year	Indication	Results
Yang et al.[18]	2015	PONV in gynecological patients undergoing laparoscopic surgery	NV ora within 24 h after operation: a) 28% (95% CI: 15–41%) of patients in the Acu group (dexamethasone+TEAS) b) 26% (95% CI: 14–39%) of patients in the Trp group (dexamtethasone+tropisetron) c) 50% (95% CI: 36–64%) of patients in the Dxm group (dexamethasone alone)
Liu et al.[19]	2008	NV after laparoscopic cholecystectomy	in the study group compared with the research group: a) incidence of NV was significantly lower ($p < 0.05$) b) doses of anti-emetics were significantly lower ($p < 0.05$) c) occurrence rate of severe nausea was significantly lower ($p < 0.01$)
Zheng et al.[20]	2008	NV induced by intravenous analgesia with tramadol	The incidence and scores of NV in group A (with TEAS) was significantly lower than in group B (without TEAS) ($p < 0.05$ or $p < 0.01$).
Xu et al.[21]	2012	postoperative NV after infratentorial craniotomy	in the study group compared with the research group: a) incidence of NV was significantly lower ($p < 0.05$) b) doses of anti-emetics were significantly lower ($p < 0.05$) c) occurrence rate of severe nausea was significantly lower ($p < 0.01$)
Ho et al.[22]	1990	emesis after laparoscopy	The incidence of postoperative emesis in the TENS group was slightly lower compared with the control group (9/25 compared to 11/25).
Xie et al.[23]	2017	chemotherapy-induced NV	The differences in occurrence rates and severities of NV after TACE were not significant ($p > 0.05$).

TENS – transcutaneous electrical nerve stimulation; PONV – postoperative nausea and vomiting; 95% CI – 95% confidence interval; TACE – transarterial chemoembolization.

Xie et al. evaluated whether a combination of TEAS and palonosetron might alleviate nausea and/or vomiting in patients undergoing intravenous chemotherapy for late-stage liver cancer. Results showed a reduction of NV incidence and nausea intensity, although the correlation was not statistically significant ($p > 0.05$).[23] In conclusion, the majority of the studies showed a positive role for TEAS in the reduction of NV after surgery. However, not all RCTs clearly present significant differences between TEAS and non-TEAS groups in this regard. Table 2 presents a summary of RCTs assessing the effectiveness of TEAS in NV prophylaxis.

Effectiveness of TEAS in pain alleviation

Tu et al. demonstrated that in comparison to the control group, patients receiving TEAS were characterized by lesser pain, as evaluated using the visual analog scale (VAS) in the 4th ($p = 0.01$), 12th ($p = 0.03$) and 24th ($p < 0.01$) hour after surgery. The same study showed that patients in the TEAS group received fewer analgesics during the first 48 h post-operation compared to the control group (127.14 ±28.46 compared to 415.27 ±86.37, $p < 0.01$).[24] AminiSaman et al. investigated the relationship between TEAS and pain reduction after mechanical lung ventilation. Compared to the control group, the TEAS group was less prone to pain ($p < 0.05$) and, simultaneously, received fewer analog-sedatives in the postoperative period.[25] Similar results were obtained by Yeh et al.[26] and Wang et al.[27] On the other hand, Liu et al. evaluated whether the use of TEAS could affect the course of anesthesia and analgesia in patients undergoing supratentorial craniotomy. The results showed that in comparison to the control group, the TEAS group received fewer anesthetics during surgery (sufentanil: 95.6 ±21.76 μg compared to 117.7 ±37.95 μg, $p < 0.05$; propofol: 216.3 ±67.72 mg compared to 234.1 ±71.30 mg). Moreover, the researchers reported a lower pain rate (described using VAS) in the TEAS group on the 1st day after surgery ($p < 0.001$). Nevertheless, the same study showed that VAS rates were higher in the 2nd and 3rd days following surgery in the TEAS group than in the control group.[28] Chen et al. assessed pain levels after the application of TEAS in patients undergoing colonoscopy. Compared to the control group, the TEAS group showed a statistically significant reduction of pain ($p = 0.007$) after the procedure – in the TEAS group, only 13/114 (11.4%) showed symptoms of pain compared to 29/115 (25.2%) in the control group.[29] Furthermore, TEAS, in combination with other therapeutic methods, might have an impact on patient outcomes. Zhan and Tian evaluated whether combining TEAS with transversus abdominis plane block (TAP) impacts postoperative pain alleviation. The study showed that the TAP and TEAS combination significantly reduced pain 24 h and 48 h after an abdominal operation ($p = 0.01$, $p < 0.0001$; $p = 0.004$) compared to the control group. Moreover, the same study demonstrated that in the TAP and TEAS groups, pain levels of VAS were lower than in the standalone TAP group ($p = 0.03$).[30] Lan et al. showed that patients in the TEAS group required lower doses of fentanyl 24 h and 48 h after surgery compared to the control group (respectively: 360 ±117 μg compared to 572 ±132 μg, $p < 0.001$;

