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FIELD OF VISION

Simulating the mind and applications – a theory-based chance for understanding psychic transformations in somatic symptom disorders

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Abstract

With the new category of somatic symptom disorder/bodily distress disorder in ICD-11, research into pathogenetic and therapeutic pathways is stimulated. By turning away from the definition of somatoform disorders as "the lack of something physical explaining everything", this new classification might offer a way to put the focus on the individual patient's psychodynamic balance and conflicts and their condensation in the symptom. Modelling and simulation have a long history in science to gain insight also into complex phenomena. Considering the evolution of precision medicine many different parameters are meanwhile operationalised and ready for consequent process research. Calculation models have to fit to the complexity of this disorder category. In an interdisciplinary discourse between computer and medical/psychoanalytic scientists a multilayer, fine grained calculation model is elaborated. Starting from a clinical case history, within iterative discussion, by acknowledging the demand for interdisciplinary synergy and cooperation in science, psychoanalytic theory served as the basis for computer-scientific information technique. A parallelisation with the Mealy model helped to establish a meaningful calculation possibility for further process research. How psychic transformations can be understood properly in order to provide meaningful treatments, the respective training, and to conduct appropriate process- and outcome-research is established

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in simulating the mind and applications.

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Core tip: For the sciences of the mind, especially to understand psychic transformations, a profound interdisciplinary discourse is necessary to bridge the gap between the brain–mind interface, the physical and the information technology fields. The Mealy model guarantees an exact merging of the neurological and the mental domains according to strict scientific principles. The domain of somatic symptom disorders offers a way to put the focus on the individual patient's psychodynamic balance and conflicts and their condensation in the symptom. To understand psychic transformation, to simulate pathogenetic and therapeutic pathways, the simulating the mind and applications model is helpful to provide further process- and even translational research.

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INTRODUCTION

Diagnostic concepts: A reconceptualisation of somatic symptom and related disorder

The new category of somatic symptom disorder/bodily distress disorder replaces somatoform disorder (ICD-10, F45.0) and neurasthenia (ICD-10, F48.0)[1]. Hypochondria remains a separate category. With the new ICD-11 category for bodily distress disorder (ICD-11 MMS)[2], the threshold for the diagnosis of disorders with burdensome somatic concerns has been lowered. This might be justified by the fact that single symptoms can be as distressing as multiple symptoms[3]. In the ICD-10 classification, the central feature of the definition was the one that in somatoform disorder the symptoms are not explained by physical causes; this has now been changed. Excessive attention on the nature and progression of a medical condition contributing to a symptom, not alleviated by appropriate diagnostics, also can be classified as bodily distress disorder (ICD-11-Guidelines)[3]. Thus, the reaction of the mind to bodily symptoms is acknowledged to be connected to the body itself. Relevant for the diagnosis are also duration (at least several months) and impairment in important areas of functioning.

By turning away from the definition of somatoform disorders as the lack of something (*i.e.*, the lack of a medical condition explaining everything), this new classification might offer a way to put the focus on the individual patient's psychodynamic balance and conflicts and their condensation in the symptom. This change in classification manuals also can serve as an opportunity for treatment and process and outcome research.

From a case history and psychoanalysis to artificial intelligence models: Acknowledging the demand for interdisciplinary synergy and cooperation in science

Defining factors to be considered to understand and develop therapies for somatic symptom disorder from an interdisciplinary point of view is one of the steps necessary for developing adequate artificial intelligence models, again rendering research. However, getting started in clinical research often begins with a question arising from a clinical case leading to a hypothesis. The following case report illustrates the complexity of the disorder and how a symptom in this patient diagnosed with somatic symptom disorder causes disproportionate levels of stress. Clinicians dealing with

patients affected by a somatoform disorder are used to dealing with a very heterogeneous syndrome rather than a distinct disease. This complexity often makes it difficult to study treatments and predict prognosis. Much discussion focuses on how artificial intelligence models could contribute to predicting therapy outcomes and simulate the therapeutic processes and effects of different interventions including delayed treatment effects. Thus, the second part of this paper will focus on the presentation of such a model and these aspects.

CASE REPORT

FS is a 52-year-old newspaper carrier working night shifts, who lives with his wife; they have three children. FS was referred from the pain clinic to the psychiatric clinic (tertiary centre, Medical University of Vienna) after 36 years of chronic pain and visiting multiple physicians (general practitioners, neurologists, anaesthetists, and orthopaedists) with the same health problem without any somatic explanation despite a repeated and thorough examination of systems – no therapy had worked. His present complaints are pain throughout the body, especially in the joints, the scalp, (papillary) breast. Furthermore, he complained about gingival paraesthesia, difficulties swallowing, digestive problems, and chronic sleep disturbances. FS is talkative, self-confident, friendly, and outgoing. At the age of 16 years, he met his first love, his admired wife-to-be, and he had his first pain attack, a terrible headache. As a child of divorced parents, he had been separated from his brother and was raised by his mother, grandparents, aunt, and in foster care. In his childhood, FS's emotional and physical needs were consistently ignored, being beaten, and emotionally neglected in foster care. However, he took advantage of the laissez-faire style of education, truanted from school, and stayed out late in the evening. He did not finish clarinet education and began his work as a newspaper carrier, to finance a house and his family. For about 2 years, he suffered from a new kind of fierce pain attack, in his description evocative of this first attack, only deeper and affecting the whole body like "a flash resulting in a pillar of pain from head to toe". The attacks appear about three times a month, in his free time. However, the most annoying complaint is recurrent left-sided hemiplegia in the morning, preventing him from starting the day without the help of his wife. Thus, only after his wife helps him out of the bed, he walks with a limp to the bathroom and takes an extensive shower, with the hemiplegia remitting spontaneously. FS has no explanation for his symptoms and behaviour. However, he had sought comfort in explaining them with the lunisolar tide, with changes in the weather pattern, and with tension and disconnection in family relationships. The relationship with his wife had worsened lately, together with his sexual disturbances. Due to the marital conflicts, he now is motivated for a new check-up and appears excited about any possible help[4].

OVERCOMING THE FOURTH NARCISSISTIC OFFENCE – THE CHANCE FOR AN EFFICIENT COOPERATION

Narcissistic personalities show an extreme over-reaction if confronted with a minor offence, because their grandiose views of themselves, built as a defence against any feeling of vulnerability, are threatened. Unlike a person with narcissistic personality disorder, FS acknowledges him needing help but seems prone to idealising his new saviour, which can quickly turn into devaluation when feeling vulnerable or threatened. For the physicians and therapists now confronted with FS's chronic condition, this means to handle insecurities and to build a therapeutic relationship stable enough to endure expectable alliance ruptures, should the solution to FS's problem not be straightforward. Due to the complexity of the somatoform/somatic symptom disorder, understanding is challenging and often requires the acceptance of not being able to know every detail but to accept the subjective burden of the patient, without giving up on trying. This implies the acceptance of limitations and a confrontation with the gap between ideal and reality. With this reasonably foreseeable loss of the feeling of omnipotence, a narcissistic wound for patients and doctors might result in the feeling of being hurt with a tendency of withdrawal resulting in a repairable interpersonal rupture. Also, interdisciplinary collaboration, needed in clinical work and research when dealing with such complex diseases, can be challenging as it requires a realistic view of one's limitations to participate in a

dialogue with experts and acknowledge contributions to the research topics from other scientific and clinical fields. Nevertheless, diversified collaboration might shed light on this complexity to reach possible treatment strategies, as well as strategies towards precise understanding and process-outcome research. But a closer differentiated look is necessary.

THEORETIC BACKGROUND: THE SOMATIC DISTRESS DISORDER

"[…] The two foes of human happiness are pain and boredom." (Schopenhauer A)[5].

The somatoform pain disorder/somatic symptom disorder/bodily distress disorder is characterized by severe, persisting chronic pain, with a marked psychological strain and pronounced reduction of the patient's quality of life. Furthermore, its origin as well as its overall longitudinal symptom dynamic and severity are linked to emotional conflicts and psychosocial risks and distress. With a prevalence of 9%–20% in the general population, somatoform/somatic symptom disorders' relevance for the health care services is quite high as patients usually are heavy users of services[6-8].

Pathogenesis

Psychiatry and neurology are both sciences dealing with disorders concerning the brain–mind interface. Exploring the interdependence of psychological and biological phenomena, either in a conscious or in an unconscious way, again, might influence both biological and psychological processes, and their interrelations (*e.g.*, psycho-neuroimmunology: depression and different profiles of the immune system[9]).

Neuronal circuits active in psychosocial distress are also associated with physical pain; emotional and physical pain exist on the same continuum[10,11]. In early childhood, somatisation is regarded as a normal and necessary developmental phase, as a reaction to stress and distress, and diminishes with maturation[12]. The same reaction already in older children is regarded as a pathological one, and the need for a classification that does justice to these developmental steps, thus avoiding over-pathologising, has been highlighted[13-15]. Interpersonal and intrapersonal emotional distress is felt as agonising pain and suffering instead. In such patients, adequate ways of communicating distress are not well developed; dysfunctional interpersonal affect regulation between caregiver and child is at the origin of the disorder[12]. On a neuronal level, networks associated with interpersonal distress are also associated with the neuronal circuits responsible for pain[16-18].

Development of affect regulation

However, at the core of the problem are dysfunctional affect regulation abilities and attachment patterns, developed in early interpersonal experiences, thus being transmitted from one generation to the other[12]. Especially in vulnerable persons with genetic predisposition and in unfavourable environmental conditions and contexts such deficits might lead to somatoform disorders[12]. In the above-mentioned case report, the educational style was characterised by emotional neglect and rejection. FS developed rejection sensitivity and a high level of worry[19]. His discomfort with closeness shows up in the relationship with his wife. He is caught in repetition: his pain expression, an expression of his distress and inner tension, leads to his wife having to care for him. Thus, in a way, he is asking for closeness, but at the same time, it seems that he feels uneasy about his wife caring for him.

While in early childhood, especially in the perinatal period, arousal and excitation are experienced and regulated somatically[12], later more sophisticated ways of regulating external and internal stimuli should rise, together with the development of cognition and higher mental functioning.

As separation from the caregiver might provoke fear in early childhood, this is one of the challenges to master in this developmental period. Diverse strategies and ways to deal with this and similarly distressing events (*e.g.*, hunger, tiredness, pain or fever) need to be developed, partly depending on the availability of care). In psychodynamic theory this process especially has been investigated in Winnicott's theory of holding (importance of the external object), Bion's theory of container/contained, and Klein's theory with a particular focus on the internal and phantasmatic experience of the child [20]. At first, the child needs soothing and satisfaction of needs by the caregiver in case of distress (compare Figure 1).

Additionally, postevent processing of distress varies and is relevant (see[21] for social distress). However, with time, it learns to soothe itself together with an understanding of the situation together with its own and the others' affective and

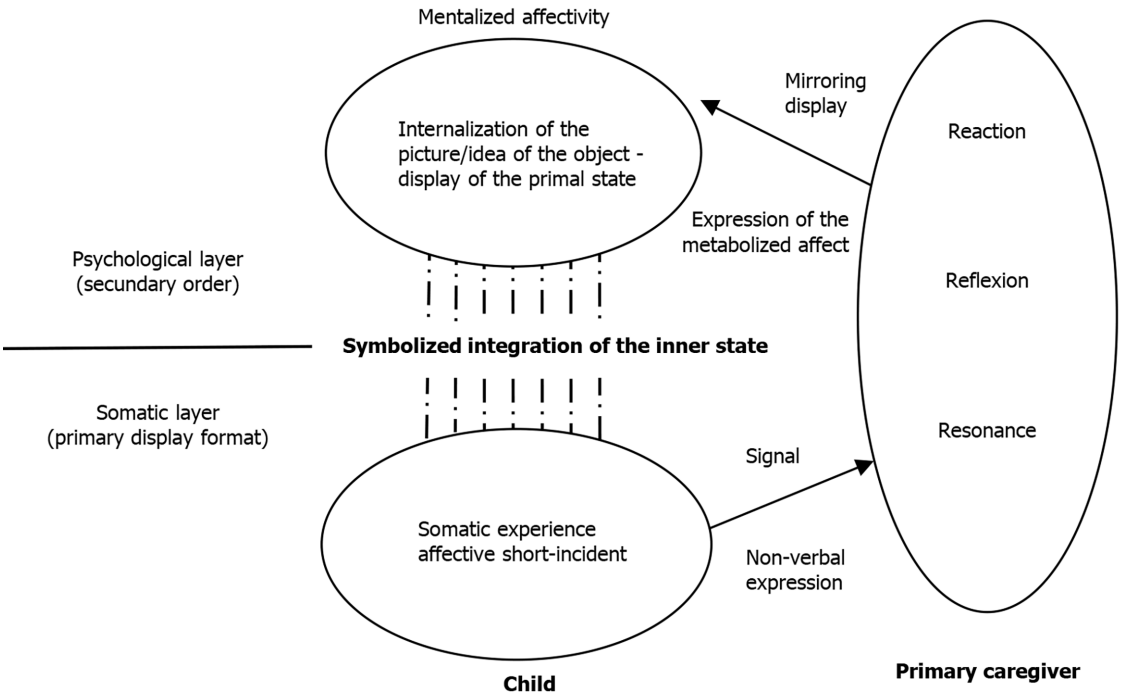

Figure 1 The emergence of mentalized affectivity. Mutual interactions between caregiver and child are shown as a process where the caregiver infers the inner thoughts and affective states through signals expressed by the child (and the contextual information and knowledge available). At first, the child notices a somatic experience and expresses it nonverbally to the caregiver as a signal. Then, the caregiver metabolises this signal (resonance, reflection, reaction) and communicates an expression of this metabolized affect to the child (mirroring display). In the next step, the child internalises this reaction of the caregiver in response to its own experience as a picture/idea of the primal state. In such a way a transformation from a somatic experience to an integration of the child's inner state is acquired *via* symbolisation in repeated social interaction.

mental states. Intention mirroring was found to be more frequent in securely attached mothers, based on well-attuned, affect-mirroring communication[22]. Self-compassion has been shown to be lower in patients with somatoform symptoms when confronted with healthy subjects[23]. In perpetuating the somatoform disorder, a learned bias to shift one's attention on somatic/bodily processes leads to an exaggerated experiencing and perception of somatic signals, increasing the risk of misperception and misinterpretation of such sensations. Furthermore, also a link between attachment patterns and somatisation has been shown for adults (insecure attachment[22,24], fearful and preoccupied attachment[25]). The case history discussed earlier in this paper also shows how "doctor shopping" can be perpetuated by the patient's insecure attachment pattern and the need for the symptom as a sign and symbol, with high costs for the health care system and a multitude of examinations for the patient.

To conclude, affect regulation is linked to the attachment pattern and mediated by social interaction (see also[12], compare Figure 2). An insecure-avoidant attachment pattern is associated with problems to trust someone and the intolerance of closeness [26]. Thus, interventions addressing attachment in therapy have the potential to improve symptoms.

Predisposing factors for somatoform disorders/somatic symptoms/bodily distress disorders

A higher lifetime prevalence of somatoform disorders has been observed for individuals with early trauma[27], lacking parental care[28], negative experiences/stressful live events[29,30], and child abuse[31]. However, the manifestation of a somatoform disorder also depends on the parenting style[28] (*e.g.*, avoidance/rejection, conflicts or emotionally unstable parents, lack of discipline and inconsistent discipline, or controlling and overprotective parents)[28]. Continuity of somatic symptoms between adolescence and early adulthood has been shown and points to the high tendency of chronicity in this group of patients. Earlier research has shown that the level of somatic symptoms in a patient's parents might be associated with the ones in their children [32,33]. In twin studies, a genetic predisposition was suggested[34].

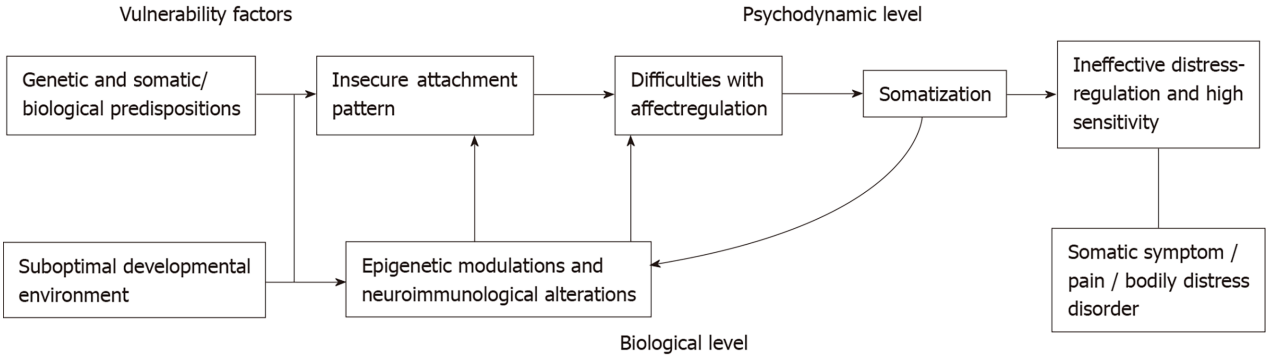

Figure 2 Vulnerability and psychodynamics.

Biological perspectives on somatoform disorders

High subjective distress, in severity not fitting the present objective findings, is one of the main symptoms of somatoform disorders (ICD10)[35]/somatic symptom/bodily distress disorders (ICD11)[2]. Unfortunately, although somatoform disorders encompass a heterogeneous group of diseases, some unrecognised diseases, misclassified as somatoform disorders might be relabelled after a repeated, sufficient examination. Furthermore, some potentially curable diseases still might lack the techniques to study them[36].

As somatoform symptoms are not considered strictly mental/psychological events, biological processes play an important role[36,37]. The underlying physiology of distress has been studied, explaining to some extent the genesis, and the experiencing of symptoms[36]. Especially research on inflammation-associated symptoms has gained promising results lately. The association between subjective health complaints and inflammation has been shown[38,39]. Raison and colleagues[39] have argued that depression is an accessory phenomenon that comes together with adaptive advantages due to genes promoting inflammation. The field of psychoneuroimmunology investigates environmental influences on the development of the immune system. Repeated exposure to danger leads to immune reactions (proinflammatory state) with pro-inflammatory cytokines and the possibility of immune sensitisation. For somatoform disorders and for sickness behaviour, specific cytokine patterns have been identified [40].

Social exclusion has been shown to be associated with somatic complaints, especially with pain. Social rejection and physical pain both are distressing, and they share a common somatosensory representation[41]. Experiments investigating the pain threshold in children/adolescents with somatic symptoms showed a divergent reaction regarding their sensory threshold after social exclusion when contrasted with controls[42]. While the group with somatisation showed a stable sensory threshold, the controls showed a decreased threshold[42]. Adolescents with functional abdominal pain showed increased parasympatic activation when exposed to induced social exclusion, whereas healthy controls showed no such activation pattern[43].

As mentioned above, the pain network is linked to the neuronal network concerned with the regulation of distress provoked by interpersonal stimuli. Important structures involved in distress regulation (emotional and the affective component of somatic pain) are the medial prefrontal cortex (antinociceptive effects, including biopsychological pain management but also chronification[44]), the dorsal anterior cingulate, and the anterior insula[41]; these regions are also involved in processing social rejection. While the brain regions concerned with the somatic representation of physical pain include the operculo-insular region; these regions are not activated by social rejection[41].

As pain is associated with depression and antidepressants influence pain (*e.g.*, tricyclic antidepressants such as amitriptyline, and selective serotonin reuptake inhibitors such as citalopram), common biochemical mechanisms are likely[45].

The role of oxytocin in social cognition, including the development of attachment and trust as well as in pain has been investigated, but there are still many open questions[46].

Exogenous factors influence human life and health. Evidence suggests that social interactions might have an influence on the expression of genes[47,48], while epigenetic and genetic predispositions modify the response to environmental factors [48,49]. Epigenetic changes have been shown to be influenceable by separation and

traumatisation with potentially permanent and profound changes. Mechanisms underlying epigenetic changes include DNA methylation, histone modification, RNA silencing, regulation of genes, *etc.*[49]. For example, epigenetic changes are one key mechanism of how stressors interact with the genome[50]. Influences on the reaction to stress in patients with depression have been shown to be associated with a changed expression of cortisol receptors in the hypothalamus (methylation and modification of histones), leading to a prolonged and pronounced stress reaction due to higher cortisol levels (changed glucocorticoid signalling). The hypothalamic–pituitary–adrenal axis activation is common in major depressive disorder[51].

Reconsidering the main aspects from the biological, psychological, developmental, and social domains it often remains open, how psychic transformations can be understood properly to provide meaningful treatments, the respective training, and to conduct appropriate process and outcome research.

UNDERSTANDING PSYCHIC TRANSFORMATIONS

One problem in psychotherapy research is finding answers to the questions of how psychotherapy works, and what works for whom, how do psychic transformations happen at all, and how to make them last? Patients requiring psychotherapy are a heterogeneous group (differences in medication, social context, comorbidities, previous therapy, age group, *etc.*), this makes interpretation of data difficult in investigations with small sample sizes. It is not possible to run the same experiment in the same patient twice under different environmental or social conditions, because each intervention changes the investigated individuum and context. Investigating long-term data is expensive, but long-term results matter as much as process research does. Comparison of new therapeutic approaches with the best known and best available care is particularly problematic in long-term therapies with real patients, because of good enough evidence for existing therapies. Randomisation to new therapeutic approaches and innovative interventions is problematic due to ethical concerns (potentially unknown side effects and unknown long-term outcome, freedom of choice for the paying and informed patient).

SIMULATING THE MIND AND APPLICATION

Modelling and simulation have a long history in science to gain insight into complex phenomena and conduct virtual experiments when real ones are not possible. This approach could be useful here. Artificial intelligence models of the mind–brain interface, rending exploration of machine learning capabilities possible, also could allow for investigations that are ethically or technically not possible in humans. To that end, the simulating the mind and applications (SiMA) model was developed. Far more than a mathematical toy, it supports the exploration of theoretic and abstract concepts so challenging as the connections between psyche and body.

The model of Mealy: bridging the gap

Coupling of the neurological system and the psyche: When searching for a scientific description of a model that considers a coupling of the neurological system and the psyche, one is inevitably confronted with the contradictions in the nomenclature of the different scientific communities. When we use the term "brain", we often hear the accusation that parts of the nervous system, as well as the associated sensory and actuators systems, are being excluded. The term "mental apparatus" is quickly pushed into the corner of neurological reductionism, and the term "nervous system" is hardly associated with the psyche. Therefore, for the purpose of this article, the term Ψ-organ will be used. The term "organ" indicates that the information system of the human being can be regarded as a unified system like all other organs. The term "Ψ" indicates that mainly psychoanalytical knowledge is used to describe the psyche of the Ψ-organ.

With this foundational understanding, the challenging scientific question of how the physical and the psychological can be described without contradiction in a common and unified model can be posed.

There have been a few approaches to this question in the past; all of which have been unsatisfactory. Two of the most frequently cited are that of Peterfreund and that of Turkle[52,53]. Peterfreund was too focused on the mathematical considerations and barely made the connection to neurology. He did not achieve a complete model based

on neurological as well as psychological concepts[52]. Turkle did not find a unified model between neurology and psyche, either, but focused mainly on the collaboration between artificial intelligence and psychoanalysis[53]. A relatively new idea from Solms[54] must be viewed sceptically from an information technology (computer technology) perspective. His attempt to build a bridge between the mental and neurological description is based on the assumption that one can merge the methods and laws of physics with those of information technology in one mathematical equation system, without substantiating this experimentally.

The model concept of SiMA is different. Dietrich uses Mealy's theory, which is generally applied in computer science, to bridge the gap between the physical and the information technology fields[55,56]. It guarantees an exact merging of the neurological and the mental domains according to strict scientific principles. The experimental simulation results confirm his approach.