Table 3. Characteristics of randomized clinical trials (RCTs) in pain indication

Study	Publication year	Indication	Results
Tu et al.[24]	2019	postoperative analgesia after ureteroscopic lithotripsy	In the study group, the VAS scores were significantly lower when compared with the control group at 4th h (3.68 ±0.68 compared to 4.79 ±0.82, p = 0.01), 12th h (2.64 ±0.72 compared to 3.92 ±0.88, p = 0.03), and 24th h (2.21 ±0.88 compared to 3.38 ±0.74, p < 0.01). In the study group, the total analgesic consumption was significantly lower when compared with the control group (127.14 ±28.46 compared to 415.27 ±86.37, p < 0.01) within 48 h postoperatively. In the study group, the incidence rates of vertigo (6.7% compared to 18.3%, p < 0.01), nausea and vomiting (11.7% compared to 21.7%, p < 0.01), and constipation (10.0% compared to 20.0%, p = 0.03) were lower when compared with the control group.
AminiSaman et al.[25]	2018	pain under mechanical ventilation	Level of pain was lower in the study group compared with the sham group (p < 0.05); the amount of analgesic and sedation drugs used was less significant in the treated group compared with the sham group (p = 0.01, p = 0.04).
Yeh et al.[26]	2018	post-hemorrhoidectomy pain	Level of pain measure using VAS score in the study group was lower compared with the control group and trend differences (time-by-group interactions) were significant at time 2 (p = 0.004), time 3 (p < 0.001) and time 4 (p < 0.001).
Wang et al.[27]	1997	postoperative analgesic required	In the study group, the amount of analgesic medication used was lower compared with the control group (by 65% for high-TEAS, by 34% for low-TEAS, by 23% for control group).
Liu et al.[28]	2015	anesthetic and analgesic effect during and after supratentorial craniotomy	In the study group, the amount of sufentanil and propofol used were lower compared with the control group (sufentanil: 95.6 ±21.76 μg compared to 117.7 ±37.95 μg, p < 0.05; propofol: 216.3 ±67.72 mg compared to 234.1 ±71.30 mg); level of pain measured using VAS score was lower in the study group on day 1 after surgery compared with the control group (p < 0.001), but on days 2 and 3 the level of pain was lower in the control group.
Chen et al.[29]	2015	pain after colonoscopy	In the study group, the level of pain was lower compared with the control group (p = 0.007).
Zhan and Tian[30]	2020	TEAS and TAP for postoperative analgesia in abdominal surgery	The combination of TEAS and TAP significantly reduced postoperative pain at 24 h and 48 h after surgery (p = 0.01, p < 0.0001; p = 0.004) compared with the control group.
Lan et al.[31]	2012	postoperative pain after total hip arthroplasty	In the study group, the amount of fentanyl used was lower compared with the control group at 24 h and 48 h after surgery (360 ±117 μg compared to 572 ±132 μg, p < 0.001; 712 ±184 μg compared to 1022 ±197 μg, p < 0.00); in both groups, no differences in the level of pain were observed.

TEAS – transcutaneous electrical acupoint stimulation; TENS – transcutaneous electrical nerve stimulation; TAP – transverse abdominal plane block; VAS – visual analog scale.

712 ±184 μg compared to 1022 ±197 μg, p < 0.001). No significant difference in pain level has been observed in both groups.[31] Taken together, all of the aforementioned studies demonstrated the positive role of TEAS in alleviating pain after surgery and reducing drug doses. Table 3 presents a summary of RCTs assessing the effectiveness of TEAS in pain alleviation.