Mealy published his idea in 1955[57]. He succeeded in developing a modelling method named after him for the calculation of electronic digital circuits. The digital circuit is transferred into a two-layer model. The lower layer contains the functions that are described physically, while the upper layer contains the functions that are described in terms of information technology (Figure 3). Both layers are connected *via* a clearly described interface. The description is a mathematical process. It can only be imagined abstractly. One must be aware that the real circuit is conceptually split into two layers: the physical layer and the information layer (not comprehensible from a physical point of view). The interface between both layers is defined by the information flowing through them. In the lower layer, the information is described physically, and in the upper layer, the same information is described information technologically.

The physical layer in Figure 3 can be diverse. In digital electronic circuits, functions can be transistors, resistors, or diodes, and information can be expressed by physical quantities such as voltages or currents. Accordingly, neurons can be described based on such functions. Referring to Figure 3, the information quantities entering the lower layer (information a) are quantities that act on the human sensors and thus must be described physically. The same applies to the output quantities h. The information quantities acting within the lower layer (the information quantities b, c, f and g) are therefore to be described by electrical properties: voltages, currents and temporal behaviour of the neurons. If one changes to the upper layer, *i.e.*, all quantities (the information quantities d and e) become independent of their underlying physics and are described purely in abstract terms. Figuratively speaking, electrical signals turn into bits and bytes. The passage of all information through the functions of the upper layer is also independent of time, it happens instantaneously. If information d in Figure 3 is generated by information c, it is simultaneously present as a quantity at the output of the upper layer and thus as a quantity f at the lower layer.

Mealy and his team were able to prove through experiments that this model – also known as the Mealy machine – was not just a mathematical gimmick. Scientists also developed modified mathematical description methods that went beyond this. Today, every design language for the development of hardware (like computer components), is based on this very abstract principle. Nowadays, no computer is conceivable without Mealy's theory.

A generalization of the Mealy principle is just as important. Imagine that several computers are to communicate with each other. The first international standardised model of this kind was the International Organization for Standardization (ISO)/open systems interconnection (OSI) model. The layer described in terms of information technology can be subdivided as required and the functions it contains can be allocated within it according to their specific tasks. The layers are arranged hierarchically. Each column in Figure 4 represents such an ISO/OSI model. The lowest layer (layer 1) is always the physically described layer, which defines the (physical) connection to the other computers. The upper layers of Figure 4 are the units described in terms of information technology. This means that the upper layer in the Mealy model is subdivided into several, further layers. The basic principle is identical in both models.

The original Mealy model (Figure 3) was developed for the design of digital circuits, *i.e.*, to obtain a holistic, functional model for the physical and informational description. The generalization of this principle, *i.e.*, the abstraction of physical signals and states into information symbols that can be further aggregated and processed on higher system layers, leads to models such as the ISO/OSI model (Figure 4) or, in SiMA, a holistic, functional model of the Ψ-organ.

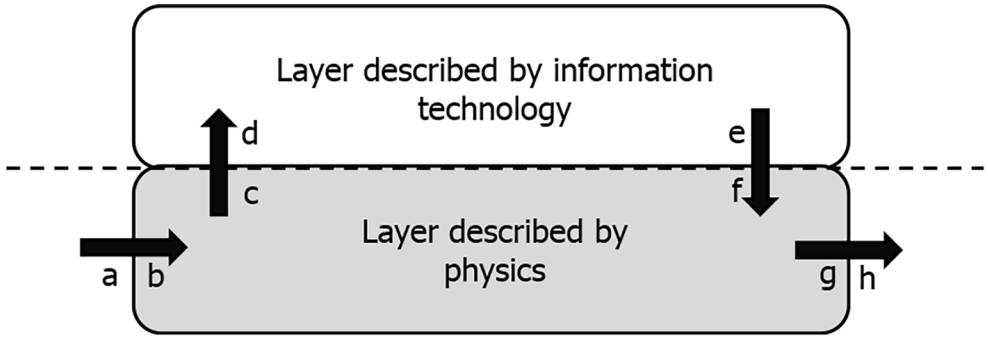

Figure 3 Mealy model. Current state and current inputs determine the Mealy model's output values. A mealy machine consists of a finite set of states, inputs and outputs and a transition as well as an output function. The model produces an output (h) while an input (a) is received (in the same simulation step). In this figure the real circuit is conceptually split into two layers: the physical layer (rectangle shaded in grey) and the information layer (rectangle shaded in white). The interface between both layers (dashed line) is defined by the information flowing through them. An abstract concept of the transition between the two layers (information technology and physics) is shown.

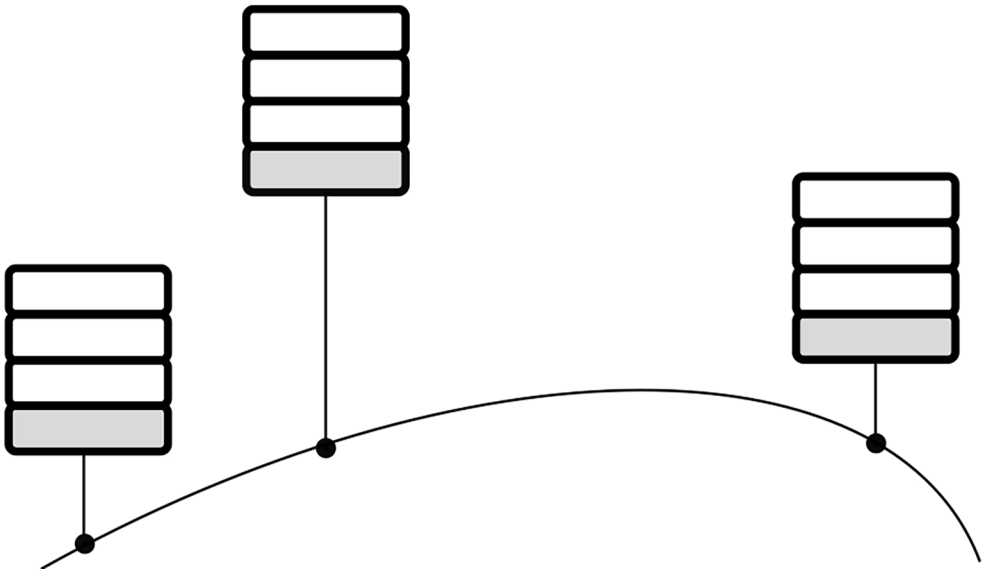

Figure 4 Possible coupling of computers according to ISO/OSI. Each column in this figure represents such an ISO/OSI model with hierarchically arranged layers. The lowest layer (rectangle shaded in grey) is always the physically described layer, which defines the (physical) connection to the other computers. The upper layers of the columns in this figure are the units described in terms of information technology (rectangles shaded in white: the upper layer in the Mealy model is subdivided into several layers as required, with functions allocated within according to the specific tasks). ISO: International Organization for Standardization; OSI model: Open Systems Interconnection model.

To bridge the gap to the Psy-sciences to overcome the fourth narcissistic offense of not being able to understand somatic symptom disorders / bodily distress disorders good enough and provide meaningful treatment options for patients, a precise stepwise collaborative working model must be considered to simulate possible ways of understanding.

SOMATIC PAIN IN SiMA AND ITS POSSIBILITIES FOR BRIDGING THE GAP

In structuring and describing functions of the mind, SiMA builds on psychoanalytic principles and concepts[56]. Functions and their inter-relations are grouped in layers according to their association with physical, unconscious and conscious processing of information. To avoid systematic errors in the development of the technical implementation and to ensure a consistent and holistic model, the concept was developed in an interdisciplinary discourse.

A central point in the model is the feedback from the body of what in SiMA is called an agent. To that end, interfaces for sensory inputs are foreseen[58], and the body of the agent is modelled. The concept of pain, however, was not originally included[59] because in psychoanalysis, pain is only seen as a pseudo drive. "The goal of this pseudo drive is just the cessation of the organic change and the associated unpleasure. Other, direct pleasure cannot be derived from the stopping of the pain. Pain is also imperative…"[60,61]. This served as guideline for including a model of somatic pain. Its implementation was tested *via* simulations, and the results discussed in an interdisciplinary review.

Originally, the SiMA model did not comprise feedback to the psyche when the agent experienced pain. Consequently, the agent would not show a reaction if its health status worsened. The inclusion of the pain concept necessitated an extension of the existing model structure and the inclusion of interfaces across the layers to allow for representing somatic pain in the psyche. The source of pain are sensory inputs of the body signalling a change. In contrast to drives, pain relief cannot generate pleasure. Therefore, pain cannot be treated in the drive track of the model. Rather, it must be included in the perception track and produce corresponding unpleasure there. This unpleasure is used in turn to create rated memories and emotions.

Figure 5 shows the functions added to the model (relevant for the processing of somatic pain[62]). Functions F12 generate the sensory inputs to the body. They register changes, *e.g.*, when the agent is hurt. The sensor value is then transferred to F13 which extracts symbol's that can subsequently be processed by the psyche. These symbols contain actual values as well as connections to the symbol health status. All this information is provided to the mental layer and further processed in F14 which generates a factual perception related to the representation of pain (Figure 6)[62]. The amount and rate of change of the symbols translate into a level of the somatic pain. Moreover, F14 generates unpleasure correlated to the pain level. This unpleasure is factored into the calculation of the emotion and added to the existing unpleasure stemming from the current drive situation. It is thus available to the functional model for subsequent decision-making and memory creation.

Calculation of the unpleasure in F14 accounts for changes in the health status as well as the difference to the optimal health,

$$U_i = (G_{i-1} - G_i) \times G_i + (1 - G_i) \times (1 - G_i)$$

with U_i being the new unpleasure value, G_i the current health status, G_{i-1} the health status from the previous simulation cycle, and $1 - G_i$ being the difference between the current and the maximum health. The health value is from the range [0..1], where 1 represents optimal health and 0 represents the death of the agent. The resulting unpleasure is also scaled to [0..1]. The equation is split up into two parts, where the first part is the change of the current health to the previous state and the second part is the difference between current and the maximum health. Both parts are weighted by the current health status (G_i). If the health status is high, the change of health will be weighted more than the difference to the maximum health. These equations were derived in interdisciplinary discussions and proved reasonable in simulation experiments.

Optimal health and its definition are challenging, as it presents an ideal but subjective state, characterized by the absence of disease but furthermore by being at one's optimum and being balanced in all aspects of existence. Thus, a difference ($1 - G_i$) between this ideal and the current health states (G_i) is the normal state, always including a certain but variable amount of unpleasure (U_i). The tricky part is quantifying the amount of the difference.

THERAPEUTIC OPTIONS

Psychoanalysis and focal psychodynamic-interpersonal therapy

The influence of early mother–child relationships on the predisposition for the manifestation of a somatoform/somatic distress disorder led to a psychodynamic therapy based on this finding. While at the beginning of treatment, understanding, and legitimation of somatic complaints and relaxation training is the focus, later, the differentiation between affects and perception of sensations of the body is trained. Links between affect experience and regulation and symptoms/body perceptions are drawn. The technique uses interpersonal experiences for this approach, a new interpersonal sphere (for transformation from layer 1 to layer 3) is created within the therapeutic relationship. Dysfunctional health attitudes (Layer 3), associated with the symptoms (from body or F12) and the experiencing of body sensations (F14 *via* F13

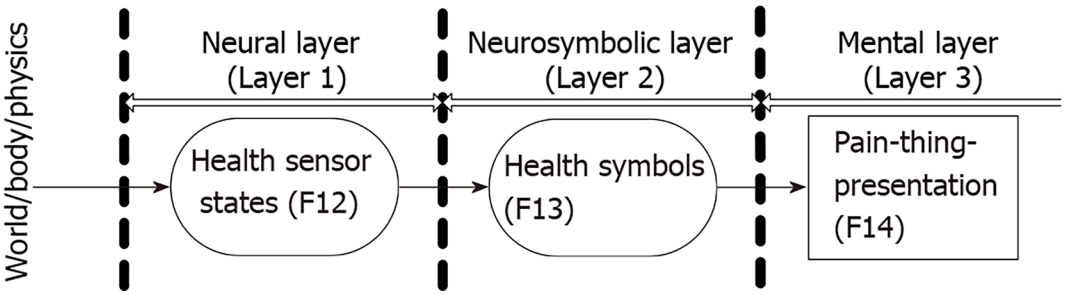

Figure 5 Excerpt from the simulating the mind and applications model. The functions relevant for the processing of somatic pain are shown (marked in dark grey).

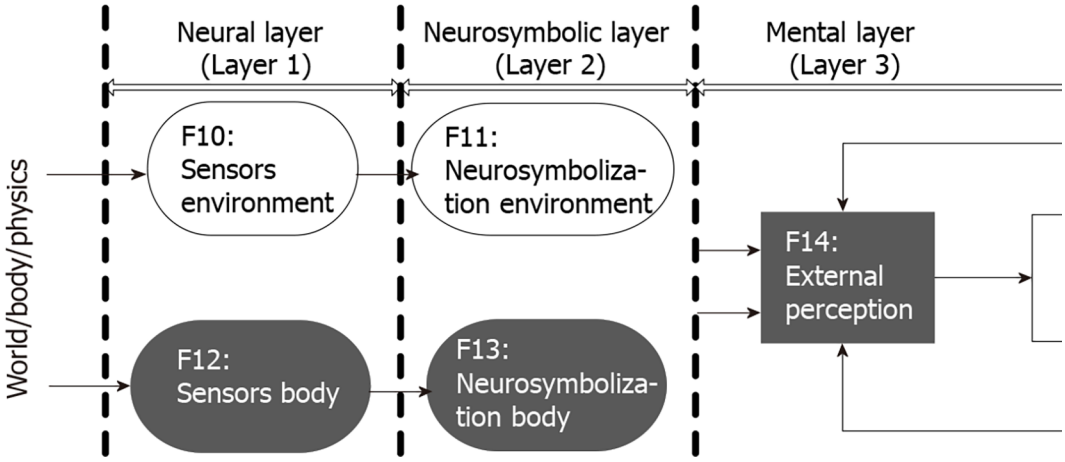

Figure 6 Creation of pain perception from the body sensors of the agent[62] **Neural layer (layer 1): functions F12 generate the sensory inputs to the body (health sensor states/registering changes)**. Neurosymbolic layer (layer 2): this sensor value is then transferred to F13 which extracts symbols, containing actual values as well as connections to the symbol health status. Mental layer (layer 3): all this information is provided to the mental layer and further processed in F14 which generates a factual perception related to the representation of pain (*i.e.*, unpleasure correlated to the pain level).

and F12), are also addressed to consolidate the therapy progress. Complex analysis and interpretations are not the focus of this focal therapy approach. At the end of therapy, the patient gets a letter from the therapist summarizing the content of the therapy. Beginning with the focus the patient then can transfer this re-consolidation and corrective emotional experience to other difficulties in life and can utilize psychoanalysis[63,64].

CONCLUSION

Outlook for further research

To facilitate a second order of meaning and understanding, a symbolized integration of somatic pain has to be established. This is normally provided in interaction circles with the primary caregiver (Figure 1) – and for therapeutic purposes then with the psychoanalyst. The affective signal is affectively marked ("resonance" in Figure 1, in Mealy's model Figure 3 "c"), then mentalized, reflected by the caregiver ("d" in Figure 3 or in psychoanalytic theory "alpha function", "dream work", "reverie"[65-67]) and a reaction is set with a metabolized affect ("f" in Figure 3). Such processes serve as essentials for establishing meanings, words, and understanding symptoms. With the Mealy model, these processes can be operationalised in a meaningful way. As an affectively meaningful second-order layer can arise and develop in a relationship, the second person with their reflective function must be considered. This conceptualisation is saturated with systems-theoretical considerations[68] and is currently empirically investigated (*e.g.*, embodiment literature[69] or epistemic trust investigations[70]).

For further research calculating operationalised psychic parameters is not enough, the weight or underlying structure/function often must be additionally considered. Therefore, the SiMA-Model can give advantages for further meaningful process research. This collaboration and iterative efficient cooperation can serve as a possibility for overcoming the fourth narcissistic offense to facilitate meaningful research for burdened patients and their therapists.

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OPINION REVIEW

Is dose modification or discontinuation of nilotinib necessary in nilotinib-induced hyperbilirubinemia?

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Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): 0

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Abstract

Nilotinib is a specific breakpoint cluster region-Abelson leukemia virus-tyrosine kinase inhibitor that is used as an effective first- or second-line treatment in imatinib-resistant chronic myelogenous leukemia (CML) patients. Hepatotoxicity due to nilotinib is a commonly reported side effect; however, abnormal liver function test (LFT) results have been reported in asymptomatic cases. When alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels are more than five-fold the upper limit of the normal (ULN) or when the serum total bilirubin level is more than three-fold the ULN, dose modification or discontinuation of nilotinib is recommended, resulting in decreased levels of hematological indicators in certain patients with CML. Nilotinib-induced hyperbilirubinemia typically manifests as indirect bilirubinemia without elevated ALT or AST levels. Such abnormal liver functioning is thus not attributed to the presence of a true histologic lesion of the liver. The underlying mechanism may be related to the inhibition of uridine diphosphate glucuronosyltransferase activity. Therefore, nilotinib dose adjustment is not recommended for this type of hyperbilirubinemia, and in the absence of elevated liver enzyme levels or presence of abnormal LFT findings, physicians should consider maintaining nilotinib dose intensity without modifications.

Key Words: Tyrosine kinase inhibitors; Nilotinib; Chronic myelogenous leukemia; Hyperbilirubinemia; Drug induced liver injure; Liver injury

Core Tip: Hepatotoxicity due to nilotinib is a commonly reported side effect; however, abnormal liver function test (LFT) results have been reported in asymptomatic cases. Nilotinib-induced hyperbilirubinemia manifests usually as indirect bilirubinemia

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without observation of elevated alanine aminotransferase or aspartate aminotransferase levels. The underlying mechanism may be related to the inhibition of uridine diphosphate glucuronosyltransferase activity. Therefore, in the absence of elevated levels of liver enzymes or presence of abnormal LFT findings, physicians should consider maintaining nilotinib dose intensity without modifications.

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INTRODUCTION

Chronic myelogenous leukemia (CML) originates in hematopoietic stem cells of malignant hyperplastic diseases and accounts for 15%–20% of all leukemia cases, with an incidence of 1–2/100000[1]. CML is one of the most common types of leukemia and is marked by the presence of a breakpoint cluster region (BCR)-Abelson leukemia virus (ABL) on the (9, 22)(C34, C11) chromosome translocation fusion gene, *i.e.*, the gene encoding P210-type fusion protein which exerts a type of tyrosine kinase activity. The subsequent abnormal clonal proliferation of myeloid hematopoietic cells is the primary cause of CML.

BCR-ABL tyrosine kinase plays an important role in cell differentiation, division, adhesion, and stress response, and its constitutive activation can lead to the development of leukemia. BCR-ABL is expressed in tumor cells of 95% of patients with CML, whereas it is not expressed in normal cells. Therefore, specific BCR-ABL tyrosine kinase inhibitors (TKIs) can be used to effectively treat CML[2].

TKIs are known to induce hepatotoxicity[3]. A systematic study conducted on the basis of 12 previous studies demonstrated that TKIs are associated with a higher risk of hepatotoxicity, compared to placebos[4]. Regarding hepatotoxicity of grade 3 or higher, the instructions recommend dosage reduction and discontinuation to allow recovery of liver functioning. The objective of this study was to confirm that the distinction between true hepatotoxicity and hyperbilirubinemia alone does not require dosage changes or discontinuation of treatment for hyperbilirubinemia caused by nilotinib alone.

PRESENT STATUS OF NILOTINIB TREATMENT

Nilotinib is a molecularly targeted kinase inhibitor that competitively binds to the platelet-derived growth factor C-kit and ABL kinases, thus it is effective for treating CML. A five-year follow-up survey showed that the 5-year cumulative rate of complete cytogenetic remission was 87%, the estimated 5-year survival rate was 89%, and the rate of progression-free survival was 93%[5]. Up to 98% of the patients achieved hematological remission, and cytogenetic response was achieved in 86% of the patients at the chronic stage after treatment. After 1, 2, 3, and 4 years of continuous treatment, only 3.4%, 7.5%, 4.8%, and 1.5% of the patients, respectively, were expected to experience deterioration. Nilotinib is also considered an effective second-line treatment for imatinib-resistant cases[6,7].

HEPATOTOXICITY OF NILOTINIB

Common adverse reactions to nilotinib include dermatological, digestive, and cardiovascular side effects. Abnormal liver function is a common side effect in nilotinib-treated patients, and a considerable proportion of the patients show elevated levels of liver enzymes and bilirubin, based on analyses of phase II and III trials (Table 1). Up to 70% of the patients exhibit elevated levels of liver enzymes[8], and most patients are asymptomatic, presenting with grade 1–2 abnormal liver function. This typically occurs within 10–14 d of initial treatment, and only 4%–9% of the

Table 1 Incidence of biochemical abnormalities across the second-line and frontline nilotinib trials

Patients (%)	Second-line trials				Frontline trials							
	Phase 2		Phase 3		Phase 2				Phase 3			
	2101[35] (*n* = 321)		ENACT[36,37] (*n* = 1422)		MDACC[38] (*n* = 61)		GIMEMA[17] (*n* = 76)		ENESTnd[39] 300 mg twice daily (*n* = 279)		ENESTnd[39] 400 mg twice daily (*n* = 277)	
	All grades	Grade 3/4	All grades	Grade 3/4	All grades	Grade 3/4	All grades	Grade 3/4	All grades	Grade 3/4	All grades	Grade 3/4
Bilirubin elevation (total)	72	7	25	4	39	7	53	16	53	4	62	8
ALT elevation	69	4	14	2	48	0	42	8	66	4	73	9
AST elevation	55	3	9	1	46	0	29	3	40	1	48	3

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

patients demonstrate a five-fold upper limit of normal (ULN) alanine aminotransferase (ALT) or aspartate aminotransferase (AST) activity, which can be improved by dosage adjustment and treatment discontinuation.

In a real-world study of nilotinib, a marked elevation of ALT and AST levels was not common, and the incidence of an increase to more than five-fold the ULN was 1%–4%. Concurrently, the manifestation of jaundice was mainly attributed to indirect bilirubin levels and self-remission, and it was attributed to Gilbert syndrome (GS)[9]. However, in a nilotinib phase I and II study spanning 36 mo and conducted in Japan, 29% of the patients showed elevated bilirubin levels, 24% showed elevated ALT levels, but only 3% showed over five-fold the ULN[10]. Severe liver failure caused by nilotinib treatment is rare. For example, a 34-year-old woman with CML developed progressive jaundice (bilirubin 14.5 mg/dL, ALT 1856 U/L, and ALP 254 U/L) after 8 mo of nilotinib treatment. She was subsequently diagnosed with liver failure and underwent liver transplantation.

COMPARISON OF HEPATOTOXICITY INDUCED BY NILOTINIB AND IMATINIB

Nilotinib is clinically used because of resistance to or side effects of imatinib treatments. In the ENESTnd trial, a phase 3 randomized trial including 846 patients[11], two different doses of nilotinib showed ALT activity rates of over five-fold the ULN at 4% and 9%, respectively, compared with 3% with imatinib. Only two patients showed relatively severe liver dysfunction (data not reported), indicating that the frequency of liver dysfunction due to nilotinib treatment was higher than that of imatinib. During the 5-year follow-up of the same group of patients, the rate of abnormal liver function was 50%–60%, but liver failure was implicated in none of the reported 50 cases of death[12]. A 47-year-old woman with CML presented with liver function deterioration (bilirubin level 20 mg/dL, alanine aminotransferase level 828 U/L prothrombin time 24 s) during imatinib therapy administrated before liver transplantation, and the postoperative treatment with nilotinib did not result in liver damage[13]. In a group of 88 imatinib-resistant CML patients who received nilotinib for 3 years, 14% showed elevated ALT and ALP levels. Interestingly, five patients with imatinib-induced hepatotoxicity did not exhibit any recurrence of nilotinib-induced hepatotoxicity[14].

THE MAIN SYMPTOM OF NILOTINIB-INDUCED HEPATOTOXICITY IS UNCONJUGATED HYPERBILIRUBINEMIA

Elevated levels of bilirubin are a common adverse drug reactions to nilotinib treatment, which occurs in 3%–16% of the patients, but constitutes no evident pathological significance[15]. In a previous study, 119 patients with CML with imatinib resistance were treated with nilotinib; nine patients subsequently showed elevated levels of indirect bilirubin, and three showed elevated ALT levels. None of

the increased parameters influenced the hematological remission effect[16].

In studies on nilotinib-induced hepatotoxicity, hyperbilirubinemia is the most common etiology of hepatotoxicity and is the main reason reducing or discontinuing nilotinib (Table 1). Hyperbilirubinemia is mainly characterized by the presence of unconjugated bilirubin. The initial median time of increase was 18 d, and the duration was approximately 8 d. A spontaneous decrease in bilirubin levels may occur without medication or phototherapy.

In a case reported by Cortes *et al*[17], a 39-year-old man with CML was administered with low-dose (300 mg/d) nilotinib for continuous treatment in the frontline GIMEMA trial, and his bilirubin levels increased gradually, resulting in the development of grade-4 hyperbilirubinemia. Ten days after drug discontinuation, the hyperbilirubinemia was resolved. Moreover, the original dose of nilotinib was continued, and hyperbilirubinemia development was observed along with four recurrences. Bilirubinemia was maintained at grade 2–3, and notably, the patients demonstrated a complete molecular response 1 year after treatment. Therefore, hyperbilirubinemia is considered a benign condition[18].

NILOTINIB-INDUCED HYPERBILIRUBINEMIA IS ASSOCIATED WITH URIDINE DIPHOSPHATE GLUCURONOSYLTRANSFERASE (UGT1A1) ACTIVITY

UGT1A1 is a membrane protein that binds to the endoplasmic reticulum. It is an enzyme that plays a crucial role in phase II biotransformation of several endogenous and exogenous substances[19]. The *UGT1A1* gene is predominantly expressed in the human liver, but also in bile tissue, the large intestine, and the stomach, and it is responsible for bilirubin binding[20]. UGT1A1 is a key enzyme involved in the metabolism of bilirubin. UGT1A1 expression is correlated with UGT1A1 activity, which leads to abnormal synthesis of unconjugated bilirubin in the blood and excretion of conjugated bilirubin *in vitro*[21], resulting in hyperbilirubinemia[22]. Hyperbilirubinemia has been observed in patients treated with TKIs. A correlation between the inhibition of UGT1A1 activity and an increase in bilirubin level was observed in patients treated with TKI. The gene polymorphism of *UGT1A1* is the main reason underlying the function of the *UGT1A1* gene[23].

The promoter region of the *UGT1A1* gene exhibits a certain polymorphism, and its atypical TATA box region contains 5–8 TA repeats. The most common genotypes included six TA repeats[24]. *UGT1A1* expression decreases with increasing TA repeats. A variant of *UGT1A1* containing an untypical TATA box region of the *UGT1A1* * 28 promoter contains seven TA repeats; it is associated with decreased UGT1A1 expression and frequently causes hyperbilirubinemia of unknown etiology[25]. Furthermore, new variants have been reported, including *UGT1A1* *6/*6, *6/*28, and *28/*28[26].

Nilotinib inhibited UGT1A1 activity in a dose-and time-dependent manner. Abumiya *et al*[27] investigated 34 CML patients treated with low and high dosages of nilotinib, and hyperbilirubinemia was more common in patients treated with high dosages and over long periods. The proportion of patients harboring *UGT1A1* *6/*6 and *6/*28 genotypes (poor metabolizers) was also higher[27]. This suggests that variations in the *UGT1A1* gene should be considered when administering nilotinib.

MECHANISM UNDERLYING NILOTINIB-INDUCED HYPERBILIRUBINEMIA

Nilotinib inhibits the activities of cytochrome P450 enzymes CYP3A4, CYP2C8, CYP2C9, CYP2D6, and UGT1A1[28]. Fujita *et al*[29] have proven nilotinib to be an effective non-competitive drug for human UGT1A1 *in vitro*. In this study, SN-38 was used as a substrate, and human liver microsome and recombinant human UGT1A1 were used as enzyme sources to study the inhibitory effect of nilotinib on UGT1A1-catalyzed SN-38 glucuronidation. The results showed that nilotinib exerted a noncompetitive inhibitory effect on SN-38 glucuronization of human liver microsome UGT1A1 and recombinant human UGT1A1 with K (i) values of 0.286 ± 0.0094 and 0.079 ± 0.0029 μm, respectively.

UGT1A1 GENOTYPE DOES NOT AFFECT NILOTINIB EFFICACY AND SAFETY

TKIs are currently used for the treatment of CML. Nilotinib can increase the level of bilirubin by inhibiting UGT1A1 activity[30,31] In imatinib- and dasatinib-treated GS patients, elevated levels of bilirubin may occur; thus, it is not known whether such a condition of hyperbilirubinemia affects the efficacy and safety of nilotinib. We previously reported the case of a 24-year-old CML patient with unconjugated hyperbilirubinemia treated with nilotinib[32]. Reduction and discontinuation of treatment can improve bilirubin levels immediately, but complete cytogenetic response (CCyR) worsens. CCyR can be achieved using a normal dose, but hyperbilirubinemia without ALT and other abnormal enzyme activities can be induced again. After conducting repeated trials four times, a normal dose of nilotinib was administered, and no pathological damage was observed in liver pathology. Although high bilirubin (grade 3–4) levels persisted, the patient achieved continuous CCyR.

In a 16-year retrospective study[33], long-term hematological toxicity and hepatocellular toxicity of imatinib, nilotinib, and dasatinib were observed. The effect of the GS genotype on progression-free survival was evaluated. One hundred and five patients with CML-CP who were consecutively treated with either first- or second-generation TKIs were evaluated. Gilbert's syndrome genotypes were distributed as follows: 17 (16.2%) patients with 7/7, 44 (41.9%) patients with 6/7, and the remaining cases with wild-type genotypes. The results showed that there was no difference in either the major molecular response or complete cytogenetic response observed among the different GS genotypes. Hyperbilirubinemia was observed in 26 patients, and grade 3/4 was observed in 9 patients. However, no true endothelial toxicity was observed in patients who continued to use TKIs.

Therefore, the European LeukemiaNet recommendations state that hyperbilirubinemia caused by TKIs is more common during nilotinib treatment and that this characteristic is the most frequently reported laboratory adverse event, rather than representing a true liver injury. This phenomenon is more common in the UGT1A1-mutated population and should be monitored closely, while dose adjustment has not been suggested[34].

This type of hyperbilirubinemia does not necessitate dose adjustment to achieve improvements, but bilirubin levels may spontaneously decline, a phenomenon which has been recognized by several scholars[18].

CONCLUSION

In conclusion, nilotinib is a commonly used drug in the treatment of CML, during which hyperbilirubinemia is commonly observed, and hyperbilirubinemia with indirect bilirubin elevation is not accompanied by abnormal hepatocyte enzymes. This abnormality is not real drug-induced liver damage, but the variation in UGT1A1 leads to inhibition of UGT1A1 activity, resulting in disorders of the bilirubin metabolism [34]. Therefore, neither dose modification nor discontinuation is recommended for patients with hyperbilirubinemia at more than three-fold the ULN, and the benefits of maintaining the original dose to CML outweigh the disadvantages caused by dose reduction or treatment discontinuation. A different abnormality, mainly accompanied by elevated levels of liver enzymes, should follow the standard of reduction or drug discontinuation at more than three-fold the ULN.

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OPINION REVIEW

Preclinical safety, effectiveness evaluation, and screening of functional bacteria for fecal microbiota transplantation based on germ-free animals

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Abstract

Fecal microbiota transplantation (FMT), also known as fecal bacterial therapy, is a treatment option that can quickly reconstruct the normal composition of intestinal microbes, and it has a good therapeutic effect on *Clostridium difficile* infection, as well as on other microecological disorders. However, the causal mechanism of FMT efficacy remains to be clarified, its safety is a major problem, and the standardization and acceptability of FMT need to be improved. This review summarizes its current research status and potential research areas that need to be strengthened, and proposes to clarify the safety of FMT and the causal relationship between FMT and therapeutic effectiveness based on germ-free animals. Meanwhile, the research system is combined with multiomics technology to screen the effective bacteria in FMT, and develop standard, safe, effective and

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controllable flora of FMT.

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Core Tip: Gut microbiota plays an important role in the development and treatment of many diseases, especially gastrointestinal disorders. Fecal microbiota transplantation (FMT) has potential application in treating various diseases. However, the mechanism of FMT is unclear, its safety needs to be improved, and it is not standardized due to a variety of reasons. Therefore, in this review, we analyze the current dilemma of FMT and propose the importance of establishing a germ-free animal evaluation system, with an aim to help establish standard, safe, effective, controllable, and product-oriented formula flora of FMT.

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INTRODUCTION

Fecal microbiota transplantation (FMT) refers to the transplantation of healthy human intestinal flora into the intestinal tract of patients to treat diseases by reconstructing the normal composition of intestinal microbes[1]. The earliest record of treating diseases with human feces was written in the book "Zhou Hou Bei Ji Fang" by Ge Hong in the fourth century AD, which was used to treat severe food poisoning[2]. In modern medicine, FMT is a treatment option that can quickly reconstruct the normal composition of intestinal microbes[3]. It is recommended by clinical guidelines for treatment of recurrent *Clostridium difficile* (*C. difficile*) infection (CDI)[4-6] and acute graft-versus-host reaction (GVHD)[7]. In addition, it has been found that FMT has potential therapeutic value for inflammatory bowel disease (IBD), irritable bowel syndrome, autism, metabolic syndrome, antibiotic-associated diarrhea, hepatic encephalopathy, and other related diseases[1,8,9], even systemic diseases associated with intestinal microbiota, such as obesity[10]. Although FMT has been shown to have potential in treating various diseases, its therapeutic mechanism remains unclear[11]. Like other treatments, FMT needs to be scientifically evaluated for its advantages and disadvantages, and its safety needs to be enhanced[12]. The degree of FMT standardization is also low due to different donors for this technique[11] and separate guidelines in different regions[9]. Therefore, in this review, we discuss the use of germ-free animal systems to understand the mechanism of FMT efficacy and screen the functional strains of FMT, to discover the functional bacteria that play a role in FMT, and develop a safe, controllable, standard, and effective combination of functional bacteria.

CAUSAL RELATIONSHIP AND MECHANISM OF FMT EFFICACY NEEDS TO BE CLARIFIED

CDI is an important risk factor for exacerbation and death in hospitalized patients, with recurrent infection occurring in up to 30% of patients receiving conventional treatment, suggesting that the mechanism of antibiotic treatment for CDI is the direct killing of *C. difficile*, but it may not be eradicated. FMT is a new approach to solve this complex problem. In the treatment of recurrent CDI, the effective rate of FMT is nearly 90%[13]. Different from the direct killing effect of antibiotics, the recovery of intestinal

flora diversity is considered as the main mechanism of FMT in treating CDI[14]. FMT has been proved to be effective in treating an increasing number of diseases, and its clinical application continues to grow. For example, the efficacy of FMT in the treatment of IBD has also been confirmed. By including 75 patients with ulcerative colitis (UC), Costello *et al*[15] demonstrated that FMT was significant in relieving UC, with nine of 38 patients (24%) in the FMT group having remission and two of 37 patients (5%) in the placebo group having remission at the end of treatment.

FMT has great clinical potential, and it is a type of donor-specific treatment, with heterogeneous efficacy for different pathological conditions. At present, the whole spectrum of bacteria and randomness of donors are still used for clinical treatment [16], and there is no universal super feces[17]; it is therefore necessary to clarify the mechanism of FMT to better exert its effects in diseases other than CDI. According to existing research reports, FMT works through direct or indirect approaches. Direct approaches include the introduction of bacteriophages, bacteriocins, and metabolites (short-chain fatty acids and bile acids), and nutritional competition between normal flora and *C. difficile*. Indirect approaches include participation in the metabolism of short-chain fatty acids and bile acids, thus participating in the regulation of immune and mechanical barriers to exert efficacy[9,18]. It has been found that human FMT compensates for the microbial imbalance in the feces of colitis rats by restoring diversity and increasing the relative abundance of health-related microbes[19]. FMT also regulates the microbial composition of the colon, which leads to recovery of colon length, and significantly reduces the severity of epithelial injury and disease[19]. Natividad *et al*[20] found that ecobiotherapy rich in Firmicutes can reduce the susceptibility to colitis in a humanized gnotobiotic mouse model, and downregulate colonic inflammation and TH17 pathways in mice; it is clear that the mechanism of FMT against colitis may be the enriched Firmicutes[20].

SAFETY OF FMT NEEDS TO BE EVALUATED

Currently, FMT has been applied to clinical treatment of various diseases. However, as an emerging clinical treatment, the short- and long-term safety issues of FMT have become the focus of attention, requiring further clinical and laboratory data to confirm. China and the United States have established the China Microflora Platform (www.cmts.com)[21] and the United States FMT National registry system (FMT National Registry System)[22,23], respectively, for the follow-up and registration of all patients receiving FMT for up to 10 years to assess its long-term safety. In the systematic review reported by Zhang Faming's research group from 2000 to 2020, FMT-related adverse events were divided into transplantation-related and microecologically related adverse events for the first time, and mucosal barrier injury-associated microbiota-related adverse events were defined. The total incidence of FMT-related adverse events was 19%, with diarrhea, abdominal discomfort, pain, and spasm being the most common, and the incidence of FMT-related serious adverse events was 1.4%. The incidence of microecologically related serious adverse events was 0.99%, and all serious adverse events occurred in patients with impaired intestinal mucosal barrier function[24].

FMT is associated with a risk of infection and needs to be taken seriously. As with all treatments, the advantages and disadvantages need to be assessed[12]. For example, FMT has been used as a potential treatment option for Crohn's disease (CD), but there is still a lack of evidence for safety based on the large number of CD samples undergoing FMT. The study by Wang *et al*[25] showed that 184 FMT frequency tests were performed in 139 patients receiving FMT, and 13.6% of mild adverse events including fever, abdominal pain, flatulence, hematochezia, vomiting, abdominal distension, and increased frequency of herpes zoster occurred within 1 mo after FMT. In two separate clinical trials, after FMT, two patients developed extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* bacteremia; both of which were related to the same fecal donor, and one of the patients died. Therefore, donor screening should be enhanced to limit microbial transmission that could lead to adverse infectious events to determine the benefits and risks of FMT in different patient populations[26]. A multicenter retrospective study by Nicholson *et al* confirmed that the treatment of CDI in pediatric patients was safe and effective, but 4.7% of patients still had serious adverse events during a follow-up period of 3 mo[27]. Ianiro *et al*[28] studied the safety of FMT for recurrent CDI in 290 patients; 109 were treated with FMT and 181 with antibiotics. After 90-d treatment, 40 patients in the antibiotic group had bloodstream infection (BSI). The analysis of matched patients showed that compared

with antibiotic treatment, the risk of BSI in the FMT group decreased by 23%, but there were still five cases of BSI in the FMT group. Bilinski *et al*[29] published a case report in which the patient developed severe diarrhea and norovirus infection after FMT, accompanied by grade III nausea, weight loss, and rising blood eosinophilia. DeFilipp *et al*[30] hypothesized that FMT may restore intestinal microbiome diversity after allogeneic hematopoietic cell transplantation (allo-HCT). In this open-label single-group pilot study, 13 patients received FMT capsules. Some patients developed grade 3-4 acute gastrointestinal tract GVHD after FMT, and one of these patients developed *Klebsiella pneumoniae* bacteremia and sepsis, followed by multiple organ failure. There was one case of *C. difficile* colitis observed after FMT and six patients developed moderate-severe chronic GVHD[30]. Take into account this, the FDA issued a warning to researchers that fecal screening in FMT studies should be expanded to include specific resistant strains[31] and opportunistic pathogens should also be considered. In addition, when improving the safety of FMT to reduce the risk of systemic infection (such as sepsis caused by FMT spreading drug-resistant pathogens), besides screening for pathogens in the donor fecal bacteria, the composition and structure of the flora should also be considered, such as flora diversity, key bacterial species (such as short-chain fatty acid-producing bacteria), and the ratio of obligate and facultative anaerobic bacteria, which can improve the safety of FMT[31]. At present, there is insufficient evidence to prove that FMT is used to treat diseases other than CDI, and long-term safety still needs to be evaluated[32].

At present, FMT has not been approved as a new drug, which is primarily due to the lack of preclinical safety studies. Experimental animal research can clarify the relationship between safety and bacterial solution treatment methods, dosage, and other factors, and provide scientific data in support of the safety of FMT in clinical application. Since 2014, Zhang Faming's team has begun to use the automatic fecal isolation system (genFMTer) to ultrafilter, purify, and centrifugally wash the fecal microbiota[33,34], and select different routes for transplantation into patients, which is defined as washed microbiota transplantation (WMT)[4,35]. Preclinical studies have confirmed the safety of WMT after automatic machine isolation in experimental animals, and its clinical application can significantly reduce FMT-related adverse events without affecting the efficacy of patients[4,25,36].

STANDARDIZATION AND ACCEPTABILITY OF FMT SHOULD BE IMPROVED

The material of FMT is variable, which is caused by biological variation and sample handling. Unlike drugs, FMT products are more personalized due to different donors [11]. Human feces (microorganisms and chemical components) vary greatly from person to person. Even among healthy individuals, the diversity and abundance of microorganisms in each individual vary widely, and there is a strong niche specificity within and between individuals, and many diseases are linked to specific microorganisms and chemicals in feces[37]. Therefore, during the process of FMT, different donors and improper preparation of samples may lead to differences or variations in these microorganisms, affecting the therapeutic effect and even causing adverse events. In addition, the choice of treatment for FMT varies from region to region, resulting in low standardization of FMT[9].

However, there is no clear and universal screening standard for fecal donors. The reported screening methods are strict, and usually < 20% of volunteers meet the screening standards of blood, feces, and urine[38]. Therefore, it is obviously necessary to develop FMT substitutes for patients with microecological disorders, including bacteria, fungi, protozoa, viruses, cytokines, and metabolites, and it is necessary to determine which of them are beneficial factors, which will establish a link between the disease and beneficial microorganisms of therapeutic value[11]. In addition, the development of targeted probiotic microorganisms or combinations can be considered, and alternatives including bacteriophages should also be considered[12], which may be an important research direction.

Recent investigations of doctors, medical students, donors, and patients have shown that FMT is less acceptable compared with traditional treatment methods, especially fecal bacterial suspension prepared by the rough method[4,39,40]. Zhang *et al*[4] found that the bacteria prepared by standardized formula have greater acceptance with the same efficacy, while improving the activity of the flora, which meets the standards of modern medical aesthetics. Therefore, it is necessary to improve the standardized preparation technology of fecal suspension to improve the acceptability of FMT, and

the screening of effective bacteria or metabolites should be considered to improve the standardization and acceptability. In 2019, China's first national consensus opinion on the methodology for WMT was confirmed and published, aiming to regulate the processes such as donor screening, fecal microbiota preparation, storage and transportation, selection of transplantation route, and safety monitoring and management of FMT, so as to improve the safety of FMT and ensure its clinical efficacy[35].

Therefore, screening of the functional strain is prepared under the condition of clarifying the mechanism of action of the FMT effective strain. At the same time, FMT operation procedures are improved to reach a consensus, which makes the FMT strain preparation easier to control the production, safer, more acceptable, and more standardized.

PRECLINICAL SAFETY, EFFECTIVENESS EVALUATION, AND EFFECTIVE MICROBIAL SCREENING OF FMT BASED ON GERM-FREE ANIMAL SYSTEMS

Germ-free animals refer to animals without microorganisms living in or on them. These animals have become an irreplaceable research tool to study the relationship between a single strain or multiple strains and the host, and also an indispensable model for studying functional microorganisms in humans and animals[41,42]. Germ-free animal disease models can be used to evaluate the effectiveness of FMT, to study the causal relationship between microbiota transplantation and therapeutic effect, and clarify the mechanism of action between FMT and disease in the absence of host microbial interference. In addition, germ-free animals can be evaluated for FMT safety and provide preclinical scientific data for FMT by evaluating cardiac, liver, and renal toxicity, immune disturbance, impact on behavior, and the risk of local and translocation infection. The use of germ-free immunodeficient animals can correspond to patients with low clinical immune function, and the humanized flora can be transplanted to germ-free immunodeficient animals for FMT safety test, to enhance the sensitivity of FMT activity and ensure the preclinical safety of FMT to treat immunodeficient individuals. For example, two immunocompromised patients developed ESBL-producing *E. coli* infection after FMT and one of them died[26]. However, safety assessment with germ-free immunodeficient animals in advance may avoid this adverse event.

Papanicolas *et al*[31] reviewed that the protection of symbiotic bacteria and the reduction of pathogenic bacteria in feces could improve the safety of FMT, and the change in fecal flora might reduce the risk of sepsis. Technology based on germ-free animals and multiomics provides the possibility to modify the fecal flora. Based on the clear causal relationship between FMT and germ-free animal models of human diseases, a function-oriented system for screening and evaluating transplanted flora could be established. By using metagenomics and cultureomics, effective target bacteria of FMT are obtained, and the formula flora with a clear function can be formed, so that the functional bacteria of FMT can be visible, accessible, and available.

The use of germ-free animals to standardize the preclinical safety evaluation of FMT, establish an effective evaluation system that can clarify the causal relationship and mechanism of the efficacy of FMT, and combine multiomics technology to screen the effective bacteria can improve the safety, standardization, controllability, and acceptance of FMT. Therefore, we propose a safety and effectiveness research system of FMT based on germ-free animals, and the screening of FMT efficient bacteria (Figure 1): (1) The causal relationship between therapeutic effect and flora transplantation is validated. For patients who have been effectively treated with FMT, the flora before and after FMT was transplanted into the corresponding germ-free disease model mice to verify the causal mechanism of FMT efficacy. We can use the standard germ-free disease model for FMT effectiveness evaluation, to ensure the scientific validity and versatility of FMT effectiveness data, clarify the causal relationship between flora transplantation and therapeutic effect, and clarify the mechanism of FMT; (2) The humanized flora model is constructed based on germ-free animals to evaluate the safety of FMT. The preclinical safety evaluation of FMT is conducted in strict accordance with the international classics and standard preclinical safety evaluation specifications of new drugs, medical devices, and medical technology. In addition, germ-free immunodeficient animals were used to evaluate the safety of FMT; cardiac, liver, and renal toxicity of FMT; interference with the immune

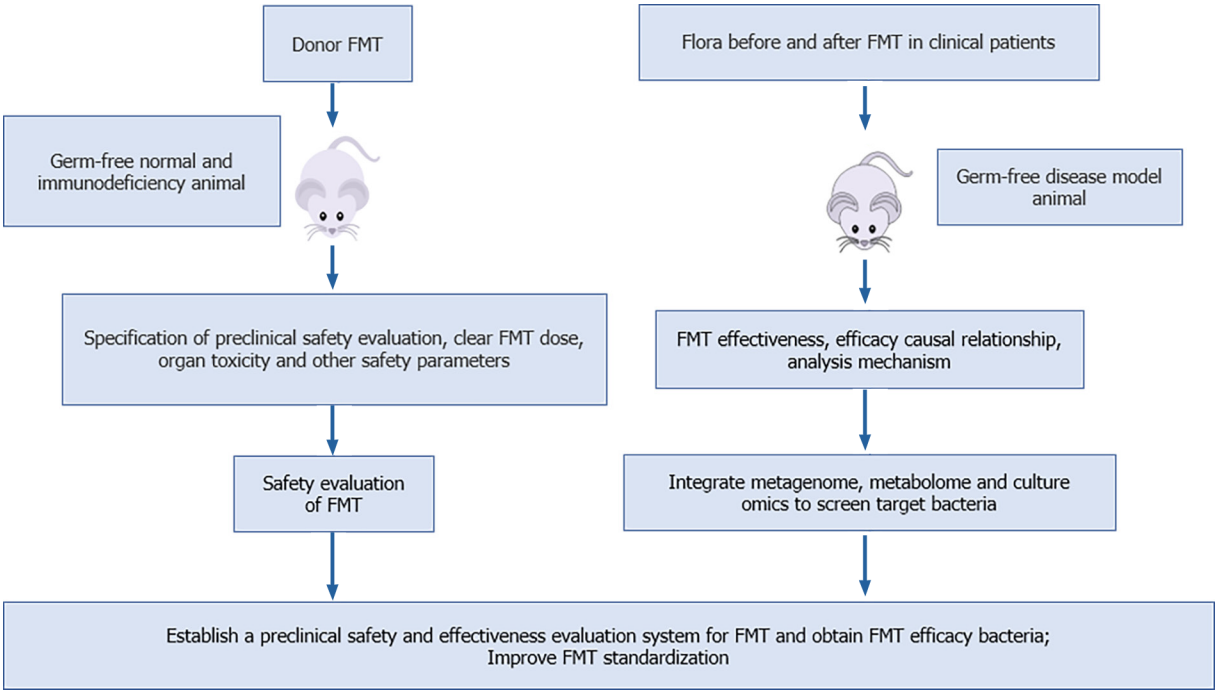

Figure 1 Preclinical safety, effectiveness evaluation, and effective bacterial screening of fecal microbiota transplantation based on germ-free animals. An FMT safety evaluation system is established based on germ-free normal and immunodeficient animals. The causal mechanism for the efficacy of fecal microbiota transplantation (FMT) is verified based on germ-free disease animal models, to clarify the effectiveness of FMT, and multiomics technology is used to screen the effective bacteria of FMT, and to improve the standardization of FMT. FMT: Fecal microbiota transplantation.

system; behavioral effects of FMT; and the risk of local and translocation infections. Germ-free immunodeficient animals are also used to enhance the sensitivity of FMT, and better identify the route and dose of FMT, and other indicators to ensure its safety; and (3) At present, there are insufficient studies to obtain the effective flora of FMT. Therefore, after clarifying the causal relationship of FMT in step one, we can combine with metagenomics and cultureomics techniques to establish a function-oriented system for screening, obtaining, and evaluating flora, and finally, the effective target bacteria of FMT can be obtained and functional formula flora can be formed. The efficacy of the formulated bacteria can be verified again by using germ-free animal models of human diseases, so as to achieve standardization of FMT, improve the acceptance level, and reduce infection risk.