Effectiveness of TEAS in other indications

Cardioprotection

Li et al. studied the possible protective impact of TEAS on cardiac muscle in patients with diagnosed coronary artery disease qualified for spinal surgery. The cardioprotective effect was evaluated by measuring the level of markers in blood serum, including troponin (high-sensitive troponin T (hs-cTnT)), C-reactive protein (CRP) and creatine kinase (CK), on the 1st, 3rd and 5th day after the surgery. In both the TEAS treatment and control groups, the levels of all of the markers were elevated (p < 0.05). However, compared to the control group, the concentration of hs-cTnT in the TEAS group on days 1 and 3 was significantly lower (p < 0.05). There was no difference in CRP and CK levels between the groups. Moreover, heart rate (HR) on days 1, 3 and 5 after surgery compared to the day before the surgery was higher in both groups, wherein the increase was lower in the TEAS treatment group.[32] Jones and Ngai assessed whether TEAS might accelerate normalization of HR in patients who were subject to physical effort. Compared to the control group, the TEAS group demonstrated a shorter time to HR normalization after physical exercise (9.98 ±4.54 min, p = 0.047, 95% CI: 0.23–19.72).[33] A study by Ngai and Jones confirms the cardioprotective properties of TEAS, as they showed a reduction of low frequency to high frequency (LF/HF) ratio by 0.37 ±0.01 (p = 0.012) compared to the control group.[34] In conclusion, all these studies show the positive impact of TEAS in cardioprotection. Table 4 presents a summary of RCTs assessing the effectiveness of TEAS in other indications.

Table 4. Characteristics of randomized clinical trials (RCTs) in other medical indications

Study	Publication year	Indication	Results
Bai et al.[9]	2018	improving postoperative recovery	In the control group, the QoR-40 scores were lower compared with the research group (164.3 ±13.7 compared to 176.9 ±11.1, p < 0.01). In the study group, cases of cough and PONV were lower compared with the control group (respectively: 10 (27.0) compared to 19 (50.0), p = 0.04; 13 (35.1) compared to 24 (63.2), p = 0.02).
Li et al.[32]	2019	cardioprotection in coronary heart disease after spinal surgery	In the study group compared with the control group, the concentration of hs-cTnT was lower on 1st and 3rd day after surgery (p < 0.05). In both groups, HR was higher after surgery than before, but in the study group the increase was lower.
Jones and Ngai[33]	2014	heart rate normalization after exercise	In the study group compared with the control group, time to normalization of HR after exercise was shorter (9.98 ±4.54 min, p = 0.047, 95% CI: 0.23–19.72)
Ngai and Jones[34]	2013	heart rate variability after acu-TENS	In the study group, LF/HF ratio was lower by 0.37 ±0.01 (p = 0.012) compared with the control group.
Chi et al.[35]	2019	improving postoperative recovery	In the control group, the QoR-40 scores were lower compared with the study group (160.1 ±5.5 compared to 170.9 ±5.0 respectively, p < 0.05).
Yu et al.[36]	2020	improving postoperative recovery	In the control group, the QoR-40 and MMSE scores were lower compared with the study group (respectively: QoR-40: 1st day – 166.07 ±8.44 compared to 175.33 ±9.66, 2nd day – 187.73 ±5.47 compared to 191.40 ±5.74; MMSE: 1st day – 24.60 ±2.35 compared to 26.10 ±2.78, 2nd day – 26.53 ±2.94 compared to 27.83 ±2.73); in treated group, the incidence of PONV was lower compared with the control group (23.3% compared to 56.7%).
Qu et al.[37]	2017	improving IVF outcomes	In the 2/100 Hz TEAS group, the clinical pregnancy, implantation and live birth rates were higher compared with other groups (p < 0.05).
Zheng et al.[38]	2015	improving pregnancy outcomes	In the TEAS group compared with the control group, clinical pregnancy rate was higher (42.31% (n = 22/52) compared to 21.57% (n = 11/51), p < 0.05).

TEAS – transcutaneous electrical acupoint stimulation; hs-cTnT – high-sensitive troponin T; TENS – transcutaneous electrical nerve stimulation; PONV – postoperative nausea and vomiting; MMSE – Mini-Mental State Examination; IVF – in vitro fertilization; QoR-40 – Quality of Recovery-40 questionnaire; HR – heart rate; 95% CI – 95% confidence interval; LF/HF – low frequency to high frequency.

Postoperative general condition

Chi et al. assessed whether TEAS treatment could affect the general condition of a patient after knee surgery. For this, they used the Quality of Recovery-40 questionnaire (QoR-40). Compared to the TEAS treatment group, the control group scored lower in QoR-40 on the 1st day after surgery, and the relationship was statistically significant (170.9 ±5.0 compared to 160.1 ±5.5, p < 0.05, respectively). The same study showed that CRP in the TEAS group was notably lower (p < 0.05).[35] Bai et al. achieved comparable results, showing a statistically significant relationship in QoR-40 score in the TEAS group compared to the control group (176.9 ±11.1 compared to 164.3 ±13.7, p < 0.01). Moreover, they showed that patients receiving TEAS less often suffered from cough (10 (27.0) compared to 19 (50.0), p = 0.04) and NV (13 (35.1) compared to 24 (63.2), p = 0.02).[9]