CONCLUSION

Gut microbiota plays a major role in the development and treatment of many diseases, especially gastrointestinal disorders. FMT is a therapeutic option that can rapidly reconstruct the normal composition of intestinal microorganisms. FMT has potential to treat various diseases, such as CDI, IBD, constipation, and cancer. However, the therapeutic mechanism of FMT is unclear, its safety needs to be improved, and it is not standardized due to a variety of reasons. Germ-free animals, as the most appropriate models for studying the interaction between microorganisms and hosts, are applicable research tools for studying the mechanism of action of FMT, screening the functional bacteria of FMT, evaluating the safety of FMT, and improving standardization of FMT. Therefore, evaluation of the safety and effectiveness of FMT animal experiments based on germ-free animals will be set up. By using a function-oriented *in vivo* experimental system, combined with clinical analysis and culturomics technology, the functional strains of FMT for microbial diseases can be obtained and verified. And the standard, safe, effective, controllable, and product-oriented flora of FMT can be developed.

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REVIEW

Gastrointestinal tumors and infectious agents: A wide field to explore

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Abstract

Infection is currently one of the main contributors to carcinogenesis. In fact, the International Agency for Research on Cancer has categorized eleven biological agents as group I carcinogens. It is estimated that around 16% of the 12.7 million new cancers diagnosed in 2008 were attributable to infectious agents. Although underdeveloped regions carry the highest incidence rates, about 7.4% of infection-related cancer cases occur in developed areas. Physicians are increasingly aware of the potential carcinogenic role of common virus like the Human Papilloma virus in cervical cancer, or the hepatitis B and C viruses in hepatocarcinoma. However, the carcinogenic role of several other infectious agents is less recognized. Given that gastrointestinal malignancies carry an overall poor prognosis, a better understanding of the carcinogenic mechanisms triggered by infectious agents is key to decrease the rate of cancer related deaths. Preventive measures directed to such infections would ideally impact survival. In this paper

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we review the main pathogenic mechanisms related to the development of gastrointestinal malignancies induced by infectious microorganisms and other pathogens which are currently under investigation.

Key Words: Gastrointestinal tumors; Infectious agents; Bacteria; Virus; Prevention

Core Tip: Except for pathogens with well-known carcinogenic potential, such as Human Papilloma virus or Hepatitis C virus, physicians are usually unaware of the relationship among other infectious agents and tumors. The identification and subsequent eradication of these pathogens might help to prevent the development of a large number of tumors. Gastrointestinal malignancies are usually related to a very poor outcome. Therefore, detection of carcinogenic pathogens in this population might help to increase overall survival. Screening strategies and further research is required to face with these preventable diseases.

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INTRODUCTION

During the last decades the causal relation between several pathogens and the onset of cancer has been firmly established[1]. However, the relationship between pathogens and cancer has been subject of research since the end of the XIX century. Rous[2,3] (1879-1970) discovered that sarcomas affecting domestic fowls could be transferred to other fowls through a viral vector, later known as Rous sarcoma virus (RSV). This finding was awarded with the Nobel Prize in 1966 and demonstrated for the first time in history that a malignant tumor could be induced by an infectious agent. In fact, the RSV was considered the first oncogenic retrovirus. Subsequently, other tumor-inducing viruses affecting animals were identified[4-6]. In 1964, the first oncogenic virus affecting humans, the Epstein Barr virus, was identified[7].

At present, a great effort is being done in order to elucidate the underlying connection between infectious agents and cancer. In 2018, an estimated 2.2 million infection-attributable cancer cases were diagnosed worldwide, corresponding to an age-standardized incidence rate (ASIR) of 25.0 per 100.000 person-years[8]. Currently, 11 infectious agents have been classified as Group 1 Carcinogens by the International Agency for Research on Cancer (IARC)[1]. *Helicobacter pylori* (*H. pylori*) (810.000 cases, ASIR 8.7), Human Papilloma virus (690.000, 8.0), Hepatitis B virus (360.000, 4.1) and hepatitis C virus (HCV) (160.000, 1.7) comprise the primary causes of infectious-related tumors, accounting for more than 90% of infection-related cancers worldwide[9].

VIRAL INFECTIONS

Viral chronic infection can promote cancer *via* three different pathogenic mechanisms:

(1) Induction of chronic inflammation provoked by the immune response to a persistent infection; HCV-related liver cancer is a paradigmatic example, in which persistence of viral replication within the liver parenchyma maintains a state of local chronic inflammation involved in cancer development[10]; (2) Virus-induced genome transformation secondary to viral persistence in infected cells; This mechanism is typically found in Epstein-Barr virus (EBV) associated Burkitt's lymphoma. EBV replicating in the oral epithelium can transform resting B lymphocytes into proliferating lymphoblastoid cell lines involved in oncogenesis[11]; and (3) Promotion of chronic suppression of host immune response. Patients infected with the human

immunodeficiency virus can be deeply immune-suppressed. These individuals exhibit an increased predisposition to develop infection-driven tumors as the mechanisms of immunosurveillance are disrupted by the viral replication[12].

Although these mechanisms may occur simultaneously[13], some other yet unknown need also be involved, since many other pathogens inducing similar immune alterations do not seem to induce cancer. It is widely recognized that viruses can cause cellular malignant transformation by inducing genetic instability. In fact, viruses are able to fuse their genome with that of the host resulting in activation of multiple oncogenes and intracellular signaling pathways leading to cellular proliferation, inflammation and immune dysregulation[14,15].

BACTERIAL INFECTIONS

Recent studies have shown that certain infectious agents like molds, helminths and bacteria, capable of interacting with mammalian host cells, can induce cancer, even in the absence of genomic alteration[16]. Unlike the well-established relation between viruses and carcinogenesis, the oncogenic role of bacterial infection remains controversial[17]. The molecular mechanisms by which bacteria might promote carcinogenesis are currently under research[10], and both bacterial and host factors seem to be involved. Bacterial cell-surface components, toxins and effector proteins can interact with host cells by activating essential signaling pathways involved in cancer formation [16].

Role of bacterial surface antigens in carcinogenesis

The bacterial surface exhibits several antigens that interact with the host and activate both innate and adaptative immune responses, allowing them to escape the host immune system. For example, gram-negative bacteria can coat their outer surface with a polysaccharide capsule, thus preventing the activation of the Complement cascade and the phagocytic process[18]. Other bacteria are able to modify some surface molecules, like lypo-polysaccharides, flagella and peptidoglycans, to avoid immune recognition[19]. Additionally, some other bacteria can express a variety of surface proteins that facilitate attachment to host cells and subsequent internalization[20]. These strategies aim to improve bacterial survival by eluding the immune response. However, definitive cellular malignant transformation needs to be induced by specific intracellular signaling cascade activation driven by certain bacterial molecules. For example, *H. pylori* can directly activate the RAS/mitogen activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway resulting in malignant transformation *via* the CagL protein (an adhesin molecule) that binds to the beta5-integrin expressed by host cells[21]; *H. pylori* also expresses the OipA protein that binds to the EGFR host receptor resulting in AKT and beta-catenin signaling activation, which also promotes carcinogenesis[22].

Immune cell elimination

To ensure survival among host cells, pathogenic bacteria have developed several strategies to attack the immune system. For example, they can secrete cytolytic toxins through their outer membranes that can affect the endosomal system. Bacterial toxins may induce carcinogenesis altering the host cell environment by inducing genome instability, promoting resistance to cell death signaling, and enhancing proliferative signaling[23].

Induction of genome instability: Bacterial toxins are capable of inducing host cell damage in the DNA double helix. Cytolethal Distending Toxin, Collibactin, Shiga toxin and endonucleases are some examples[24]. DNA damage causes immune host cells to arrest the cell cycle at the G1-S or G1-M stages.

Resistance to cell death signaling and induction of proliferative signaling: Toxins produced by some pathogens, like *Bacteroides fragilis*, can bind to intestinal epithelial cell receptors and induce cellular proliferation and differentiation. *Bacteroides fragilis* can silence the tumor suppressor protein E-Cadherin, resulting in the activation of beta-catenin/Wnt and nuclear factor kbeta (NF-kB) signaling pathway[25].

Host cells transformation secondary to bacterial proteins

To induce malignant transformation, bacteria must ensure a persistent infection within the cells of the new organism. They need to be internalized, must control their own

Table 1 Pathogens related to gastrointestinal malignancies

Ref.	Primary tumor	Pathogen	Molecular mechanisms
von Knebel Doeberitz *et al*[30], Syrjänen *et al*[31], Liyanage *et al*[36]	Esophageal	HPV	Oncoproteins E6 and E7
Wu *et al*[41], Zhang *et al*[42], Wang *et al*[43]		EBV	Oncoproteins EBER and LMP-1
Yang *et al*[44], Tan *et al*[45], Han *et al*[46]		Herpes Simplex and CMV	Unknown
Xu *et al*[56], Mueller *et al*[57]	Gastric	Helicobacter pylori	Cag A *H. pylori* protein
Rassow *et al*[58], Jain *et al*[59]			VacA
Lamb and Chen[61], Brandt *et al*[62]			Interleukins, NFbeta, p53
Haghjoo and Galán[73]	Gallblader	Salmonella typhi	Cytolethal Distending Toxin
Belzer *et al*[75], Maurer *et al*[76]		*Helicobacter* spp.	Urease production
Lax and Thomas[10]		Escherichia coli	Cytotoxic necrotizing factor I
Sripa and Pairojkul[78], Kurathong *et al*[79]		Opisthorchis vierrini and Clonorchis sinensis	Poorly known
Lau *et al*[88], Minami *et al*[89]	Liver	Hepatitis B virus	HBx protein
Raimondi *et al*[92], Akuta *et al*[93]		Hepatitis C virus	HCV core protein
Tahara *et al*[97], Abed *et al*[98]	Colon	Fusobacterium nucleatum	Fap2 protein
Choi *et al*[110], Roxas *et al*[111]		Escherichia coli	CEACAM6, MIC-1
Arumugam *et al*[112], Sears *et al*[113]		*Bacteroides fragilis*	BFT
Del Bel Belluz *et al*[116], Kang *et al*[117]		Salmonella enterica	Typhoid toxin, AvrA effector protein
Palefsky[125], Dyson *et al*[127]	Anal cancer	HPV	E6, E7, p53

HPV: Human Papilloma virus; EBV: Epstein Barr virus; CMV: Citomegalovirus; VacA: Vacuolating cytotoxin; BFT: *Bacteroides Fragilis* toxin.

growth and facilitate their release. Usual non-pathogenic bacteria are typically phagocyted in the phagosome, and then merged with the lysosome and turned into a phagolysosome, where they are finally destroyed. However, some bacteria have developed a number of mechanisms that avoid the formation of the phagolysosome, allowing them to freely enter the cytosol[26]; some bacteria, for example, secrete proteins that induce pore formation in the organelle[27]; while others have developed different mechanisms to avoid destruction inside the phagolysosome[28].

The ultimate explanation by which bacteria promote cancer and whether they obtain any survival advantage remains unknown. This is an interesting and fast-growing research field, in which prevention measures directed to infection may impact overall cancer development. The oncogenic role of various infectious agents regarding gastrointestinal tract malignancies are now described. Since these tumors carry relevant mortality rates, screening programs directed to identify such pathogens may be beneficial.

ESOPHAGEAL CANCER

The incidence of esophageal tumors differs substantially across countries. The highest rates are found in Southern and Eastern Africa and Eastern Asia. Although the histopathological squamous variant has been associated to several viral subtypes, the pathogenic role of viruses in the development of adenocarcinoma is yet unclear.

Human papilloma virus

HPV family viruses are non-enveloped DNA tumor viruses transmitted by sexual contact. More than 100 subtypes of HPV have been described, of which at least 14 carry a significantly increased risk of tumor development [29]. HPV infects epithelial cells and is able to integrate itself inside the host genome. Some oncoproteins

produced by the virus (mainly E6 and E7) alter tumor suppressor pathways and promote malignant proliferation[30]. In 1982, Syrjänen *et al*[31] observed the typical morphological lesions present in HPV related-condylomas in both benign and cancerous esophageal tissues. These findings supported the hypothesis that HPV was involved in the pathogenesis of esophageal malignancies. Subsequent immunohistochemical studies demonstrated that HPV structural proteins were present in malignant lesions of patients from different continents[32]. In fact, the oncogenic potential role of HPV in the development of squamous esophageal carcinoma has been the subject of several metanalysis[33-35]. The first metanalysis of case-control studies investigating the role of HPV in esophageal tumors was conducted in 2013 by Liyanage *et al*[36]. They gathered 21 studies, including 1223 patients and 1415 controls. The authors calculated a pooled OR for HPV and squamous esophageal tumors of 3.04 [95% confidence interval (CI): 2.20 to 4.20]. Noticeably, countries with a low to medium esophageal cancer incidence showed a stronger relationship (OR 4.65, 95%CI: 2.47 to 8.76) than regions with higher incidence (OR 2.65, 95%CI: 1.80 to 3.91). The authors concluded that HPV infection was associated with a 3-fold risk of squamous esophageal cancer and made an urgent call to the IARC to promote the use of vaccines against HPV in high-risk populations.

In 2016, Wang *et al*[37] published a systematic review and meta-analysis on the association between HPV 16 and 18 subtypes and esophageal cancer worldwide. They included 32 studies and showed that HPV infection rate in the tumoral cohort was 46.5% *vs* 26.2% in controls (OR = 1.62; 95%CI: 1.33–1.98), providing strong epidemiologic evidence supporting the association between HPV infection and esophageal tumors[37].

EBV

EBV is an oncogenic virus that belongs to the gamma-herpes virus family. It is a widespread pathogen carried by 95% of the population worldwide. Fortunately, the majority of EBV infections are subclinical[38]. Although, the EBV typically infects B lymphocytes, recent research has shown that it can also infect epithelial cells, and suggests a potential association with esophageal carcinoma, yet to be confirmed. Jenkins *et al*[39] detected for the first time EBV in esophageal tumors, however, only in 5 out of 60 tumors. Awerkiew *et at*[40] demonstrated the presence of EBV-DNA in 35% of squamous cell carcinomas and 36% of adenocarcinomas, using nested polymerase chain reaction (PCR) diagnostic techniques.

In areas with higher incidence of esophageal carcinoma, the association between EBV and esophageal cancer has been investigated. Wu *et al*[41] studied the coinfection of EBV and Herpes Simplex virus (HSV) and detected, by in situ hybridization and immunohistochemistry, the EBER and LMP-1 proteins in 6.1% of carcinoma specimens, preferentially among poorly differentiated squamous cell carcinomas and undifferentiated carcinomas with intense lymphoid infiltration. Other authors have contributed to elucidate the role of EBV in the carcinogenesis of esophageal carcinoma [42,43]. In summary, EBV infection has been related to the etiology of some variants of esophageal carcinoma, at least in countries with increased prevalence. However, more studies are needed to establish the exact pathogenic role of EBV in esophageal carcinogenesis.

Herpes simplex and Citomegalovirus

HSV-1 infection occurs mainly in the mouth and has been related to oral cancer[44]. Citomegalovirus (CMV), a common human pathogen has been associated with cervical and non-melanoma skin cancer[45,46]. Zhang *et al*[42] reported an increased predisposition of esophageal carcinoma in patients infected with HSV-1 (OR 10.3 with 95%CI: 3.331.6). HSV infection is primarily related to esophagitis, which usually precedes esophageal carcinoma. However, no CMV was detected in any of the samples[42]. Wu *et at*[41] found HSV DNA and HSVI, II protein expression in 52 (31.7%) of the 164 tumors analyzed supporting the relationship between HSV and esophageal carcinoma cell differentiation with lymphocyte infiltration in the tumoral stroma. However, other authors have failed to demonstrate the relation between HSV/CMV and esophageal carcinoma, therefore leaving the question open[47].

GASTRIC CANCER

H. pylori

Gastric cancer can be generally classified in cardia (upper stomach) and non-cardia (lower stomach) subtypes. These entities differ in terms of risk factors, carcinogenesis and epidemiologic patterns. Chronic *H. pylori* infection is considered the principal cause of non-cardia gastric cancer, being nearly all cases attributed to this bacterium [48,49]. The worldwide prevalence of *H. pylori* infection is extraordinarily high, affecting around 50% of population[50], and its geographic variability correlates with the incidence of gastric cancer. Yet, less than 5% of infected persons will develop cancer, likely because of differences in bacterial and host genetics, age of infection and environmental factors[51]. In 1994, the World Health Organization categorized *H. pylori* as a Carcinogen type I for gastric cancer on the basis of observational studies reported from the International Agency for Research in Cancer[52].

H. pylori infection has been associated with an increased risk of all variants of adenocarcinoma, whether diffuse or intestinal, and whether located in the body of the stomach or in the antrum; actually, it is related to tumors distal to the cardia. Conversely, tumors growing in the esophagogastric junction, usually arising from the altered mucosa in Barrett's esophagus, have not been linked to *H. pylori* infection[53].

H. pylori survives in the gastric acid microenvironment, being able to damage the mucosa. It induces a chronic inflammatory response that results in chronic gastritis and peptic ulceration. Development of gastric cancer is one of the long-term consequences of *H. pylori* infection[54]. Chronic *H. pylori* infection has been also related to gastric MALT (Mucosa associated Lymphoma), which is an extra-node lymphoma variant consisting of a morphologically heterogenous small B cells neoplasm[55].

H. pylori is able to dysregulate host signaling pathways and to promote oncogenesis by two main mechanisms: Citotoxin-associated gene A (CagA) and its pathogenic island (Cag PAI); and vacuolating cytotoxin A (VacA). CagA *H. pylori*-protein is encoded by one of the genes located in the CagPAI island. CagA binds to the ectodomain of alfa5beta1 integrin allowing its internalization. Once it has been translocated, CagA binds to the inner surface of the cell membrane and undergoes tyrosine phosphorylation. Both the phosphorylated CagA and the unphosphorylated CagA can interact with a number of host proteins to activate downstream signaling pathways such as the RAS/MEK/ERK pathway, the NF-kB pathway, and the beta-catenin pathway. Such pathways activation enhances the proliferation of gastric epithelial cells[56,57].

The VacA is secreted by *H. pylori* and has a variety of biological functions. It binds to host cells and is internalized creating vacuoles (large vesicles) inside the host cells. This *vacuolization* process leads to the activation of endosomes and early lysosomes. Additionally, VacA can also be transferred to the mitochondria where it alters the membrane barrier that releases cytochrome c which finally activates the pro-apoptotic factor Bcl-2 associated X protein (Bax) leading to apoptosis. Therefore, the alteration of the membrane impermeabilization ends up in preventing apoptosis of gastric cells [58]. Inside the mitochondria, VacA can also activate the dynamic related protein 1 which inhibits the activation of Bax mitochondria outer membrane impermeabilization that prevents death by apoptosis[59]. Further, it can also disrupt the gastric epithelial cell-to-cell unions, which prevents T lymphocyte activation and proliferation in the lamina propria, and ultimately induces inflammation and carcinogenesis by disrupting autophagy in gastric cells[60].

In addition, *H. pylori* induces a chronic inflammatory state by upregulating many pro-inflammatory cytokines, like IL-1, IL-6, IL-8, TNF-alfa, and NF-kB[61]. Among them, activation of NF-kB and upregulation of IL-8 are crucial[62]. Suppressor gene P53 also plays an important role in gastric inflammation and carcinogenesis. Mutations of p53 have been related to the development of gastric cancer. In fact, inactivation of p53 gene is present in 40% of gastric tumors and has been specifically found in patients infected with the CagA positive strain of *H. pylori*[63]. *H. pylori* infection has also been related to epigenetic modification during gastric carcinogenesis by promoting hypermethylation of O6-mehylguanine DNA methyltransferase (MGMT). MGMT protein is essential for the repair of O6-methylguanine, which prevents mutations during DNA replication. Therefore, reduced levels of MGMT can increase mutagenesis in the gastric epithelium[64]. Finally, *H. pylori* has been linked to the oxidative stress that promotes DNA damage in gastric tissue by stimulating the production of intracellular reactive oxygen and nitrogen species in gastric tissues, which can damage DNA tumor suppressor genes like p53 and contribute to gastric carcinogenesis[65].

In summary, the *H. pylori* infection is one of the leading factors in the development of gastric carcinoma. Chronic inflammation induced by *H. pylori* is related to cell proliferation, apoptosis, epigenetic changes of tumor suppressor genes, and alterations of the oxidative stress mechanisms. Eradication of *H. pylori*, especially in endemic areas, should be a healthcare priority in order to decrease gastric cancer-related casualties.

GALLBLADER CANCER

Gallbladder cancer (GC) is a common hepatobiliary malignancy that carries very poor prognosis. It is the fifth commonest gastrointestinal tract cancer and is endemic is several countries, with the highest incidence rate reported in Delhi (India), Pakistan and Quito (Ecuador). It shows female preponderance[66], and genetic, infectious, and lifestyle factors have been associated with GC[67]. Infection leads to chronic gallbladder inflammation that seems to contribute to carcinogenesis. Two species-Salmonella Typhi and Helicobacter Pylori- and a liver parasite -Clonorchis sinensis-, among others, have been specifically linked to GC.

Salmonella Typhi

The association between chronic typhoid infection and GC was first reported in 1971 [68] and confirmed by ulterior studies. For example, in 1964, during the typhoid outbreak in Aberdeen (Scotland), Caygill *et al* studied the mortality rate of infected population and reported a 6% of risk of death attributable to GC among chronic carriers[69].

Further studies from Northern India reported a 7.9-fold increased risk of GC among infected persons. Species of Salmonella typhi and paratyphi-A could be isolated from bile, gallbladder tissue and stones in patients with GC. Bacterial serologic detection was related to a 9.2-fold increased risk of developing GC.

Another study from India, using nested PCR technique in hepatobiliary specimens, detected a 67.3% of typhoid carriers among GC patients compared to 8.3% of controls. The authors concluded that the liver served as a preferential survival niche for *Salmonella* Typhi (*S*. Typhi)[70]. As *Salmonella* spp. are excreted in the gallbladder, the multiplying bacteria secrete toxins and metabolites that favor mutational changes. Several carcinogens have been proposed: bacterial glucuronidase, bacterial enzymes and the production of nitrous compounds from nitrates[71,72].

Additionally, *S*. Typhi produces a genotoxin, the cytolethal distending toxin (CDT) that allows bacteria to internalize inside the host cells. Then, they are able to avoid the usual endocytic pathway that leads to the formation of destructive lysosomes and finally reaches a specific intracellular compartment where it can survive and replicate [73]. Once in the cytosol, the CDT facilitates the persistence of infection thanks to its immunomodulatory activity. Later on, it can reach the nucleus causing irreversible DNA damage[74].

Helicobacter spp.

H. bilis, *H. pullorum*, *H. hepaticus,* and *H. pylori* have been suspected to cause biliary tract diseases. *Helicobacter* spp. cause persistent infection in the biliary tract inducing chronic inflammation and gallstone formation, mainly *via* urease production[75,76]. *Helicobacter* spp. can produce several carcinogenic toxins and metabolites that initiate malignant cell transformation within the gallbladder[77]. Helicobacter bilis specific DNA sequences have been amplified in 27.2% of gallbladder and in 33.3% of biliary duct cancers[77].

Escherichia coli

Escherichia coli (*E. coli*) is part of the regular human intestinal microbiota but may become highly pathogenic following the acquisition of virulence factors, like the cytotoxic necrotizing factor I -CDT I-. The presence of genotoxin colibactin, related to *E. coli* infection, is known to be capable of inducing double stranded DNA breaks[10].

Opisthorchis viverrini and Clonorchis sinensis

Liver fluke *Clonorchis sinensis* (*C. sinensis*) is a high-risk pathogenic parasitic helminth endemic in some Asian countries. This parasite is classified among the Group I of human carcinogens by the IARCC[1]. Fresh water snails and several species of fish serve as secondary intermediate hosts. Humans and other fish-eating animals are infected through the ingestion of raw or undercooked freshwater fish that contains

meta-cercariae. After ingestion, the meta-cercaria excysts in the duodenum and ascends the biliary tract through the ampulla of Vater[78].

Infection with *Opisthorchis viverrini* has been related to cholangiocarcinoma upon the results of several cross-sectional and case-control studies[79]. The association between *C. sinensis* infection and cholangiocarcinoma is even better substantiated and the IARCC has already classified it as a probable carcinogenic for humans (Group 2A) [80,81].

Liver fluke pathogens cause mechanical injury and inflammation in the biliary tree, leading to metaplasia of mucin-producing cells, periductal fibrosis and hyperplasia of epithelial cells[82]. The severity of these changes correlates with the duration of the infection, the parasite load and the susceptibility of the host. The molecular mechanisms involved in the development of cholangiocarcinoma are poorly known, and a multistep process has been proposed: chronic inflammation damages cholangiocytes which in turn promotes cell proliferation and genetic and epigenetic mutations, finally transforming cholangiocytes into malignant cells[83]. Other carcinogenetic mechanisms include genomic instability, transcriptomic, proteomic and microRNA profile alterations and dysregulation of immune response.

LIVER CANCER

Worldwide, liver cancer is the sixth most common cancer, yet the second leading cause of cancer-related death in men, with 745000 deaths per year[84]. Hepatocellular carcinoma (HCC) represents approximately 90% of all primary liver cancers. It preferentially affects males and presents a wide geographical variation. However, chronic Hepatitis B virus and HCV infections are the leading cause of HCC, being responsible for 60%-80% of all these tumors worldwide, especially in developing countries.

Hepatitis B virus (HBV) is a partially double-stranded DNA virus that replicates *via* reverse transcription. It is highly contagious and is transmitted by percutaneous and permucosal exposure to infected blood and other body fluids. HBV particles package the incomplete double-stranded DNA into the nucleus of the host, where the virus is recognized as damaged and induces a DNA repair response, resulting in virus replication[85]. Chronic HBV infection is one of the leading causes of HCC worldwide. HBV typically replicates inside the hepatocytes. Virions bind to the surface of the hepatocytes and the nucleocapsid is released into the cytoplasm and translocated by microtubules to the microtubule-organizing center near the nucleus. Once inside the nucleus, HBV can lead to histone degradation, which enhances chromatin dynamics and may promote genetic instability, chromosomal alterations, and can also initiate oncogene mutation[86]. However, HBV integration into the hepatocytes occurs randomly -at one or multiple sites- and occasionally may promote direct oncogene activation or inactivate tumor suppressor genes[87]. While viral integration is an early event, selective clonal amplification of hepatocytes occurs throughout progression of the disease[88]. It is known that both the HBV-encoded envelope and the regulatory HBx protein directly contribute to hepatocyte transformation: HBx regulates expression of many genes involved in signal transduction pathways, cell cycle control, metastases, and avoidance of immune response. Several cohorts and case-control studies have assessed the risk of HCC among individuals infected with HBV[89,90]. The ability of antivirals to inhibit HCC progression is limited, likely because hepatocarcinogenesis seems to occur prior to the onset of liver fibrosis/cirrhosis. Therefore, once the disease is established, there is no turning back.

HCV is a single-stranded, positive sense RNA virus that encodes a single polyprotein that can form structural -which constitute the viral particle, such as the core protein- and non-structural proteins which support viral genomic replication. Seven genotypes have been described. Epidemiological studies show than infection with genotypes 1b and 3 is associated with an increased risk of developing HCC[91]. HCV replicates in the cytoplasm of hepatocytes and is unique among cancer-causing viruses by not encoding oncoproteins or integrating its genome into the host chromosomal DNA. In fact, HCV core genes variants have been associated with HCC even in patients in which the infection had already disappeared[92]. This suggests that viral factors influence progressive liver disease. HCV associated carcinogenesis includes increased hepatocyte proliferation and steatosis; virus-induced inflammation and oxidative stress which induces genomic mutations and genome instability; mitochondrial damage and induction of reactive oxygen species; and inhibition of host immune responses[93]. HCC is associated with the development of multifocal, genetically distinct tumors throughout the liver, suggesting that the entire organ is

altered.

COLON CANCER

Colorectal cancer is the leading cause of mortality regarding gastrointestinal neoplasms worldwide[94]. Pathogenic microorganisms able to induce intestinal dysbiosis have become the spotlight of current research in this area, as they carry the potential for colorectal tumorigenesis. Fusobacterium nucleatum, *E. coli*, *Bacterioides fragilis* (*B. fragilis*) and *Salmonella enterica* (*S. enterica*) have been reported as high-risk oncogenic pathogens.

Fusobacterium nucleatum

Fusobacterium nucleatum (*F. nucleatum*) is an adherent and invasive Gram-negative anaerobic bacterium frequently found in the oral cavity. Current research relates *F. nucleatum* to the development and progression of colon cancer, and has been found in primary lesions and stools of patients with colon cancer[95], especially those located in the cecum and rectum[96]. Tumoral cells over-express Gal-GalNAc molecules, which promote bacterial adhesion through the Fap2 protein[97].

Additionally, *F. nucleatum* infection has been related to a decreased survival rate among colon cancer patients and an increased resistance to chemotherapy agents[98, 99]. The presence of *F. nucleatum* in colon cancer patients varies worldwide, ranging from 15% in North American to 60% in Chinese patients[100]. Interestingly, tumors infected with *F. nucleatum* show three similarities: they are microsatellite unstable, show a methylation phenotype of CpG island, and exhibit mutations in the BRAF/KRAS genes[101]. Microsatellite instability is responsible for the ability of infected cells to elude the immune response[102] and has also been related to the activation of beta-catenin signaling pathway, commonly unregulated in colon cancer [103]. *F. nucleatum* also promotes inflammation, by increasing TNF-alfa and IL-10 Levels in adenomas and Il-6 and IL-8 in carcinomas, both regulated by the NF-kB transcription factor[104].

E. coli

E. coli is a widely distributed Gram-negative bacterium that can alter the intestinal microbiome. The B2 *E. coli* strains have been related to colon cancer[105]. *E. coli* promotes colon pathologic inflammation -as in Chron's disease- which seems to be a relevant factor in colon cancer formation[106]. However, the exact role of *E. coli* in the pathogenesis of colon cancer is not completely known. Recent studies have identified two potentially pathogenic *E. coli* strains: Adherent-invasive *E. coli* (AIEC) and entero-pathogenic *E. coli* (EPEC). During infection, AIEC binds to CEACAM6 (a cellular adhesion receptor associated to carcinoembryonic antigen- CEA- which is overex-pressed in colon cancer cells[107]. Infection stimulates IL6 production, which together with the increased expression of CEACAM6 can promote carcinogenesis. AIEC also secretes colibactin, a secondary metabolite associated to DNA damage acting as an alkylating agent and promoting tumor development[108]. EPEC is thought to stimulate macrophage-inhibitory cytokine-1 production - a cytokine related to metastasis- and inducing autophosphorylation of EGFR receptor[109,110].

B. fragilis

The *Bacteroides* spp. are regularly found in the human intestine and comprises 30% of the microbiota[111]. *B. fragilis* is an anaerobic Gram-negative bacterium colonizing about 0.5%-2% of the entire human intestine. A toxigenic *B. fragilis* strain -also known as ETBF- has a pathogenicity island encoding a metalloproteinase, the *B. fragilis* toxin (BFT) which is associated to increased inflammatory bowel disease and colitis, both considered high risk factors to develop colon cancer[112]. *B. fragilis* has also been detected in stool from cancer patients. Although the exact role of enterotoxigenic *B. fragilis* has not been completely described, it is believed that carcinogenesis is either induced by BFT toxin secretion or through host immune system dysregulation[113]. It can also trigger carcinogenesis through the beta catenin pathway activation by disrupting the adherent e-cadherin gap unions[114].

S. enterica

S. enterica includes serotypes of Salmonella typhi, Salmonella paratyphi, Salmonella enteritidis and Salmonella typhimurium. In recent years, it has been found that bacteria may modulate host immune response in two different ways: First, it can

promote carcinogenesis inducing both DNA damage and increasing cell abnormal proliferation; and second, it can induce cell migration as a result of chronic inflammation[17].

Two proteins of *S. enterica* have been associated with an increased risk of developing colon cancer: typhoid toxin and AvrA effector protein. Typhoid toxin is a cyclomodulin that, similar to *E. coli* CDT, increases cell survival and promotes intestinal dysbiosis, resulting in the development of inflammatory bowel disease and colon cancer[115,116]. AvrA is secreted by bacteria and has been found in stool samples from colon cancer[117]. AvrA may promote inflammatory and immune dysregulation through several mechanisms: the inhibition of NF kappa-beta signaling pathway, the inhibition of IL-12, INF-c and TNF-α secretion, the inhibition of IL-6 transcription, and the increase of IL-10 transcription[118,119]. On the other hand, AvrA might also activate the Wnt/β catenin pathway inducing cellular proliferation, by both β catenin phosphorylation and deubiquitination[120].

ANAL CANAL

Anal cancer refers to the malignancy of the intestinal mucosa arising in the anatomic anal canal, defined as beginning at the dentate line and ending at the anal verge. Eighty-five percent of anal tumors are of squamous cell origin, approximately 10% are adenocarcinomas, and the remaining 5% are other neoplasms (melanoma, small cell carcinoma…)[121]. Although it comprises 2.7% of all gastrointestinal malignancies, anal cancer incidence has been increasing over the last few decades[122].

As previously highlighted in the paper, HPV are small DNA viruses that infect various epithelial tissues. They also include the anogenital tract, and HPV is recognized as the causative agent of more than 80% of cases of anal cancer[123]. The difference in their ability to promote malignant transformation is the basis for the classification of HPV into low and high-risk variants. Mainly two HPV oncogenic subtypes, 16 and 18, are related to the development of squamous anal cancer[124]. HPV can integrate into the host DNA. Epithelial cells that harbor integrated HPV 16 DNA have a selective growth advantage over cells that carry normal extrachromosomal viral genomes; this growth advantage correlates with the increased expression of two viral genes in particular, E6 and E7[125]. The early proteins, E6 and E7, bind and inactivate the tumor-suppressor gene p53, and the retinoblastoma tumor-suppressor protein (pRb), respectively[126-128].

GUT MICROBIOTA

Most of the literature summarized in the present paper has been published previous to 2016 as research over the last years is focused in the role of gut microbiota in human carcinogenesis. Although it is not the scope of our review, we cannot conclude our review without summarizing intestinal microbiota key points. Gut microbiota consists of viruses, fungi and more than 1000 bacteria which are crucial for maintaining the gut barrier, metabolism and immunity. Disruption of the host relationship with the microbiota might result in oxidative stress, chronic inflammation and dysbiosis which can finally promote carcinogenesis[129]. The influence of gut microbiota relies mainly on cancers in the gastrointestinal tract, and the regulation of microbiota by diet, prebiotics, probiotics, symbiotics and antibiotics are proposed as new strategies to be explored in the future[130]. Gut microbiota is thought to play an important role in colon -stool samples of patients with CRC have higher proportions of Escherichia and Fusobacterium and lower concentrations of Firmicutes and Actinobacteria[131], HCC [132] and GC[133], Interactions between gut microbiota and GI cancers are likely to yield new opportunities to reduce cancer morbidity and mortality (Table 1).

CONCLUSION

Cancers arise from the transformation of a single cell so that its behavior is no longer under the control of normal regulatory pathways. The relationship between some pathogens and gastrointestinal tumor carcinogenesis has been well stablished. However, this relation is difficult to determine with many other agents as cancer is a multistep process. Besides, the presence of bacteria at the site of a tumor does not itself

implies causation. There is a long time period between the onset of carcinogenesis and the development of overt disease, and randomized studies are expensive and extremely difficult to make (one cannot, for example, infect a person with an agent and then wait to see if cancer develops). However, the occurrence of cancer attributable to infections is a global concern especially in underdeveloped regions. Chronic infections can mimic precancerous lesions that could be treated with antibiotics or antiviral treatment and therefore prevent the onset of carcinogenesis. Greater understanding of the consequences of long-term infections will help to elucidate the exact pathogenic processes involved in the development of some pathogen-related neoplasms. Preventive measures (like vaccines and antimicrobial agents) aimed to eradicate these oncogenic microorganisms should ideally interrupt the carcinogenetic pathways and avoid the formation of a relevant proportion of gastrointestinal cancers. Only global effort and an international strategy might help to decrease gastrointestinal cancer related deaths secondary to infectious pathogens. New molecular techniques are needed to identify new infectious agents in tissues. However, caution is requested not to overemphasize the association between pathogens and gastrointestinal malignancies without a proper causation proof.

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REVIEW

Newer developments in viral hepatitis: Looking beyond hepatotropic viruses

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Abstract

Viral hepatitis in the entirety of its clinical spectrum is vast and most discussion are often restricted to hepatotropic viral infections, including hepatitis virus (A to E). With the advent of more advanced diagnostic techniques, it has now become possible to diagnose patients with non-hepatotropic viral infection in patients with hepatitis. Majority of these viruses belong to the Herpes family, with characteristic feature of latency. With the increase in the rate of liver transplantation globally, especially for the indication of acute hepatitis, it becomes even more relevant to identify non hepatotropic viral infection as the primary hepatic insult. Immunosuppression post-transplant is an established cause of reactivation of a number of viral infections that could then indirectly cause hepatic injury. Antiviral agents may be utilized for treatment of most of these infections, although data supporting their role is derived primarily from case reports. There are no current guidelines to manage patients suspected to have viral hepatitis secondary to non-hepatotropic viral infection, a gap that needs to be addressed. In this review article, the authors analyze the common non hepatotropic viral infections contributing to viral hepatitis, with emphasis on recent advances on diagnosis, management and role of liver transplantation.

Key Words: Hepatitis; Non hepatotropic viruses; Cytomegalovirus; Herpes simplex virus; Coronavirus-2019; Liver transplant

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Core Tip: With growing numbers of patients receiving solid organ transplantation including liver transplant, and subsequent immunosuppression there is an increasing incidence of non-hepatotropic viruses causing hepatitis. Several gaps exist in the diagnosis and management of such patients. Through this review article we attempt to outline the important non hepatotropic viruses causing liver injury. We also address the challenges in diagnosis, current and future prospects in treatment as well as prevention of these infections.

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INTRODUCTION

Viral hepatitis in the entirety of its clinical spectrum is vast and most discussion are often restricted to hepatotropic viral infections, including hepatitis virus (A to E). With the advent of more advanced diagnostic techniques, it has now become possible to diagnose patients with non-hepatotropic viral infection in patients with hepatitis. Non hepatotropic viruses do not infect the liver as the primary organ. These infections can present as hepatitis as a part of systemic infection. In a study performed in India, 10.5% of the patients with acute hepatitis and acute on chronic hepatitis were found to be secondary to non-hepatotropic viral infection[1]. Majority of these viruses belong to the Herpes family, with characteristic feature of latency. With the increase in the rate of liver transplantation globally, especially for the indication of acute hepatitis, it becomes even more relevant to identify non hepatotropic viral infection as the primary hepatic insult. Immunosuppression post-transplant is an established cause of reactivation of a number of viral infections that could then indirectly cause hepatic injury. Antiviral agents may be utilized for treatment of most of these infections, although data supporting their role is derived primarily from case reports. There are no current guidelines to manage patients suspected to have viral hepatitis secondary to non-hepatotropic viral infection, a gap that needs to be addressed. In this review article, the authors analyze the common non hepatotropic viral infections contributing to viral hepatitis, with emphasis on recent advances on diagnosis, management and role of liver transplantation.

ETIOLOGY OF VIRAL HEPATITIS

The most important and common cause of viral hepatitis is infection with hepatotropic virus, including hepatitis A-E. However, a small percentage of individuals exhibit signs and symptoms of hepatitis without testing positive for any of the hepatotropic viruses. In such patients, the differential diagnosis should be expanded to include other non-hepatotropic virus, listed in Table 1.

CYTOMEGALOVIRUS

Epidemiology

Cytomegalovirus (CMV) or human herpes virus-5 (HHV-5) belongs to the herpesvirus family. It is an enveloped, double-stranded DNA virus that remains latent in the body in two-thirds of the patients after primary infection. The capacity of the virus to remain latent in the host cells leads to risk of endogenous reactivation in a susceptible host, in addition to the risk of exogenous transmission. It is one of the most common viruses causing chronic infections as reflected in seroprevalence rates (ranging from 40%-100%) in adults and increases with age[2]. Demographic variability exists; women, non-white population and people belonging to the lower socioeconomic strata exhibit a higher prevalence[3-5]. CMV infection and disease are defined as distinct entities. CMV infection is any evidence of replication of the virus regardless of

Table 1 Example of non-hepatotropic viral infection causing hepatitis

Herpesvirus	HSV1, HSV2, HHV6, HHV7, HHV8, EBV, CMV, VZV
Adenovirus	
Enterovirus	Coxsackie B virus, Echovirus
Paramyxovirus	Measles
Togavirus	Rubella
Parvovirus	Parvovirus B19
Coronavirus	COVID-19

COVID-19: Coronavirus disease 2019; HSV: Herpes simplex virus; HHV: Human herpes virus; EBV: Epstein-Barr virus; CMV: Cytomegalovirus.

symptoms whereas CMV disease is infection along with symptoms that are explained by the virus[6]. CMV hepatitis is exceedingly rare in immunocompetent individuals and is more prevalent in immunocompromised patients, particularly post liver transplant (LT)[7]. Based on data available from population-based studies, 1%-4% of adults with acute hepatitis are due to CMV in developed countries[1].

Pathogenesis and clinical features

The transmission of CMV is through multiple routes including sexual exposure and close contact with bodily fluids such as saliva and breast milk[8]. The virus initially infects mucosal epithelial cells, and has broad cellular tropism allowing it to interact with a myriad of cell surfaces. Systemic dissemination occurs hematogenously with polymorphonuclear leukocytes, macrophages in the gastrointestinal and pulmonary tissues, and infected monocytes, all playing a role[9-12]. CMV has a predilection for hematopoietic, connective tissue and parenchymal cells; it specifically infects hepatocytes and macrophages in the hepatic tissue[11,13]. The virus then has complex interactions with the immune system leading to the repression of the primary infection, which is often followed by the stage of latent infection[14]. It plays a role in modulating both the humoral and adaptive immune responses in humans[15]. The sinusoidal endothelial cells of the liver, instead of being a barrier, provide an ideal environment for viral dispersion through the organ and act as sites for latency and reactivation[16,17]. The sinusoidal cells also facilitate immune activation in the liver by modulating T cell recruitment and activation *via* trans endothelial migration of CXCL10 and ICAM-1 dependent CD4+ T cells[18]. Notably, the sinusoidal cells play a role in viral latency, reactivation and dissemination within the liver but have a limited capacity for viral replication. Hepatocytes, on the other hand, play a major role in viral reproduction but have a limited role in latency. The pathogenesis of CMV disease is summarized in Figure 1.

Various factors lead to the reactivation of the virus such as allogeneic transplantation (especially those receiving anti-lymphocytic drugs), ischemia/ reperfusion, sepsis, immune cell depletion, injury and other inflammatory states[19].Immunosuppressant medications like corticosteroids and cyclosporine do not directly cause reactivation but can facilitate viral replication[20-22]. Allograft rejection is an important risk factor as well a consequence of CMV disease[22,23].

CMV causes indirect cytotoxicity in the liver *via* cytotoxic T cell activation and alterations in vasculature, subsequently causing necrosis[24-26]. Additionally, it also has a direct cytotoxic effect on hepatocytes as evidenced in a study by Sinzger *et al*[11] that demonstrated lysis of CMV infected hepatocytes. Thus, in contrast to other herpetic infections such as Epstein-Barr virus (EBV), CMV affects the liver both indirectly *via* continuous immune activation and cytokine release as well as with direct cytotoxicity.

The clinical features vary according to the patient's immune status. Both stages, acute and chronic stages, are seen with the viral infection. In immunocompetent patients, a mononucleosis-like syndrome is seen with splenomegaly and hepatic dysfunction. Only case reports exist describing uncommonly seen CMV hepatitis in immunocompetent hosts[11,13]. Immunocompromised patients, especially LT patients, have a high incidence of CMV tissue invasive disease including hepatitis, esophagitis, gastritis, enteritis and/or colitis[21,23,27]. The risk of CMV hepatitis occurs with the highest frequency in the combination of seropositive donor/ seronegative recipient patients (incidence estimate of 44%-65%), followed by the

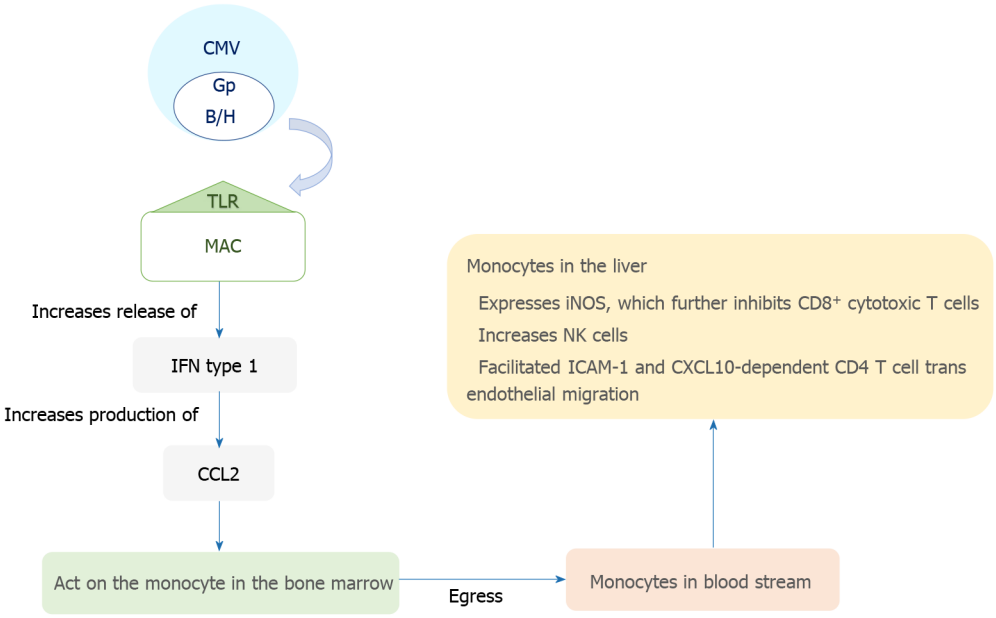

Figure 1 Pathogenesis of cytomegalovirus disease in the liver. CMV: Cytomegalovirus; Gp: Glycoprotein; TLR: Toll-like receptors; MAC: Macrophages; IFN: Interferon; CCL2: Cytokine; Inos: Inducible nitric oxide synthase.

combination of seropositive donor/seropositive recipient patients or seronegative donor/seropositive recipient (8%-18%), and with the least frequency in the combination of seronegative donor/seronegative recipient patients (1%-2%)[23,28].