Yu et al. analyzed the influence of TEAS on the perioperative period in patients after laparoscopy due to gynecological diseases. The study included not only QoR-40 but also Mini-Mental State Examination (MMSE). Compared to the control group, scores in the TEAS group were significantly higher in both scales on day 1 and 2 after surgery (QR-40: 1st day – 166.07 ±8.44 compared to 175.33 ±9.66, 2nd day – 187.73 ±5.47 compared to 191.40 ±5.74; MMSE: 1st day – 24.60 ±2.35 compared to 26.10 ±2.78, 2nd day – 26.53 ±2.94 compared to 27.83 ±2.73). In addition, the incidence of PONV was considerably lower in the TEAS group (56.7% compared to 23.3%).[36]

Obstetrics

Qu et al. examined the implication of TEAS on in vitro fertilization (IVF). The authors identified 4 groups: controls and 3 TEAS groups with various frequencies: 2 Hz, 100 Hz and 2/100 Hz. Compared to all other groups, the number of pregnancies, successful implantations and live births in the 2/100 Hz group was significantly higher (p < 0.05).[37]

Zheng et al. showed that TEAS therapy in women with lowered ovarian reserve notably increased the number of pregnancies after IVF in comparison to the control group (respectively: 42.31% (n = 22/52) compared to 21.57% (n = 11/51), p < 0.05).[38]

Discussion

Our review is the first to identify and analyze TEAS trials in several medical indications, providing an evaluation

of TEAS effectiveness in comparison to placebo or standard therapy. Out of 22 chosen RCTs, 9 studies evaluated the effectiveness of TEAS in the treatment of NV, demonstrating superior clinical outcomes in TEAS-treated patients in comparison to standard therapy. Eight of those studies were focused strictly on PONV. Those RCTs bear promising results, as PONV, a direct consequence of total opioid dosage, occurs in up to 27% of surgical patients, contributing to prolonged hospital stays and resulting in higher hospitalization expenses.[39] Another 8 RCTs evaluating pain management found TEAS to be more effective in pain reduction. The studies showed lower VAS and/or total analgesics doses in the TEAS study group when compared with other study arms. Most of those studies evaluated the effectiveness of TEAS in surgical pain management exclusively. However, there was 1 notable study on the efficacy of TEAS in decreasing pain related to mechanical ventilation.[25] Such application of TEAS might be particularly valuable in the face of the coronavirus disease 2019 (COVID-19) global pandemic that is associated with a significant percentage of patients requiring prolonged mechanical ventilation depending on disease severity. Despite ample evidence on the effectiveness of TEAS on both PONV and pain control, there are some studies that show contrasting results. One RCT showed significantly worse outcomes of TEAS compared to control in pain alleviation at days 2 and 3.[28] However, this study primarily evaluated the impact of TEAS on anesthetics use, and the modality ended upon culmination of the surgery. Besides the influence of TEAS on pain management, it might have a broadly defined beneficial impact on patients recovering from surgical interventions. Three RCTs demonstrated that patients receiving TEAS scored higher in either QoR-40 or MMSE. Such findings seem to be crucial, as quality of recovery is directly associated with satisfaction and quality of life up to 3 years after surgery.[40] Another 3 RCTs display the cardioprotective properties of TEAS by either lowering the HR, reducing the HR normalization time or lowering the LF/HF ratio. Furthermore, the results of 2 RCTs showed improved pregnancy and IVF outcomes after TEAS therapy – a crucial feature given the rising infertility rates worldwide.[41]

Only one previous systematic review assessed the effectiveness of acupuncture and, as a separate subgroup, acupuncture-related methods in treating postoperative pain. Wu et al. included only 5 studies that compared TEAS with standard treatment. Similar to our results, they found that patients receiving TEAS suffered less pain on the 1st day after surgery, as evaluated by VAS ($p = 0.0020$). Also, consistent with our findings, they found that the TEAS group required a lower total opioid dose on the 1st postoperative day than the control group ($p < 0.001$).[42]

Although the results of these studies are encouraging, they have limitations, primarily with respect to methodology. The biggest concern is insufficient blinding of care providers. In all of the studies that included a placebo group, the care provider knew whether they applied a true or sham treatment modality. A considerable amount of properly executed RCTs are required to establish TEAS as a noninvasive alternative to standard therapy in the aforementioned indications and other ones. To do so, researchers need to adhere to appropriate methods of intervention. Sizeable cohorts including the study group, along with control and placebo arms, as well as proper randomization are necessary. Means of double-blinding should be developed, as informing the research staff might cause bias. For publication purposes, much like the Standards for Reporting Interventions in Clinical Trials of Acupuncture (STRICTA),[43] the Consolidated Standards of Reporting Trials (CONSORT) has been developed to avoid unnecessary mistakes and hindering otherwise useful studies from being recreated.[44]

Limitations

We recognize that our review has a number of limitations. Firstly, we only included trials published in English, possibly significantly reducing the database. Even though a substantial effort was put into extracting every available RCT on the subject, we cannot guarantee that the search was all-inclusive. The most significant factor limiting the quality and conclusiveness of this review is selective publishing. Since only 1 included RCT showed a negative outcome of TEAS, RCTs with negative results likely remain unpublished, misrepresenting the outcome of TEAS overall.

Conclusions

The results of this systematic review demonstrate TEAS as an emerging, noninvasive modality with distinct advantages over classical acupuncture and needle-based electrostimulation, regardless of its application (as a standalone TEAS therapy or as an adjuvant to standard therapy). Our study suggests this approach may be useful in clinical practice, particularly for pain and NV management. However, considering the methodological quality of most RCTs, rigorous large-scale clinical trials of TEAS are needed to evaluate the clinical utility of this technique.

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A comparison of different symptomatic reflux esophagitis treatments: A real-world study

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Abstract

Background. Proton pump inhibitors (PPIs) are currently the reference drugs for gastroesophageal reflux disease (GERD), but symptoms often recur after their withdrawal. Moreover, whether prokinetics or barrier drugs used alongside PPIs are more effective remains under debate.

Objectives. The aim of the study was to assess the efficacy of different therapeutic approaches to GERD treatment.

Materials and methods. We enrolled 211 grade A reflux esophagitis patients who consented to participate in this non-randomized, open-label trial. The study consisted of 6 sequentially administered medical treatments for GERD, lasting 2 months, with a 3-week washout period between each drug schedule:
Group A: PPI (esomeprazole 40 mg/day before breakfast);
Group B: mucosal protective drugs (a combination of hyaluronic acid, chondroitin sulfate and poloxamer 407, or a combination of hyaluronic acid, chondroitin sulfate and aluminum, 3 times daily after a meal);
Group C: prokinetics (levosulpiride 25 mg or domperidone 10 mg, 3 times daily before a meal);
Group D: barrier drug (alginate 3 times daily after a meal);
Group E: PPI (esomeprazole 40 mg/day before breakfast) and mucosal protective drugs (a combination of hyaluronic acid, chondroitin sulfate and poloxamer 407, or a combination of hyaluronic acid, chondroitin sulfate and aluminum, before sleep);
Group F: PPI (esomeprazole 40 mg/day before breakfast) and prokinetics (levosulpiride 25 mg or domperidone 10 mg before lunch and dinner).
Symptoms were evaluated using the visual analogue scale (VAS) and global symptomatic score (GSS), as follows: heartburn: 0–3; retrosternal chest pain: 0–3; regurgitation: 0–3.

Results. All but 2 treatments (groups C and D) significantly improved VAS and GSS, with group E showing the most significant GSS improvement. Group C had the highest number of dropouts due to treatment failure and reported more side effects.

Conclusions. Using PPIs and mucosal protective drugs resulted in significant symptom alleviation. However, the administration of prokinetics caused higher dropouts due to treatment failure.

Key words: GERD, PPIs, prokinetics, barrier drugs, protective mucosal devices

Background

Gastroesophageal reflux disease (GERD) is one of the most frequently diagnosed digestive disorders in primary care.[1–4] The prevalence of reflux disease varies depending on the geographical area.[5] The first line of medical treatment for GERD consists of proton pump inhibitors (PPIs).[6–9] However, other drugs are used in GERD therapy, including antiacids pro-kinetics and alginates.[10–15]

Recently, new solutions have been proposed for GERD therapy, such as hyaluronic acid, which promotes re-epithelization of the upper gastrointestinal (GI) mucosa, epithelial cell turnover and ulcer healing.[16–20] Also, chondroitin sulfate, a glycosaminoglycan secreted in the upper stomach, inhibits pepsin-induced gastric and duodenal mucosa damage.[21–24] Using these 2 compounds in addition to PPIs alleviates symptoms in non-erosive reflux disease (NERD) patients.[25] A melt-in-the-mouth tablet containing hyaluronic acid, chondroitin sulfate and aluminum (HYCHSA: 1100 mg) has been administered to subjects with poor or no response to alginates and/or PPIs and induced satisfactory symptom relief.[26] A possible hypothesis was suggested by an experimental model of esophageal mucosa.[27] A multi-center study on NERD patients using a hyaluronic acid–chondroitin sulfate-based bioadhesive formulation, including poloxamer 407, a hydrophilic non-ionic surfactant, showed symptom alleviation.[28]

One major criticism is the variability between the samples, the targets (esophagitis or NERD) and the drugs used. However, studies comparing different drugs in the same sample are lacking.