A study by Toghill *et al*[29] studied 70 patients with cirrhosis due to a variety of causes including alcoholic cirrhosis, primary biliary cirrhosis, secondary biliary cirrhosis, hemochromatosis, congenital hepatic fibrosis and cryptogenic cirrhosis. The authors did not find evidence of CMV disease as the cause for the liver cirrhosis and the antibody titers in these patients were similar to that of the general population. CMV disease was, however, found to be an important cause of chronic rejection in post-LT patients and associated with increased mortality in patients with cirrhosis[30-32]. Liver involvement in CMV is varied and can manifest as mild hepatitis, necrotizing hepatitis, granulomatous hepatitis or even portal vein thrombosis.

Diagnosis and treatment

The diagnosis of CMV is starts with serological testing to detect CMV IgM and IgG antibodies, antigenic testing of CMV pp65 that detects CMV antigens in leukocytes, polymerase chain reaction (PCR), culture and biopsy. Serological testing can provide risk assessment prior to LT but its role is limited in the diagnosis of CMV in immunocompromised patients due to the inability of these patients to mount an immune response[20,33,34]. In immunocompetent patients, serological tests may be falsely positive due to cross reactivity with other herpetic viruses, persistence of antibody levels after primary infection, reactivation or presence of rheumatoid factor[32,35].

Serological tests may provide quick diagnosis in immunocompetent patients after other etiologies of hepatitis have been ruled out. The pp65 antigen assay has a sensitivity of 64% and a specificity of 81% but since it detects antigens in leukocytes, it may not be reliable in patients with leukopenia[35,36]. The utility of viral culture is limited due to the long turnaround time, with one study demonstrating sensitivity of only 52% with cell culture[37]. The use of shell vial assay has the advantage of faster turnaround time (12 h), similar specificity to traditional culture and higher sensitivity [38]. PCR has a high sensitivity and specificity, ranging from 61%-92% and 75%-99%, respectively[39,40]. It can provide both quantitative and qualitative measurements from body fluids or tissue samples and is particularly useful in immunocompromised patients to determine the need for preemptive therapy and monitoring disease response[35,41]. While liver biopsy is not mandatory for diagnosis, it may be required when the diagnosis is uncertain. It is also required in LT patients to distinguish between acute graft rejection and CMV infection since CMV is a risk factor for rejection[8,28]. CMV hepatitis has characteristic histology with cytoplasmic and intranuclear inclusion bodies, nonspecific hepatocellular necrosis, mononuclear cell infiltrate and micro abscesses[42,43]. The degree of inflammation on the biopsy

depends on the immune status of the patient. To increase the sensitivity, immunohistochemistry and/or DNA hybridization can be added to the liver biopsy[6,44].

Agents acting on CMV DNA polymerase including ganciclovir, valganciclovir, foscarnet and cidofovir are recommended for treatment of CMV hepatitis in immunocompromised individuals[6,28]. Immunocompetent patients usually have self-limited infectious mononucleosis (IM) like syndrome which does not require treatment. In immunocompetent patients with severe disease, limited data suggests using the above mentioned anti-viral agents[45-47]. There also have been reports of acute liver failure (ALF) from CMV hepatitis requiring LT[47-49].

LT

About 18%-29% patients receiving LT are affected by CMV disease and it remains one of the most common infectious complications following solid organ transplant (SOT)[28,50]. Infection in the LT recipient can either be a primary infection, re-infection or reactivation of the latent virus. CMV disease in LT patients leads to other co-morbidities such as acute or chronic rejection, graft loss, post-transplant lymphoproliferative disorders (PTLD), increased infections, vascular thrombosis and increased mortality[28,51]. Increased rates of bacterial infection, invasive fungal infection such as Nocardia and viral co-infection such as EBV, HHV6, HHV7 and HCV has been described in literature[28,52-54].

Two basic approaches have been proposed to prevent CMV disease post-liver transplantation: Prophylactic and pre-emptive. The prophylactic approach refers to treatment which is immediately started post-transplant and continued for three to six months while the pre-emptive therapy refers to close monitoring for evidence of CMV replication with prompt initiation of antiviral therapy upon detection[23]. Both approaches have been shown to have comparable efficacy [0.34, 95% confidence interval (CI): 0.24-0.48 with prophylactic approach *vs* 0.30, 95%CI: 0.15-0.60 with preventative approach] in a meta-analysis. Notably, the population used in this meta-analysis was treated with ganciclovir as opposed to preferred alternative, valganciclovir[55].

For high-risk recipients (seropositive donor/seronegative recipient), prophylactic therapy is preferred with acyclovir, valacyclovir, intravenous ganciclovir and valganciclovir, if available for use[6,22]. Valganciclovir has demonstrated better efficacy, lower incidence at 6 mo and 12 mo follow up and better safety profile in multiple studies[21-23]. Preemptive therapy requires resource intensive monitoring which may not be achievable in all clinical settings. It can still be employed for high-risk LT patients (seropositive donor/seronegative recipient) and intermediate risk LT patients (seropositive donor/seropositive recipient, seronegative donor/seropositive recipient). Intermediate risk LT patients can also be managed with prophylactic therapy[13]. Low-risk LT patients (seronegative donor/seronegative recipient) do not require routine prophylaxis. Table 2 outlines the strategies for CMV prevention in LT patients based on risk stratification.

Ongoing research and future directions

Another high-risk patient population for CMV disease are patients undergoing hematopoietic stem cell transplant patients (HSCT). This field is rapidly evolving with ongoing research on multiple strategies for management of disease and risk mitigation. The concept of adoptive transfer of T-cells with protective effects against CMV is currently being studied[56-58]. Letromovir, a viral terminase complex inhibitor, has been approved for prophylactic CMV treatment for HSCT transplant patients and acts against both viral replication as well as latent infection[59]. Maribavir, an inhibitor of the viral kinase UL97, is also being evaluated in patients undergoing HSCT and has shown better safety profile with regards to hematologic side effects as well as nephrotoxic effects when compared to ganciclovir and valganciclovir[60]. A phase III trial comparing maribavir and placebo did not show any difference in patients with HSCT[61]. However, the trial used low-dose maribavir and repeating the trial with higher doses may reveal different, perhaps, positive results[62]. Maribavir is also being evaluated in an ongoing phase III clinical trial as a treatment for CMV disease in transplant recipients with resistance to ganciclovir, cidofovir and foscarnet (NCT02931539). The therapies used in HSCT patients may have a future in patients undergoing liver transplantation, given the overlap in immune status. Therapies against CMV latency can have significant clinical benefits. As indicated by *in vitro* studies, vincristine has the potential to be a therapeutic agent with the ability to kill latent infected cells; however, its use is limited by the extensive adverse effect profile[63]. A protein named F49A-fusion toxin protein (FTP) which kills infected cells has been developed, which may be a possible future therapeutic agent to

Table 2 Strategies for cytomegalovirus prevention in liver transplant patients based on risk status

Risk status	Donor/Recipient CMV serological status	Prevention strategy
High risk	Donor positive/recipient negative	Prophylactic therapy for 3-6 mo
		Or
		Pre-emptive therapy requiring close monitoring
Intermediate risk	Donor positive/recipient positive	Prophylactic therapy for 3 mo
		Or
		Pre-emptive therapy requiring close monitoring
Intermediate risk	Donor negative/recipient positive	Prophylactic therapy for 3 mo
		Or
		Pre-emptive therapy requiring close monitoring
Low risk	Donor negative/recipient negative	No routing prophylaxis

CMV: Cytomegalovirus.

target latent disease[13,64]. Apart from this, studies have also suggested using immunotherapeutic strategies which force the virus to be partially reactive only to be detected and demolished by the host immune system[14,65].

Several vaccine candidates have been developed including live attenuated viral vaccines, and subunit vaccines against CMV phosphoprotein 65 and glycoprotein[13,66]. Till date, the most efficacious results are from a subunit recombinant vaccine against CMV glycoprotein with MF59 adjuvant indicating 50% efficacy in young mothers as well as in recipient negative/donor positive transplant patients[67].

EBV

Epidemiology

The most common presentation of primary EBV is IM which manifests as fever, cervical lymphadenopathy, tonsillitis and splenomegaly. In 90% of these cases, abnormal liver function tests are noted with hepatomegaly observed in about 14% cases[68]. However, a much smaller percentage of the population, estimated to be 0.85%-1% in population-based studies, are diagnosed with EBV hepatitis[69,70]. According to the available literature, the incidence of ALF secondary to EBV is estimated to be 0.21%[71]. In a recently published Russian study, EBV DNA was detected in 58.1% of the patients with viral hepatitis and correlation indicated worse outcomes in hepatitis C patients, coinfected with EBV[72]. The median age for EBV hepatitis in a British population-based study was noted to be 40 years and 41% of the individuals were above the age of 60 years[70]. Subsequently, another population-based study indicated the median age of patients to be 17 years, overlapping the age group most commonly affected by IM[69]. The scarcity of data and the difficulty in determining causation of EBV in patients with viral hepatitis or hepatitis of unknown etiology stems largely from lack of a diagnostic criteria. This forms the basis for the need to develop better diagnostic tools to identify these patients and initiate early treatment.

Pathogenesis and clinical features

EBV or herpes human virus 4 belongs to the family of herpesvirus and has predilection for epithelial cells of the oropharynx and B lymphocytes. Once the virus infects B lymphocytes, it causes polyclonal expansion of T lymphocytes (specifically cytotoxic CD8 T cells). As EBV does not directly infect hepatocyte, vascular or biliary epithelium, the primary mechanism of damage is mediated indirectly through cellular immune responses. In majority immunocompetent patients (approximately 90%), hepatic involvement is subacute, mild, anicteric and self-limiting. In rarer cases, despite immunocompetence, the involvement can be acutely severe, recurrent or chronic[69,70]. In immunocompromised individuals, severe hepatitis with icterus is more commonly seen[72].

Another important concern in immunocompromised individuals following transplantation is the development of PTLD. EBV has been recognized as the cause for development of PTLD in 70% cases and occurs due to unregulated replication of EBV infected B cells in an environment of T cell immunosuppression. Depending on the source of EBV infected B cells that generate the clone pathognomic of PTLD in these patients, the disorder can be classified as host-derived PTLD and donor-derived PTLD. In patients receiving hematopoietic stem cell transplant, PTLD is often systemic and secondary to activation of latent EBV infection in the host[73,74]. Following LT, one study showed latent EBV infection in the donor as a likely cause[75]. The clinical manifestations range from constitutional symptoms to extra nodal lymphadenopathy and organ dysfunction (including allograft dysfunction)[76].

Liver involvement as a result of EBV infection can also be a manifestation of hemophagocytic lymphohistiocytosis (HLH)[77-79]. This rare life-threatening clinical entity occurs as a result of excessive immune system activation, primarily of lymphocytes and macrophages, that results in severe cytopenia, coagulopathy and splenomegaly in addition to hepatitis[80].

Diagnosis

Liver enzymes, aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) are elevated up to 5-fold in majority of patients with subacute hepatitis presentation[81]. In the rare case that acute severe hepatitis develops, transaminase levels can exceed 5 times the upper normal limit. Serum bilirubin levels are elevated in only 5%-10% of the patients[68]. Cholestatic pattern of injury [elevated alkaline phosphatase (ALP) and gamma glutamyl transpeptidase (GGT)], compared to other viral etiologies, is seen in some patients with EBV[70]. As part of initial blood work, lymphocytosis with atypical lymphocytes is characteristically seen[82]. In the subgroup with HLH, additional laboratory abnormalities of note are bicytopenia (92% patients), hyperferritinemia (> 500 mcg/L in 94% patients) and hypofibrinogenemia (90% patients)[83]. Heterophile antibodies, although nonspecific, is rapid and has reasonable sensitivity ranging from 85%-100%, depending on assay used. The Paul Bunnell test (against sheep erythrocytes), the Monospot test (against horse erythrocytes) and the enzyme linked immunosorbent assay against other substrates such as ox or goat erythrocytes are some examples of widely available confirmatory tests for EBV infection[84]. In individuals with negative heterophile test but high clinical suspicion, further testing with specific antibody assays against EBV can be used. The immunogenic components of EBV used as basis for antibody testing are viral capsid antigen (VCA) and EBV nuclear antigen (EBNA). Given that 90%-95% of the general adult population in the United States is seropositive for anti-VCA IgG, it is difficult to use it as a diagnostic test in clinical practice[81]. The presence of anti-VCA IgM antibodies in the serum is considered to be a more reliable marker of active EBV infection and lasts for 4-6 wk after infection. IgG antibodies against EBNA, on the other hand, are established 6-12 wk after infection and are a marker for latency or convalescence. Thus, the combination of presence of anti-VCA IgM antibodies and with the absence of anti-EBNA-1 IgG antibodies is key to diagnosis of active EBV infection[85].Additionally, autoantibodies such as anti-nuclear antibodies, anti-smooth muscle antibodies may be seen in EBV infection due to cross reactivity of EBV proteins with cellular antigens. As a result, in immunocompromised individuals, autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus could hypothetically be triggered, further confounding the etiology of hepatitis[86].

In patients diagnosed with IM, the presence of elevated transaminase is sufficient to diagnose EBV hepatitis. However, the diagnosis of isolated EBV hepatitis in the absence of IM is trickier. Liver biopsy is indicated in these patients to establish etiology. The interpretation of the biopsy is challenging as a small percentage of EBV infected lymphocytes may be present in the liver in seropositive individuals without hepatitis. The diagnosis, thus, requires serum testing to establish the context for interpretation of the histopathological features in liver biopsy. Typically, portal and intra-sinusoidal lymphocytic infiltration (B and T cells) with few apoptotic cells is seen in EBV hepatitis. The most common lymphocytic population visualized in EBV hepatitis was CD3 positive cytotoxic T cells[71,87]. The diagnosis is further confirmed by either EBV-DNA PCR or EBER-RISH (EBV encoded RNA in situ hybridization), both methods demonstrating comparable sensitivity[88].

Management

In an analysis published in 2015, ALF secondary to EBV was shown to have a high case fatality rate. While the study population was treated with antivirals (acyclovir, famciclovir and ganciclovir) and high dose steroids, the efficacy of either treatment

option (alone or in combination) is not clearly established[89]. Antivirals such as ganciclovir have shown efficacy in both immunocompromised and immunocompetent individuals[90]. Oral valganciclovir has also been used in immunocompetent individuals, although with uncertain benefit[91]. While steroids have been used in acute hepatitis to limit inflammatory response, steroid use has been proposed to be associated with EBV reactivation, most likely at the time of withdrawal of high dose steroids. This mechanism is possibly the rebound increase of suppressed cytotoxic T cells that attack infected B cell infiltrate in latent EBV[92].

The definitive treatment for management of ALF currently remains LT. Orthotopic liver transplantation has been described in cases of fulminant hepatitis secondary to EBV infection[89,93]. Subsequent treatments with antivirals such as acyclovir has been suggested in a few case studies of patients developing fulminant hepatitis requiring orthotopic liver transplantation. The rationale behind it is similar to the concern outlined with steroid use in management of ongoing hepatitis. While immunosuppression is required post-transplant, reinfection of graft liver remains a possibility, warranting antiviral therapy[94].

Another important concern related to transplantation, both liver and HSCT, is the risk of development of PTLD. This risk can be lowered by cautious use of immunosuppression post-transplant (in terms of dosage, duration and choice of drug regiment), careful donor-recipient matching (in term of avoiding serodiscordant match) and antiviral prophylaxis. While there is no clear data supporting efficacy of antiviral prophylaxis for EBV in adults, oral acyclovir and intravenous ganciclovir have been used in patients receiving liver allograft. The milder spectrum of PTLD is seen to resolve with the cessation of tacrolimus[95]. In patients who cannot tolerate tapering or changing of immune suppression regiment or persist to have PTLD despite cessation of immune suppression, treatment with anti CD20 agent rituximab has shown success. Single agent treatment with rituximab has shown 40%-50% remission. Other therapeutic options for these patients include surgical removal of affected organ (if localized PTLD) or chemotherapy[96-101].

HERPES SIMPLEX VIRUS

Epidemiology

Herpes simplex virus (HSV) 1 and 2 affect the majority of adults in the western world with prevalence being 80% and 30% respectively. Like other members of the Herpesviridae family, the virus exhibits latency in the human body persisting in the neurons. Majority of patients who suffer from HSV hepatitis are immunocompromised such as organ transplant recipients, patients on immunosuppressive medications, patients with acquired immuno-deficiency syndrome, neonates and pregnant women in their second and third trimesters[42,102,103]. A study on HSV hepatitis with 137 patients revealed 24% patients were immunocompetent, 23% were pregnant and 53% patients were taking immunosuppressant medications either for organ transplantation or for other reasons[42,104]. HSV hepatitis can also affect immunocompetent patients[105]. Interestingly there have been case reports suggesting reactivation of latent HSV by inhaled anesthetic agents such as enflurane, isoflurane, desflurane and nitric oxide [105,106].

Pathogenesis and clinical features

A multitude of theories exist regarding HSV pathogenesis in causing hepatitis. As herpes is known to be a neurovirulent virus, studies have shown hepatovirulent strains of HSV that can cause fulminant hepatitis. Another theory suggests an acute infection superimposed on a latent HSV reactivation as causing liver failure. With regards to viral dissemination to the liver, one hypothesis suggests that the virus spreads to the liver from the herpetic lesions in the setting of impaired immunity and delayed type hypersensitivity reaction. While another suggests that during initial infection, a large inoculum of the virus may overwhelm the innate host defenses leading to dissemination to the visceral organs including the liver[107,108].

HSV hepatitis occurs during the primary infection and rarely as a reinfection in immunocompromised individuals. It presents with non-specific features such as fever, abdominal pain in the right upper quadrant, nausea/vomiting with jaundice rarely present. The characteristic herpetic skin rash is present in only about 18% to 50% of patients. Patients also present with leukopenia, thrombocytopenia, markedly elevated liver enzymes, and mild bilirubin increase[103]. Cases of fulminant hepatitis present with aminotransferase levels 50 to 100 times the upper limit of normal.

Patients may also develop acute kidney injury, disseminated intravascular coagulation, multi organ failure and eventually death. Up to 6% of fulminant hepatitis is associated with HSV with favorable outcomes after treatment[109]. With regards to viral related ALF, up to 2 % of cases are attributed to HSV hepatitis and less than 1% of all ALF are due to HSV[110]. These patients typically have a high mortality of up to 90%[111]. Risk factors associated with increased mortality are age > 40 years, immunocompromised status, coagulopathy, encephalopathy, degree of AST elevation and male gender[104].

Diagnosis and treatment

A thorough physical examination of the skin and pelvis should be conducted in patients with suspicion for HSV infection to detect characteristic herpetic lesions. HSV serology (IgM and IgG antibodies) have limited utility due to false negative and false positive results. PCR of HSV DNA utilizing blood samples is rapid, with a better yield than serology and even viral cultures[112,113]. A liver biopsy is imperative in the diagnosis of HSV hepatitis with typical biopsy findings of intranuclear inclusions (Cowdry Type A) occurring in the foci of coagulative or sometimes extensive hemorrhagic necrosis which are irregular in distribution[103]. There is a characteristic scarcity of inflammatory cells in the portal veins or the parenchyma under light microscopy[107]. Due to risk of increased bleeding with the percutaneous approach in patients with ALF, a trans jugular approach is preferred with consideration of administering factor VII recombinant to reduce the risk[113-115]. Computed tomography may reveal diffuse hypodense lesions along with hepatomegaly due to areas of focal necrosis but this is a nonspecific finding also seen in candida hepatitis, lymphoma, sarcoidosis. However, the clinically acute course along with the characteristic skin rash (if present) can help[116-118].

The disease is curable and carries a high mortality, hence treatment must be initiated as soon as possible. While no standardized guidelines or prospective studies exist, literature exists that has shown reduction in mortality and the need for LT from 88% to 51% in patients receiving treatment[104]. The most important aspect is that in patients with high suspicion of HSV hepatitis, empiric acyclovir should be considered until it is ruled out *via* PCR and/or biopsy. Cidofovir and foscarnet can be used in cases of acyclovir resistance which are quite uncommon about 0.27% in immunocompetent patients and 7% in immunocompromised patients[119,120]. Expert consensus recommends treatment from 2 wk to up to 4 wk[104]. Very limited data exists on the use of therapeutic plasmapheresis which theoretically works by removing infectious particles, reducing viral load and buying time for the immune system to mount a stronger response[121].

LT

An urgent LT is indicated in patients not responding to antiviral therapy as above as a final treatment option. Although disseminated HSV is not a contraindication for transplant, thorough evaluation is necessary since sepsis, and multi organ failure is usually present in these cases which can make it difficult to initiate an immunosuppressive regimen post-transplant. Patients who do receive transplant have a higher risk of HSV recurrence , and require life-long acyclovir which contributes towards acyclovir resistance[119,122,123].

In patients who have received LT, HSV tends to occur in the early post-operative period from 20 ± 12 d and is associated with increased mortality[124]. The early recurrence of HSV in LT patients may be due to acquisition of the virus from the donor or due to immunosuppression. A very high index of suspicion is to be maintained since acute cellular rejection or biliary complications are the commonest issues in the early post-operative period. Early diagnosis improves survival and patients should empirically be started on acyclovir as soon as the suspicion arises[125,126]. In patients with LT who are not receiving CMV prophylaxis which also has activity against HSV, prophylactic treatment is associated with low incidence on clinical disease[127].

Ongoing research and future directions

The first attempt at the HSV vaccine was in 1964 by Kern and Schiff[128]. Since a live attenuated vaccine was developed for varicella zoster virus, a member of the alphaherpesvirus, there was a possibility to develop a vaccine against HSV-2 as well[129]. Currently no effective vaccine exists for HSV-2, however, Heprevac- a truncated glycoprotein D2 (gD2) vaccine did show efficacy for prevention of genital HSV-1 disease (58%) and HSV-1 infection (32%) in a clinical trial[130].

Various types of vaccines including whole killed virus, attenuated virus, subunit vaccines (glycoprotein) as well as DNA based vaccines have been attempted to come up with a preventative/therapeutic vaccine against HSV-2[131]. A promising candidate comprising of HSV-2 glycoprotein D2 and infected cell particle 4 mice with matrix-M2 adjuvant provoked a humoral as well as a cell mediated response with acceptable safety profile in a clinical trial. Antiviral therapy along with the above-mentioned vaccine seems to be a promising approach for HSV-2 treatment[132].

SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS 2 OR CORONAVIRUS DISEASE 2019

Epidemiology

The coronavirus disease 2019 (COVID-19) pandemic has ravaged the world affecting over 3 million people worldwide, as of July 12, 2021. Case studies from China, where the pandemic emerged, indicated that 2%-11% patients affected by COVID-19 had prior liver comorbidities and abnormal levels of liver enzymes (ALT and AST) were seen in 14%-53% cases[133]. Prothrombin time abnormalities signifying synthetic function of liver were also seen in COVID-19 patients with gastrointestinal symptoms [134]. Another large study of 1099 patients across 552 hospitals in China demonstrated that patients with severe COVID-19 infection had abnormal liver enzyme levels as compared to those with less severe disease[135]. Li *et al*[136] conducted a study among COVID-19 patients and found that patients with elevated C-reactive protein levels greater than 20 mg/L and lymphopenia with counts less that 1.1×10^9 per liter were related to ALT elevation thus highlighting the fact that COVID-19 disease severity correlates with liver dysfunction[136].

Pathogenesis and clinical features

The pathogenesis of COVID-19 induced liver injury continues to evolve as we learn more about the virus. The virus is known to cause immune dysregulation causing systemic inflammatory response syndrome which causes release of inflammatory mediators including interleukins causing a cytokine storm causing hepatocellular injury with the intrahepatic cytotoxic T cells as well as Kupffer cells playing a role[137, 138]. The virus probably also has direct cytotoxic effect but the ACE2 receptors, which the virus has an affinity for, is expressed in the bile duct cells more than the hepatocytes[139]. It would thus be expected that patients would have elevated ALP levels but patients with COVID-19 hepatitis usually have elevated AST and ALT levels. The virus predominantly affects the lung and in severe disease causes refractory hypoxemia as well as hypotension leading to ischemic liver injury which adds up to another mechanism of liver induced injury caused by the virus. Another important consideration in the pathogenesis of liver dysfunction in patients with COVID-19 is the myriad of drugs that have been tried and are currently being used for treatment that cause hepatic injury *via* hepatocellular damage and cholestasis[140,141]. Reactivation of hepatitis B is associated with use of biological agents such as tocilizumab which has been used and studied in the treatment of COVID-19[142]. Thus a multitude of factors including inflammatory mediated damage, direct cytotoxicity, hypoxemia/hypotension mediated ischemic injury and drug induced injury contribute to the pathogenesis of hepatic damage in COVID-19. Figure 2 depicts the multiple factors contributing to hepatic injury in COVID-19.

The pattern of liver injury seen in COVID-19 is typically elevated AST and ALT levels with a predominance of AST elevation[143]. Serum bilirubin levels can also be mildly increased but it's relation to disease severity is unclear in contrast the levels of aminotransferases that correlate with disease severity[144]. Hypoalbuminemia can also be seen along with increased levels of GGT in severe cases, but the levels of ALP are usually normal in mild or severe cases[139,145]. A case report of a patient initially presenting with hepatitis that was later diagnosed as COVID-19 infection, has also been reported[146]. Patients with pre-existing liver disease may be more susceptible to suffer from liver damage from COVID-19 according to the meta-analysis done by Mantovani *et al*[138]. Patients with non-alcoholic fatty liver disease (NAFLD) were demonstrated to have higher risk of disease progression, longer viral shedding time and higher likelihood of abnormal liver function tests[147]. Table 3 describes relevant studies in the context of COVID-19 and liver disease[148-157].

Table 3 Studies studying coronavirus disease 2019 infection and liver disease

Ref.	Patients	Type	Study highlight with regards to liver disease
Xie *et al* [148]	79	Retrospective study	Liver injury maybe related to systemic inflammation and liver function should be monitored in patients with severe pulmonary lesions on imaging
Zhang *et al*[149]	115	Retrospective study	Liver enzymes as well as INR significantly elevated in patients with severe COVID-19; Albumin low in severe cases
Huang *et al*[145]	41	Prospective case series	Two percent patients had chronic liver disease; 37% patients had elevated AST which was more pronounced in ICU patients
Fan *et al* [141]	148	Retrospective case series	In patients with abnormal liver function, more received treatment with lopinavir/ritonavir as compared to those with normal liver function
Wang *et al* [150]	138	Retrospective study	Of 2.9% patients had chronic liver disease, AST elevation > ALT and seen more in ICU patients
Xu *et al* [151]	62	Retrospective study	Of 12% patients had underlying liver disease; 16% patients had elevated AST
Shi *et al* [152]	81	Retrospective study	AST more elevated in patients with increasing pulmonary lesions on imaging; 9% patients had hepatitis or cirrhosis on imaging
Zhang *et al*[153]	82	Retrospective study; Jul 2020	Of 2.4% patients had underlying liver disease; 1.2% patients died due to liver disease; 30.6%, 61.1% and 30.6% had elevated levels of ALT, AST and Total bilirubin respectively
Guan *et al* [135]	1099	Retrospective study	There are 2.1% patients had hepatitis B; AST, ALT and Total bilirubin were elevated in 22.2%, 21.3% and 10.5% patients respectively
Ji *et al* [147]	202	Retrospective study	Liver injury frequent but mild in nature with mostly hepatocellular pattern; Patients with NAFLD and BMI had higher risk for persistent liver injury. Patients with NAFLD had higher risk for severe COVID-19 and longer viral shedding.
Mao *et al* [154]	6686	Systematic Review and Meta-analysis	Pooled prevalence of liver comorbidities was 3%. Pooled prevalence of liver injury was 19%; Patients with severe COVID-19 had higher risk for abnormal liver enzymes.
Singh *et al* [155]	2780	Multicenter research network study	Patients with cirrhosis and pre-existing liver disease are at increased risk for hospitalization and death
Bloom *et al*[143]	60	Prospective cohort study	Predominant AST elevation commonly seen in COVID-19 and correlates with disease severity
Wang *et al* [156]	105	Retrospective study	Elevated liver enzymes more likely in patients with severe COVID-19
Cai *et al* [157]	417	Cross sectional study	Of 76.3% patients had abnormal liver enzymes and 21.5% had liver injury during hospitalization; Patients who received lopinavir/ritonavir had higher odds of liver injury. Patients with abnormal liver tests had higher chance of severe COVID-19

INR: International normalized ratio; COVID-19: Coronavirus disease-2019; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ICU: Intensive Care Unit; NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index.

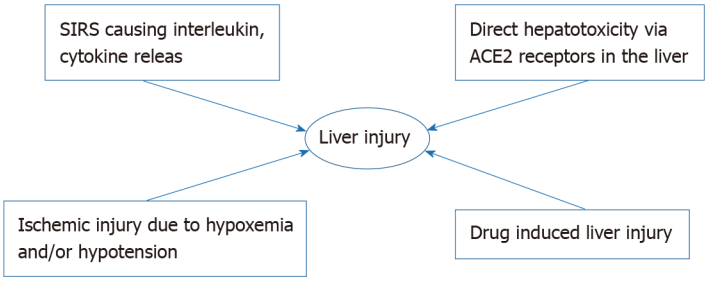

Figure 2 Multiple factors contributing to hepatic injury in coronavirus disease 2019.

COVID-19 in LT recipients

The risk of COVID-19 infection and its severity remain unclear in patients with LT, although a preliminary analysis of the SOT recipient registry from the University of Washington reported that the risk of contracting COVID-19 in SOT recipients is comparable to the general population[158]. With regards to mortality in LT patients, older patients with LT seem to have a higher mortality[159]. International voluntary registries that collect information on COVID-19 patients with underlying liver disease

Table 4 Studies evaluation coronavirus disease 2019 infection post liver transplantation

Ref.	Patients	Type	Study highlight with regards to liver transplant
Coll *et al*[168]	110	Retrospective	Higher incidence of COVID (two-fold) in solid organ transplant patients. Eighty-five percent patients had adjustment in their immunosuppression
Becchetti *et al*[169]	57	Multicenter Prospective	Of 12% overall fatality rate and 17% in-hospital fatality rate. Patients with history of cancer had poorer outcomes
Colmonero *et al*[170]	111	Prospective	LT patients with increased risk of contracting COVID-19 but lower mortality when compared with matched general population. Dose reduction/withdrawal in mycophenolate helped prevent severe COVID-19 but complete discontinuation of immunosuppressants discouraged.
Webb *et al*[171]	151	Multicenter Prospective	Need for invasive mechanical ventilation and ICU admission more in LT group when compared with a control cohort – 20% *vs* 5 % and 28% *vs* 8% respectively. LT not independently associated with death, but presence of comorbidities and increased age were
Belli *et al*[172]	240	Multicenter retrospective	Of 84% patients required hospitalization, 25% of hospitalized patients died. Use of Tacrolimus associated with increased survival probability
Bhoori *et al*[159]	111	Retrospective	Three patients died of COVID-19 and all of them were male, > 65 years with multiple comorbidities and minimal immunosuppression
Rabiee *et al*[173]	112	Prospective	Hospital and ICU mortality rates lower rates in matched patients with chronic liver disease without LT
Mansoor *et al*[174]	126	Retrospective	Higher risk of hospitalization in LT patients. No difference in mortality and need for ICU in LT patients *vs* non- LT patients
Tejedor-Tejada *et al*[175]	16	Retrospective	Post COVID-19 syndrome present with mild symptoms but no loss of liver graft or graft dysfunction noted

LT: Liver transplant; ICU: Intensive care unit; COVID-19: Coronavirus disease-2019.

and LT described 81% patients hospitalized with 30% requiring intensive care unit care and 19% expired[160]. A systematic review described a case fatality rate of 37.5% among LT recipients[161].

Organ procurement has decreased due to the limitations of the pandemic whereas telemedicine is increasingly utilized in evaluation LT recipients[162,163]. All the major societies recommend that patients with high MELD scores, risk for decompensation or HCC progression only be considered for LT[162,164,165]. AASLD recommends that patients with COVID-19 do not receive LT but the procedure can be undertaken 21 d after symptom resolution and negative test in recipient. With regards to immunosuppression in the post-transplant period, all the major societies recommend against reducing it as there has been no data to suggest immunosuppression as a risk factor for severe COVID-19[162,164,165]. AASLD however recommends lowering antimetabolite medication dosages while maintaining the same doses of calcineurin inhibitors (CNI) in LT patients with COVID-19, based on similar principles for managing an active infection in LT patients. Managing immunosuppressive therapy is challenging and should be done cautiously in patients with LT who had COVID-19 due to interactions between corticosteroids and CNI, and the liver toxicity associated with remdesevir and tocilizumab[166]. AASLD recommends vaccination preferable 3 mo after liver transplant once the doses of immunosuppressant medications have been reduced[167]. Table 4 highlights important studies in the context of COVID-19 and liver transplant[168-175].

CONCLUSION

The topic of non-hepatotropic viral infection is very broad and covers a number of infections that do not have liver as the primary site of infection. Majority of the known infections belong to the family of herpes virus infections and often require reactivation, as seen in immunocompromised individuals. Since the development of systemic disease with these infections depends on immune dysregulation, full blown disease is rarely seen in immunocompetent patients. Moreover, the infection is more severe in immunocompromised individuals, especially post-transplant (including liver transplant). These patients can also suffer from allograft rejection, in addition to hepatitis of varying degree of severity. The diagnosis, despite the presence of new testing modalities, is often based on exclusion of hepatotropic infection and liver

biopsy findings inconsistent with other etiologies of hepatitis. There is a lack of guidelines regarding management of each viral infection. Antivirals are often the first line, with or without steroid use. Patients with poor prognosis are worked up for liver transplant and studies have indicated continued use of antivirals following transplant to cover for latent infection. Lastly, there is growing literation on the involvement of liver in coronavirus 2019 pandemic and warrants it to be included in the differential diagnosis of hepatitis, once hepatotropic infection is ruled out.

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MINIREVIEWS

Prediabetes and cardiovascular complications study: Highlights on gestational diabetes, self-management and primary health care

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Abstract

International collaboration on the prediabetes and cardiovascular complications study started in 2013. In 2017, a reflection was reported. Incompleteness of documentation and screening of antenatal cases for gestational diabetes mellitus

Okuleye A and Ezugwu EC were involved in discussions throughout the study and made intellectual contributions to the study performance; Nwose EU, Bwititi PT and Agofure O drafted the manuscript; All authors revised the manuscript and approved the final version for submission.

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(GDM) was concerning. Hence, further observations have been made that warrant an update. The objective of this review is to highlight gaps between clinical knowledge and practice in GDM, diabetes self-management and primary health care (PHC) for rural dwellers. We followed a descriptive field notes method. Antenatal records of patients screened for GDM with incomplete documentation were examined to determine incompleteness of data in those that also met the criteria for GDM risk assessment. Experiences on development of a diabetes register and education and notes on behavioural change wheel were also reviewed. Other data included cross-sectional evaluation of activities of daily living at two private hospitals. Up to 29% had high GDM risk factors, which fulfilled selection criteria for laboratory screening. Demographic data was complete in all women; however, incomplete documentation was observed with as much as 98% of basic data. High levels of physical activity were found in the population, and health lectures proved effective in food choices. The workforce need for diabetes care seems underestimated, but this may be better understood with reactivation of PHC services. The observations highlight behavioural change wheel issues on GDM and PHC services that need concerted focus. Two proposals are to advance the use of a 'risk assessment and screening sheet' for GDM screening and enlightenment of stakeholders on the central hub role of PHC in diabetes management.

Key Words: Behavioural change wheel; Community health; Diabetes education; Knowledge *vs* practice gap; Lifestyle; Patient follow-up; Screening services; Telehealth

Core Tip: There is a gap between knowledge and practice among stakeholders in diabetes management. Behavioural change wheel issues on gestational diabetes mellitus (GDM) and primary health care (PHC) services need concerted focus. The necessity for GDM selective screening is recognized. 'Risk assessment and screening sheet' needs to be employed in clinical practice. Stakeholders including individuals living with diabetes, community leadership, policy makers and health care provision staff need enlightenment to deliberately use PHC centres for diabetes management. PHC centres have a central-hub role in community health. Medical records need to include patients' phone numbers to maximize potential for follow-up and telehealth.

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INTRODUCTION

Diabetes mellitus is a global challenge and public health concern that has continued to attract multifaceted research[1-3]. These interests include laboratory examinations for definitive diagnosis of gestational diabetes mellitus (GDM)[4-6], lifestyle treatment options[7-9] and use of technologies for patient follow-up and self-management[10-12].

In 2013, a proposal for an international collaboration on prediabetes and cardiovascular complications study was launched[13]. In 2017, an update with future directions was reported[14]. In the last 3 years, the program has been expounded, with niches developing from parts of the research. The Global Medical Research and Development Organization that oversees the activities at Catholic Hospital Abbi conceptualized the integration of a monthly diabetes clinic and GDM screening and registry for antenatal clinic patients[15-17]. A user-friendly GDM risk assessment and

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screening form was proposed in January 2020 in collaboration with the University of Nigeria Teaching Hospital.

An opportunity arose after the acquisition of the BRIDGES2 grant [Eli Lily and International Diabetes Federation (IDF)] for a targeted diabetes self-management education trial. This created impetus to develop the lifestyle component of the research. In addition to reports[18-20], two field notes detailing dietary options and food choices as well as physical activities of daily living (ADL) were presented.

To further add to the diabetes registry and BRIDGES2 project, the Global Medical Research and Development Organization reviewed primary health care (PHC) services. This included a discussion about telehealth capacities to enhance diabetes services[21,22]. Telehealth is becoming an invaluable resource. The lessons from the coronavirus disease 2019 pandemic have proven this. Hence, field note observations relevant to PHC and telehealth constitute part of this update, including lessons from the World Diabetes Day (WDD) 2020.

Objective and approach of review

The objective of this paper is to give a periodic review of trends[23-25]. This review is based on our current prediabetes and cardiovascular complications study research with a focus on the gap between knowledge and practice in laboratory testing for GDM diagnosis, including the use of risk assessment factors for selective screening. Other focus areas are emerging treatments (specifically nutrition) and technologies (medical record documentation and telehealth).

DESCRIPTIVE FIELD NOTES REVIEW

This review was designed to follow a descriptive field notes study method. The notes were based on the international collaboration on a prediabetes and cardiovascular complications study program. The study was carried out in Delta State, Nigeria and at health care centres/hospitals. For this update, the 'project evaluation' looked at notes and data from the ongoing study, and the goals for each section are given as subheadings.

GDM screening

GDM constitutes a subsequent health risk to the mother and child. Several risk factors for GDM are known; hence, screening is encouraged. However, the adoption of robust screening protocols in various health care facilities is lacking, and reports have indicated a similar situation in our research performance sites[15,29]. One notable observation was that selection criteria for lab tests in GDM screening were being followed[16] but arbitrarily and with rampant incompleteness of procedure. Therefore, while a screening flow sheet has been articulated[15], it was appropriate to develop another sheet with selective screening criteria (Table 1). This proposed GDM risk assessment and screening sheet was informed by two points: (1) Risk factors are generally non-invasive and can be obtained during antenatal registration and history taking[30-32]; and (2) Risk scoring systems exist. One includes gestational age[31], while the other is without gestational age[32].

Standard selection for screening: A user-friendly algorithm or chart is yet to be developed. Pending the adoption of the risk scoring chart, this evaluation would assess antenatal patients with three risk factors for laboratory investigation of GDM. Two retrospective cohort reviews have tabulated the GDM risk assessment and screening sheet (Table 1). Furthermore, the mother's blood type is a potential genetic factor for the likelihood of GDM developing overt diabetes mellitus (DM) postpartum [33]. Blood type assessments are determined for antenatal patients but may not be included in GDM screening sheets. Nevertheless, it is important to identify this as part of GDM management.

GDM screening register: In another field note, evaluation was specifically aimed to (1) Progress the development of screening registers, (2) Determine the level of attention given to or the potential of monitoring the rate of increase in body mass index (BMI) among the patients; and (3) Update the prevalence of GDM among pregnant women.

Diabetes self-management

Four items are imperative components of behavioural change included in diabetes education programs[34]. They are dietary options, physical ADL, adherence to medical

Table 1 Proposed gestational diabetes risk assessment and screening sheet

SN	Assessment stage	Factor	Yes	No
1	Initial routine data	Age > 35 yr		
2		Overweight[1] (BMI > 25)		
3		Hypertension		
4		Sedentary lifestyle[2]		
5		Family history of diabetes		
6	Previous history	GDM		
7		Miscarriage		
8		Foetal/neonatal death		
9		Polycystic ovary syndrome		
10	Antenatal monitoring	Presentation of GDM symptoms		
11		Excess gestational weight gain[3]		
12		Foetal growth		
13		Macrosomia		
14	Lab tests[5]	Urinalysis for glucosuria		
15		Blood sugar test[4]		

Keys to positive risk factors for 'Yes' response.
[1]Especially obesity (body mass index > 30), which also defines nutritional status.
[2]Activities of daily living being non-physical and/or mainly sitting position.
[3]> 1.80 kg in first trimester and/or > 0.45 kg per week during the rest of pregnancy.
[4]Interpret according to method.
[5]For antenatal GDM screening.
GDM: Gestational diabetes.

check-ups and adherence to prescription medicine. For this update, two field notes on dietary options and physical ADL are presented.

Physical ADL: Physical inactivity predisposes a person to DM. Thus, engaging in physical activities is preventive and therapeutic. The study evaluated ADL of local residents with diabetes who attended two non-government health facilities in rural communities of Delta State, Nigeria. This study was an addendum to a previous report [20]. A cross-sectional descriptive design was used and comprised 48 participants from Catholic Hospital Abbi and Novena Health Centre Amai. A semi-structured questionnaire with integrated anthropometric and blood pressure measurements (weight, height, blood pressure, and BMI) was used. ADL of participants was delineated into blue *vs* white collar jobs, depicting physically-active and -inactive occupations respectively. The data were analysed using the Microsoft Excel Analysis ToolPak. A demographic description of the respondents is presented in Table 2. Among the women, 11/21 (52.4%) were widows, and the average age of all respondents was 57 years.

Dietary options and food choices: In another study niche, the consumption of indigenous carbohydrate foods was evaluated. This niche was originally studied to determine the agribusiness and health economics nexus[35], but the basis of this field note was the controversy around cassava[36,37] that was buoyed by indigenously sourced cassava meal (eba/garri) being omitted from a hospital-based menu[19]. The study involved baseline and post-intervention surveys of community members including hospital visitors, and the intervention was public health lectures on medical nutrition therapy value of cassava. Information collected included consumption habits regarding farmed choices (cassava and yam) and an imported option (wheat).

Other evaluations

PHC needs – lessons from WDD 2020: With the invaluable support from the Public Health Department of Novena University, the WDD event was organized at 11 health

Table 2 Descriptive statistics of respondents

		Male	Female
	n	**27**	**21**
	Age in yr	59	55
Educational status	≤ Secondary school	19	14
	≥ Tertiary education	8	7
Marital status	Married	23	10
	Single	4	11

facilities, including four research performance sites, a general hospital, two PHC centres in the Delta state, one centre in the southern Nigeria, and three centres in northern Nigeria. There were 23 volunteers comprised of academic staff and postgraduate students of the Public Health Department of the Novena University who facilitated the event. An additional 79 health care personnel were recruited who delivered public health lectures and diabetes screening. A total of 286 community members benefited from the event[38]. Observations were made from this event.

Telehealth potential in rural communities of Nigeria: Development of diabetes registers was started at the research performance sites, *i.e.* health facilities. However, evaluation of the usefulness for patient recall and telehealth have yet to be reviewed. Hence, in the hospital cohort study of 123 cases, the evaluation approach for telehealth involved assessing previous antenatal patients by telephone.

PERSPECTIVES ON GDM, SELF-MANAGEMENT AND PHC IN PREDIABETES AND CARDIOVASCULAR COMPLICATIONS

GDM screening

In a hospital cohort involving 90 cases, there was no urinalysis on the antenatal patients despite various prevalent risk factors. A history of GDM was the least common risk factor, and age over 35 years was the most common risk factor (Figure 1). Considering the prevalence of risk factors at initial registration, the lack of urinalysis is inexplicable. In another hospital cohort of 123 cases, 29% of those assessed had three or more risk factors that warranted screening (unpublished data). These reports provide evidence that screening education should be provided to antenatal providers.

Level of completeness of data collected during GDM screening: We collected data from 391 antenatal cases that were seen from September 2018 to December 2018. The women's ages ranged from 15 to 46 years, and average gestational age was 23 wk, with the earliest clinic visit occurring at the fourth week. Further, several of the women were in their first pregnancy, and cohort average was less than gravida 3 (Table 3).

Among the 391 cases, the names, ages, medical record numbers and dates of hospital visits were complete, *i.e.*, 100% documentation. Information on the mother's and/or family history of type 2 diabetes and GDM were not indicated, *i.e.*, 0% documentation on hereditary risk factor. There were also no records of lipid profiles. Other relevant data that were collected are presented in Table 4. Blood sugar tests were undocumented for 98.7% of patients, and BMI was not reported for 76.7% of patients. Overall, our results show that the average level of completeness of data collection during GDM screening was 79% (Figure 2). The figure shows the percentages of completeness that were not captured in Table 4.

Level of attention given to BMI: The 301/391 cases lacking BMI measurement had missing data on height (Table 4), but only 14 Lacked weight measurements. A critical evaluation showed that 5% of the cases without BMI values were due to lack of height and weight measurements, and 95% were solely due to lack of height data (Figure 3). Further review of the potential monitoring rate of increase in BMI among the patients showed that 13% (12/90) were in the first trimester and 48% (43/90) in the second trimester. There was a noticeably higher level of BMI in the third trimester relative to the earlier trimesters, but the difference was not statistically significant.

Table 3 Descriptive statistics of patients entered in a gestational diabetes screening register

	Age in yr	Gestational age in wk	Gravida[1]
Mean	28.57	23.01	2.60
Standard error	0.30	0.41	0.08
Median	28.00	23.00	2.00
Mode	25.00	24.00	1.00
Standard deviation	5.91	7.33	1.63
Sample variance	34.97	53.72	2.67
Range	31	35	9
Minimum	15	4	1
Maximum	46	39	10
Count	391	324	377

[1]Number of times a woman has been pregnant.