Objectives

The aim of the study was to assess the efficacy of 6 different therapeutic approaches to GERD using PPIs, pro-kinetics, alginate, and hyaluronic acid combined with chondroitin sulfate, in a unique homogeneous GERD sample (all patients diagnosed with esophagitis A according to the Los Angeles (LA) classification[29]), in which every patient acts as their own control.

Materials and methods

Study design

We enrolled a sample of consecutive patients suffering from typical GERD symptoms (heartburn, retrosternal chest pain and regurgitation) who were under the care of 37 general practitioners in a primary care setting in Northeast Italy. All patients consented to participate in this non-randomized, open-label study of 6 different medical treatments for GERD, lasting for 2 months. Patients were sequentially administered drugs, with a 3-week washout period between every schedule. The drug schedules were designed as follows:

Group A: PPI (esomeprazole 40 mg/day before breakfast);

Group B: mucosal protective drugs (a combination of hyaluronic acid, chondroitin sulfate and poloxamer 407, or a combination of hyaluronic acid, chondroitin sulfate and aluminum, 3 times daily after a meal);

Group C: prokinetics (levosulpiride 25 mg or domperidone 10 mg, 3 times daily before a meal);

Group D: barrier drug (alginate, 3 times daily after a meal);

Group E: PPI (esomeprazole 40 mg/day before breakfast) and mucosal protective drugs (a combination of hyaluronic acid, chondroitin sulfate and poloxamer 407, or a combination of hyaluronic acid, chondroitin sulfate and aluminum, before sleep);

Group F: PPI (esomeprazole 40 mg/day before breakfast) and prokinetics (levosulpiride 25 mg or domperidone 10 mg before lunch and dinner).

Symptoms were evaluated before and after each treatment using the visual analogue scale (VAS) and global symptomatic score (GSS), as follows: heartburn: 0–3; retrosternal chest pain: 0–3; regurgitation: 0–3.

The number of patient dropouts for each group was considered a secondary aim. Figure 1 presents the flowchart of the study. Figure 2 summarizes the details of VAS and GSS.

Statistical analyses

Changes from baseline for VAS and GSS were evaluated with the Wilcoxon test for paired data using median and 1st quartile–3rd quartile (Q1–Q3) intervals. The Mann–Whitney test was employed to compare the differences between drug schedules. A value of $p < 0.05$ was considered statistically significant. All statistical analyses were performed IBM SPSS v. 28.0 software (IBM Corp., Armonk, USA).

Results

A total of 211 patients (98 females and 113 males with a mean age of 51 years, ranging from 27 to 73 years) were enrolled in the study. They underwent upper GI endoscopy and were diagnosed with grade A esophagitis according to the LA classification. The distribution of patients between the 37 general practitioners was homogeneous, with around 6 individuals per physician, ranging from 3 to 9. Figure 2 summarizes the results obtained from assessing symptom scores using VAS and GSS after the 6 drug schedules.

All treatments resulted in significant alleviation in VAS and GSS (Fig. 2). Group E showed the most improvement in VAS ($p = 0.001$) and GSS ($p = 0.028$), though there were no statistically significant differences between schedules for either VAS or GSS ($p = 0.082$ for both). The dropouts and improvements (%) in the different groups are shown in Table 1.

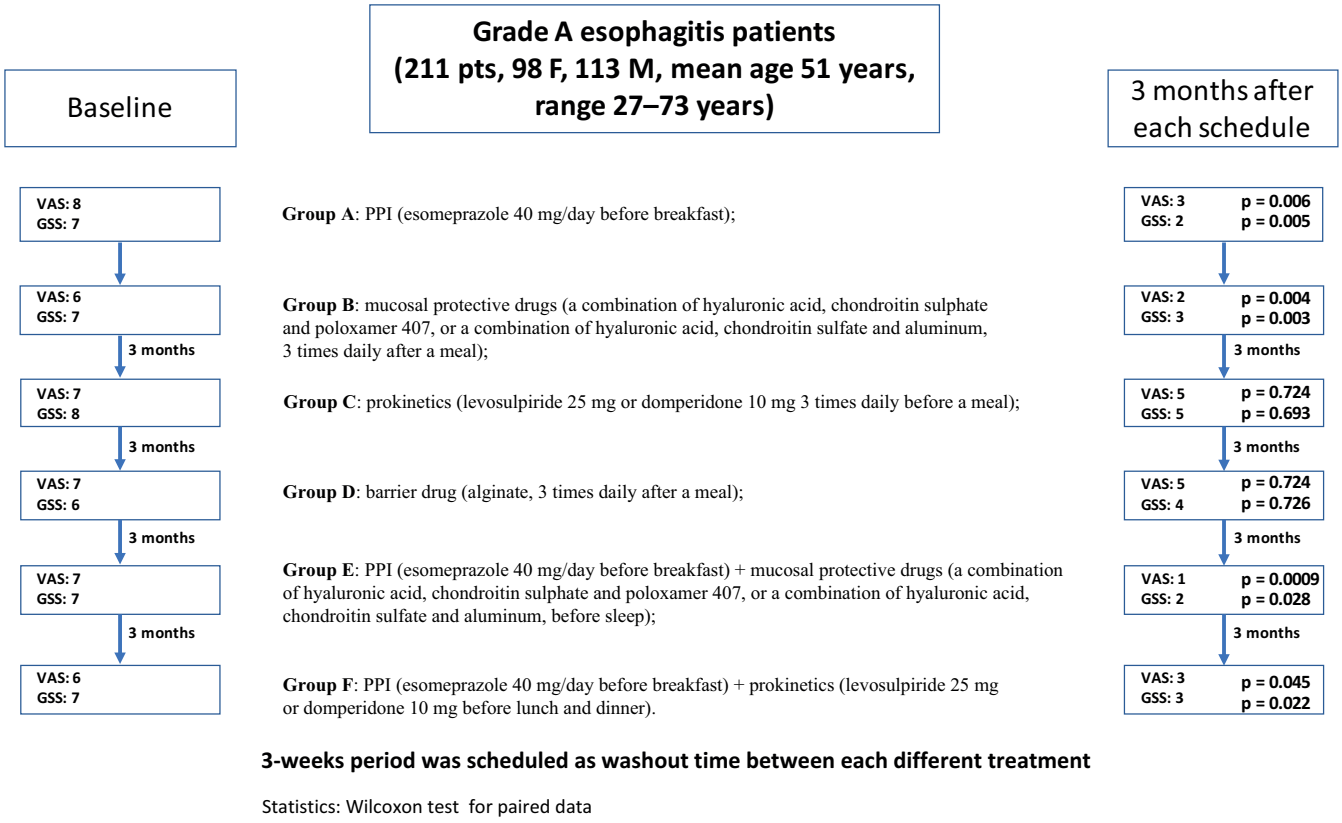

Fig. 1. Study flowchart

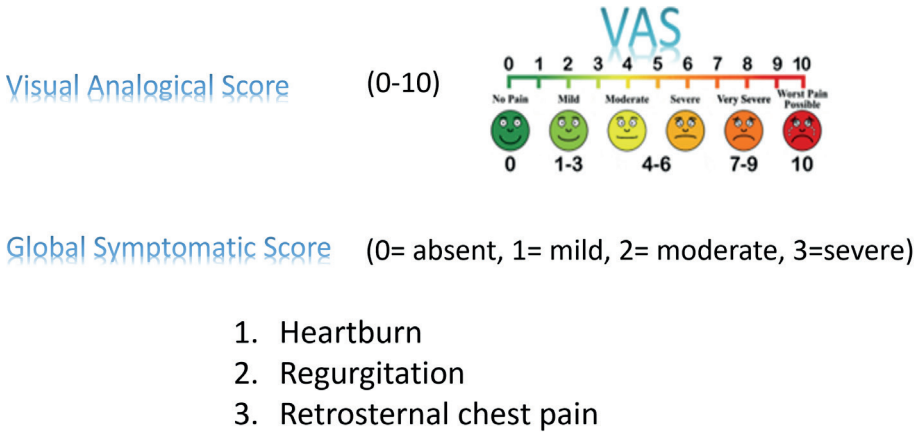

Fig. 2. Assessment of symptoms using the Visual Analogue Scale (VAS) and the Global Symptomatic Score (GSS)

PPI – proton pump inhibitor.

Table 1. Patient population, dropouts and improvements of symptoms according the systemic grouping

Group	Patients, n (F, M)	Dropouts, n	Improvements, n
A	211 (98, 113)	19	86
B	192 (91, 101)	13	81
C	179 (85, 94)	28	54
D	151 (79, 72)	8	69
E	143 (76, 67)	8	94
F	135 (74, 61)	7	90

Group C had the highest number of dropouts due to treatment failure or side effects, while group E had the lowest number of dropouts.

Discussion

Patient compliance with a long-term PPI use in the absence of symptoms is low, and asymptomatic subjects spontaneously withdraw from treatment.[30,31] The prolonged PPI use is thought to impact the intestinal microbiota.[32,33] However, almost 30% of GERD patients show an early relapse of symptoms after withdrawal of PPIs.[9]

Besides PPIs, other drugs and/or agents have been proposed, alone or in combination, to improve GERD symptoms, maintain healing and reduce relapses.[34] Novel products include levosulpiride,[10,11] domperidone,[13] alginate,[12,14,15] and mucosal protective devices (MPDs) (hyaluronic acid, chondroitin sulfate).[25–28] One of the most

cited studies on the association between PPIs and prokinetics demonstrated the superiority of using a second drug with a different mechanism of action.[35] Therefore, to test the real-world efficacy of these different compounds for GERD treatment, we designed a study based on:

a) A non-randomized population of GERD patients who were referred to a primary care clinical center and were under the care of general practitioners for GERD treatment within a well-defined geographical area. As such, 37 general practitioners enrolled between 3 and 9 patients with a confirmed GERD diagnosis;

b) A homogeneous sample of GERD patients, i.e., only subjects with typical symptoms (heartburn, regurgitation and pain), with grade A esophagitis (according to the LA Classification) at endoscopy. This point is important because GERD patients could be classified as NERD or ERD and show a variety of symptoms (such as typical, atypical and extraesophageal), with possible biases when comparing results to clinical trials. Grade A reflux esophagitis is not a conclusive diagnosis of GERD based on the recent Lyon consensus.[36] In the present study, the subjects suffering from typical GERD symptoms (heartburn and regurgitation) and erosions in the esophagus were considered true GERD patients;

c) Every patient acting as their own control. Several factors could influence the response of GERD patients in clinical trials, including individual variability, different risk factors, compliance, and type of reflux (acid, weakly acidic or non-acid). For these reasons, we asked patients to undergo 6 different medical treatments for GERD step-by-step, using the drug schedules described above;

d) Comparisons between single drugs or devices: PPIs compared to alginate compared to prokinetics compared to MPDs, and the association between PPIs (as reference drugs) and prokinetics or MPDs. Recently, important American (American Gastroenterological Association (AGA) and American College of Gastroenterology (ACG)) guidelines suggested modifications in the use of alginates, prokinetics and histamine receptor (H2) antagonists.[37–39] However, we decided to perform the study in a real-world setting that represents the daily life of those receiving GERD therapy;

e) An acceptable duration of therapy for each drug or agent (3 months), separated by a standardized washout period (3 weeks) between different schedules;

f) A validated instrument for assessing symptom modifications, i.e., VAS and GSS, administered before and after each treatment schedule by the same medical team.

The results of the study showed a statistically significant alleviation of symptoms for group A (PPI), group B (MPD) and group E (the association between PPI and MPD). We also considered compliance with different schedules by assessing dropouts motivated by both failures in efficacy and side effects. The highest number of dropouts was observed in group C (prokinetics) due to reduced efficacy and side effects such as hyperprolactinemia and extrapyramidal symptoms.[40–42]

Study strengths and weaknesses

Weaknesses

Open study design

One significant weakness of the study was its open-label design, which lacks blinding and can introduce bias. Since participants and researchers were aware of the administered treatments, there is a potential for placebo effects, experimenter bias and participant expectations to influence the outcomes, making it difficult to establish a clear cause-and-effect relationship.

Lack of randomization

Another weakness was the absence of randomization in participant assignment to the treatment groups. Without random allocation, the study's internal validity is at risk, as confounding variables may unevenly distribute among groups, leading to inaccurate attributions of observed effects to the treatments. This limitation diminishes the study's ability to confidently assert that any observed differences are solely due to the treatments and not to other factors.

Strengths

Overall, the strengths of the study are as follows: 1) each patient being their own control; 2) the homogeneous patient sample, i.e., grade A esophagitis; 3) the adequate treatment period (3 months for every schedule); 4) validated instruments for symptom assessment (VAS and GSS); and 5) the primary care setting.

Longitudinal design

An important strength of the study was its longitudinal design, which allowed for the examination of changes and trends over an extended period of 3 months, separated by a standardized washout period of 3 weeks between different schedules. By collecting data in this manner, the study could better capture the dynamics of the phenomenon under investigation, enhancing the understanding of potential causal relationships.

Large and diverse sample

The study's robustness was bolstered by its large and diverse sample, encompassing a wide range of demographics and backgrounds. Such inclusivity enhanced the generalizability of the findings, enabling us to draw more comprehensive conclusions that apply to a broader population.

Conclusions

In the present study, we demonstrated the possibility of using other drugs and agents besides PPIs to obtain comparable results in the treatment of GERD patients.

In particular, MPDs (hyaluronic acid, chondroitin sulfate, and aluminum or poloxamer 407) seemed to provide better results alone or in combination with PPIs.

The study, performed in a primary care setting with a sample of non-randomized patients, offers a potentially promising approach to the medical treatment of GERD in daily life.

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