Table 4 Level of incompleteness of data

Missing data	*n*/391	Absolute Hz	Relative Hz
Address	1	0.2558	0.0760
Rh factor	6	1.5345	0.4563
HVS, 2/385 positive	6	1.5345	0.4563
Urine glucose	6	1.5345	0.4563
ABO	8	2.0460	0.6084
Marital status	12	3.0691	0.9125
Weight in kg	14	3.5806	1.0646
SBP in mmHg	14	3.5806	1.0646
DBP in mmHg	14	3.5806	1.0646
Gravida	14	3.5806	1.0646
Packed cell volume, %	15	3.8363	1.1407
Phone number	60	15.3453	4.5627
Gestational age in wk	67	17.1355	5.0951
Occupation	92	23.5294	6.9962
Height in cm	301	76.9821	22.8897
BMI	301	76.9821	22.8897
Blood sugar, as FBS or RBS	386	98.7212	29.3536

ABO: Blood grouping system; BMI: Body mass index; DBP: Diastolic blood pressure; FBS: Fasting blood sugar; HVS: High vaginal swab; RBS: Random blood sugar; SBP: Systolic blood pressure.

ADL in the community

This analysis was based on the occupation of the participants. The indicated occupations were categorized into blue collar (manual labour, *e.g.*, agriculture, construction, *etc.*) and white collar (office or non-hard manual work professional) depending on assumptions of physical demands of the jobs. Forty-eight respondents were analysed, including four who did not indicate their occupations and were assumed to be blue collar. Evaluation showed that 73% were involved in physically demanding ADL (Figure 4).

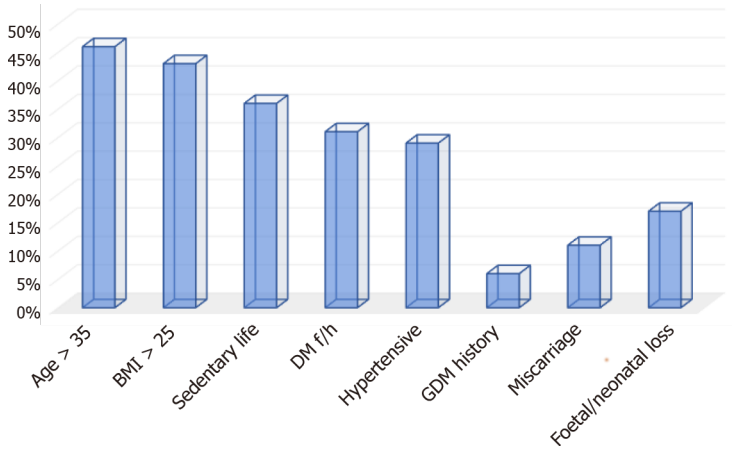

Figure 1 Prevalence of gestational diabetes risk factors in a hospital cohort from Delta State, Nigeria. History of gestational diabetes mellitus was the least common risk factor, while age was the most common risk factor. BMI: Body mass index; DM: Diabetes mellitus; GDM: Gestational diabetes mellitus.

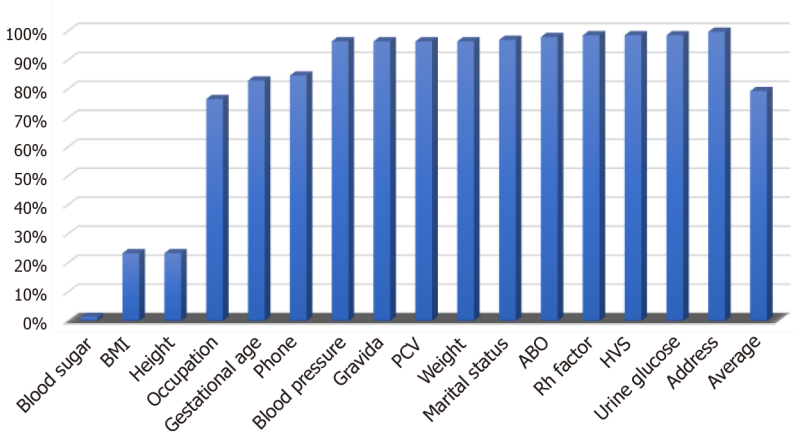

Figure 2 Translated percentage completeness of data collection. Most antenatal patients lacked blood sugar and body mass index measurements. ABO: Blood grouping system; HVS: High vaginal swab; PCV: Packed cell volume; BMI: Body mass index.

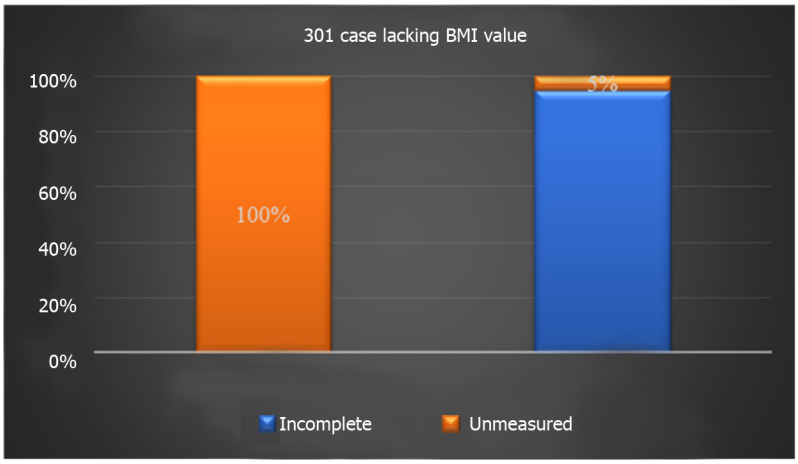

Figure 3 Proportions of missing body mass index values attributable to either missing height or weight. Most cases without body mass index data were due to a lack of height measurement. BMI: Body mass index.

Level of physical activities among participants

Evaluation of responses to exercise ADL indicated that over 50% of the respondents were not able to stretch or swim, while walking appeared to be the activity for most (Table 5). Evaluation of interference of basic ADL showed that ill health interfered

Table 5 Responses to exercise abilities, *n* = 48

Duration	Stretching	Walking	Swimming	Bicycling
None	28	15	41	24
< 30 min	11	10	2	6
30 min–1 h	4	13	3	7
1–3 h	2	6	2	4
> 3 h	3	4	0	7

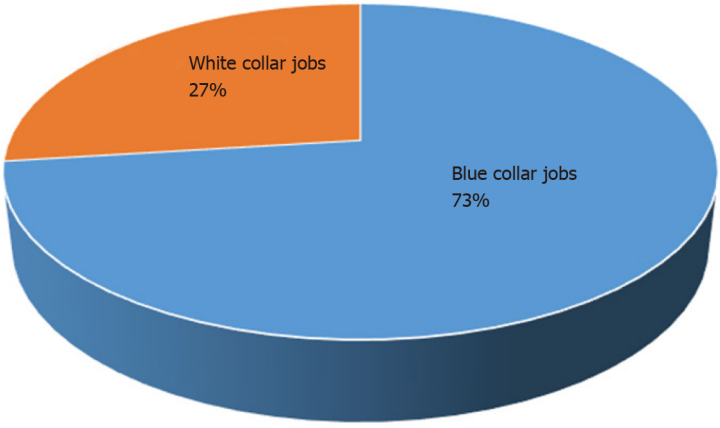

Figure 4 Distribution between manual and non-manual jobs. Most cases were in occupations with physically demanding activities of daily living.

mostly with respondents' hobbies, but generally all people engaged in a variety of physical activities.

Effect of diabetes on the ADL of participants in the community

When participants were categorized based on DM and hypertension + DM, the results showed that diabetes interfered with hobbies and work the most. However, the interference was minimal. The impact of hypertension + DM on all the surveyed ADL items was higher (moderate interference of hobbies and work) (Figure 5).

It was concluded that participants in manual jobs constituted about three-quarters of the diabetes patients. Nevertheless, the proportion of those in non-manual jobs with hypertension comorbidity was higher, which implies healthy occupational ADL as indicated by a preponderance of manual occupations in people with diabetes. The impact of ill health on leisure ADL was also indicated, especially that it was worse in those with hypertension comorbidity. This highlights that a pivotal focus on diabetes self-management regarding physical activities should include evaluation of the patient's capacity to engage in ADL, especially as the impact of diabetes on ADL translates to a barrier of a therapeutic lifestyle regimen. Given that the interference of ADL by ill health was worse among those with hypertension and diabetes, both groups require different levels of care.

Effect of public education on food consumption

A baseline survey showed that 100% of rural farmers harvest cassava. The survey also showed that only 48% consumed cassava as one of their food choices, and 10% consumed wheat only and disregarded other options (Figure 6). This observation differed from other cohorts, such as urban dwellers who were wheat consumers and included a high proportion of educated people. After the public health lecture, the post-intervention survey showed a shift in consumption patterns, in favour of cassava products[39].

WDD 2020 highlights

At WDD 2020, the IDF highlighted the following three urgent facts about Nigeria and diabetes: (1) Six million nurses are needed; (2) About 50% of adults living with diabetes are undiagnosed; and (3) Over 75% of people living with diabetes live in

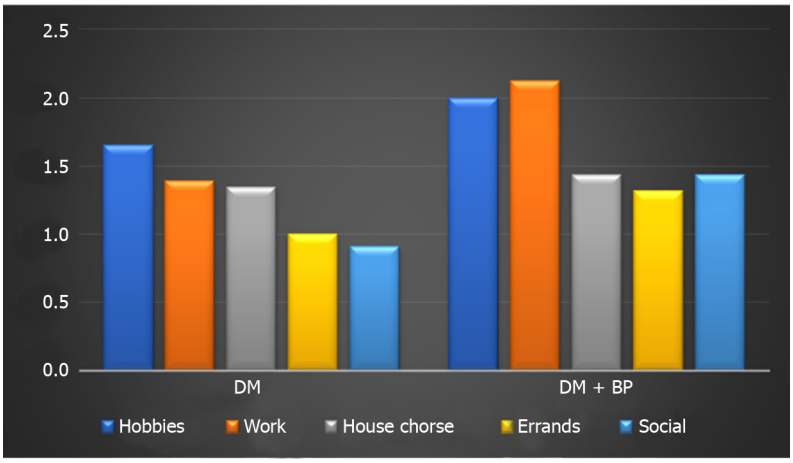

Figure 5 Average responses on interference of ill health on activities of daily living. The group with diabetes mellitus only was more responsive to physical activities of daily living. DM: Diabetes mellitus; BP: Blood pressure (indicated hypertension in this graph).

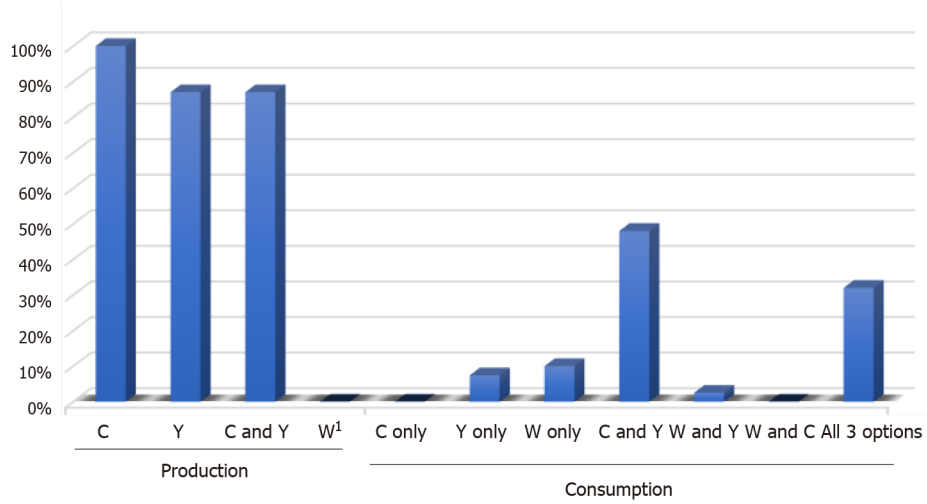

Figure 6 Consumption and production carbohydrate food choices. Most individuals undervalued their indigenous healthy food options, such as cassava and yams. [1]Never farmed in the area. C: Cassava; W: Wheat; Y: Yam.

urban areas and are of working age.

Lessons from the event can be summarized in two themes. First, there is an underestimated burden of diabetes in terms of the nursing workforce requirement in PHC. During preparation for the event, it was determined that the 6 million projection is an underestimation. This determination was based on: (1)Australia being speculated to require about 120000 nurses in the next 10 years, which is 2% of the projected 6 million need[40]; and (2) More than 75% of people in African countries being undiagnosed for diabetes[41], making the actual burden yet to be estimated. This implies that Nigeria, with a population of over 200 million, needs to train and recruit many health care workers in diabetes care. Considering the current nursing workforce in the country, Nigeria will need more than the estimated 6 million.

Non-governmental organizations run diabetes screening services at community and PHC centres, but resident staff of the health care centres do not. Therefore, the knowledge is present but is not practiced, hence the apparent gap in knowledge *vs* practice. Importantly, most of the health care workers in the rural community-based PHC facilities are unaware of the WDD diabetes educator courses. However, these courses are well-received because the general hospitals requested a reschedule of the WDD from a Saturday to a weekday, in order for the staff to benefit. In addition, at three of the hospitals, the event was carried out twice, once for the general public and again for the hospital staff.

Telehealth

Our data indicates that 20% of the cases on medical records did not have phone numbers on record. However, 80% of the patients were able to receive services *via* telehealth. A pertinent observation is that some of the phone numbers were actually owned by the patient's child who was not living with the patient. Although many of the calls were unsuccessful, 34% of the cohort received telehealth services, and 29% of the sub-cohort received health advice (Figure 7). In light of the coronavirus disease 2019 pandemic and its effects on routine health care provision, this is an area that can be further utilised and expanded.

DISCUSSION

This review highlighted some of the problems in diabetes research and practice. Specifically, the issues of GDM, nutrition in terms of carbohydrate food choices, PHC, and telehealth. Links have been made to the concept of behavioural change wheel (BCW_ regarding the gap between knowledge *vs* practice. BCW is important in managing chronic conditions. It is a guide for designing intervention programs, and it is predicated on capacity, motivation, and opportunity[26]. It is a comprehensive framework for designing interventions by explicitly integrating behaviour theory to understand and target mechanisms of action within the intervention[27], and it is mindful that individual and collective behaviour change is key to implementing new practices and to improving health outcomes. It also incorporates context, which is key to effective design and implementation of interventions[28].

There has been a lack of clarity on the universal screening of pregnant women for GDM[42-44], and the result is that this service remains under-utilised[45]. This low level of GDM screening service appears to represent a gap between knowledge and practice among the clinicians. The positive note is that GDM risk assessment for selective screening of pregnant women now exists[30-32]. The user-friendly risk assessment sheet needs to be validated for local clinical use and integrated into GDM screening services. Two notable pieces of clinical knowledge include (1) BMI may be more reliable as a risk factor when measured in the first trimester, and (2) More weight measurements should be done because gestational weight gain is imperative in monitoring well-being of the baby and mother. Therefore, most antenatal clinics assess these at every visit, but height is documented at registration. This review highlighted the proportions of cases missing different necessary measurements and/or documentation.

Our cohort of 391 antenatal patients in the register were reviewed to determine the number of conclusive GDM diagnoses. This review was an addendum to a previous observation[15], and it was observed that 385 patients had a urine glucose test, out of which 16 (4%) were positive for glucosuria. Glucosuria may be found in about 50% at some point because of the associated changes in glomerular filtration rate. However, a follow-up blood sugar test for at-risk women is still essential[46]. None of the 16 cases were followed-up with a blood sugar test. Instead, 5 other patients had blood glucose test results without urinalysis. Therefore, we do not know the incidence rate of GDM at our hospital. This highlights a knowledge and practice gap in antenatal patient laboratory test requisition and follow-up.

The four components of necessary diabetes education are: Adherence to medication, nutritional lifestyle, physical ADL, and up-to-date medical check-ups including laboratory tests[2,47,48]. This review reported on carbohydrate nutrition and highlighted that people are still unaware of the values of their indigenous foods. This lack of knowledge was identified long ago[49], hence this report suggests that the problem is yet to be resolved. Therefore, more concerted efforts are needed to advance the knowledge of the glycaemic index of Nigerian carbohydrate foods. This report also highlighted physical ADL affordances and their effectiveness in the rural community.

PHC care is a function of the state, regarding the government's policy and practice on health services. One of the issues, at least in developing communities such as Africa, is limited resources[50]. However, studies on knowledge, attitude and practice have indicated that there are patient factors to consider[51]. The Alma Ata declaration identified the necessity and potential of PHC to play a central hub role, and diabetes care services are encompassed[52]. This report highlights a known problem that is still unresolved.

Therefore, it is recommended for the World Health Organization and other stakeholders (community, community leadership, policy makers and health care provision staff) to focus on PHC for diabetes care. For instance, looking at the Nigerian

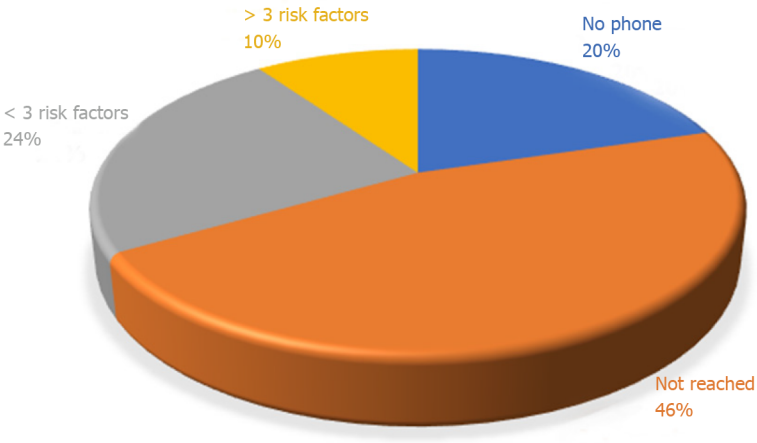

Figure 7 Distribution of cohort of cases assessed by telehealth. Most cases provided incomplete or incorrect phone numbers on their medical records.

health care system, there are PHC centres scattered and operated by local governments, but the community dwellers rarely patronize the centres. By implication, the feedback to the government is hampered, and the impression is that "the government is not doing enough." However, it is not entirely the fault of the government *per se*[18]. This requires behavioural change of the community, inclusive of the ministry of health[53]. It was reported from a BCW survey that about 40% of stakeholders agreed on the ability of PHC to offer diabetes services, whereas over 70% believed the motivation was poor[18]. Therefore, this update highlights one area of need of BCW for IDF and the World Health Organization.

Over the past 3 years, translational research efforts have included development of a Diabetes Registry in the health facilities[17,54,55]. It has been reported that a major factor to consider is concerted integration of telephone numbers of patients in medical files[54]. For this obvious reason, the Global Medical Research and Development Organization is developing telehealth services for the area, of which telephone numbers are indispensable.

There is ongoing interest to utilise accessible and affordable telecommunication tools including telephones in community health service delivery[56-58]. This review reported the potential to use the telephone for follow-up in rural communities. It also highlighted two limitations, which are non-documentation of the patient's phone number or the patient providing another person's telephone. This observation calls for awareness for health care providers to document phone numbers in patients' records as well as alert patients on the importance of providing their own numbers.

CONCLUSION

These updated field note observations from ongoing research and topical research areas include prediabetes, diabetes registry, GDM screening concerns, PHC and telehealth potential for rural dwellers. From experience on the development of a diabetes register and intensive diabetes education, notes on BCW were reviewed and this is a work in progress, especially due to the negative impact of the coronavirus disease 2019 pandemic. Other field data included a cross-sectional study of ADL at two private hospitals. One of the important topics in diabetes care is that of PHC. There is a knowledge and practice gap for the important role that PHC must play in diabetes care, and this constitutes another point on BCW. This problem is likely confounded by the IDF and World Health Organization underestimating the nursing workforce. It is possible that a proactive PHC system equipped with various registries, such as a diabetes registry, could minimize the documentation issues as well as improving screening procedures. Mobile phones and internet services are becoming affordable and accessible, and research needs to focus on their utilisation. Future research should also advance BCW of all stakeholders (community, community leadership, policy makers and health care provision staff) to maximize the impact of diabetes research.

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SYSTEMATIC REVIEWS

Hydroxychloroquine alone or in combination with azithromycin and corrected QT prolongation in COVID-19 patients: A systematic review

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Author contributions: Ashraf H, Ghafouri P, Kazemian S, Soleimani A, Karbalai S designed the research study; Ashraf H, Kazemian S, Soleimani A, Sadat Naseri A, and Kazemi Saeid A performed the research; Ashraf H and Ghafouri P contributed new reagents and analytic tools; Ashraf H, Soleimani A, Sadat Naseri A, Karbalai S, and Kazemi Saeid A analyzed the data and wrote the manuscript; All authors have read and approved the final manuscript.

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Abstract

BACKGROUND

Despite the controversies about the effectiveness of the current drug regimens for coronavirus disease 2019 (COVID-19), these drugs are still the only options available. Moreover, the safety of these drugs is yet to be confirmed. A serious concern is the occurrence of various cardiac arrhythmias, particularly QT prolongation.

AIM

To summarize the incidence and estimate the risk of QT interval prolongation in patients scheduling for conventional treatment (hydroxychloroquine alone or in combination with azithromycin) for COVID-19.

METHODS

We comprehensively searched Medline, Web of Knowledge, Google Scholar, Scopus, and Cochrane Central Register of Controlled Trials databases until October 31, 2020 for all eligible studies under the considered keywords COVID-

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19, arrhythmia, QT interval, therapy, azithromycin, and hydroxychloroquine until. The study protocols were established in compliance with PRISMA-P guidelines (Preferred Reporting Items for Systematic Review and Meta-Analysis – Protocols), and a nine-star Newcastle-Ottawa Scale scoring system was used to assess the methodological quality of all eligible studies. Outcome measures were corrected QT (QTc) prolongation, cardiac arrhythmias, or sudden cardiac death.

RESULTS

Fifteen studies enrolling 8298 patients with targeted COVID-19 therapeutic regimes were included. The eligible studies found a significant increase in the mean QTc interval following treatment with the described medications compared to baseline QTc with weighted standard differences in means of 0.766. The pooled prevalence rate of QTc prolongation was estimated to be 9.2% (95% confidence interval: 4.5% to 18.1%).

CONCLUSION

Hydroxychloroquine ± azithromycin regimen can significantly increase the risk of developing QTc prolongation.

Key Words: Azithromycin; COVID-19; Hydroxychloroquine; QTc interval

Core Tip: Given the greater importance of coronavirus disease 2019 worldwide, there is an ongoing controversy about the potential harms of anti-viral agents in which caused uncertainties in daily clinical practice. Given the unresolved debate, during this systematic review and meta-analysis, we investigated the association of Hydroxychloroquine (alone or in combination with azithromycin) with the risk of QT interval prolongation, cardiac arrhythmias, and sudden cardiac death. Although there are some studies about the effects of these agents, there are scarce systematic reviews and meta-analyses about both QT prolongation and risk of cardiac arrhythmias which is a distinguishing point for our study.

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INTRODUCTION

With the outbreak of the coronavirus disease 2019 (COVID-19) pandemic, we have seen high mortality rates from the disease in almost all countries involved[1]. Besides the pulmonary involvement in form of acute respiratory distress syndrome, one of the main features of this disease is the involvement of other systems, like cardiovascular, gastrointestinal, central nervous system, and even skin and mucosal involvement[2-4]. However, respiratory failure and subsequent cardiovascular compromise were major determinants for patients' survival[5]. So far, there are no successful and safe drug regimens for the treatment and prevention of COVID-19. Current approaches have either failed or have been withheld due to potential side effects. Thus, the side effects have added to the high mortality and morbidity caused by COVID-19[6].

Current evidence suggests that using hydroxychloroquine and azithromycin for COVID-19 increases the risk of cardiac arrhythmias[7,8]. Previous studies reported that these drugs caused corrected QT (QTc) prolongation, leading to life-threatening conditions like torsades de pointes (TdP) and sudden cardiac death[9,10]. Although both *in vivo* and *in vitro* studies recommended the combination therapy of azithromycin and hydroxychloroquine, even as the first-line approach in preventing disease, it has also led to QTc prolongation[11]. In addition to cardiac monitoring,

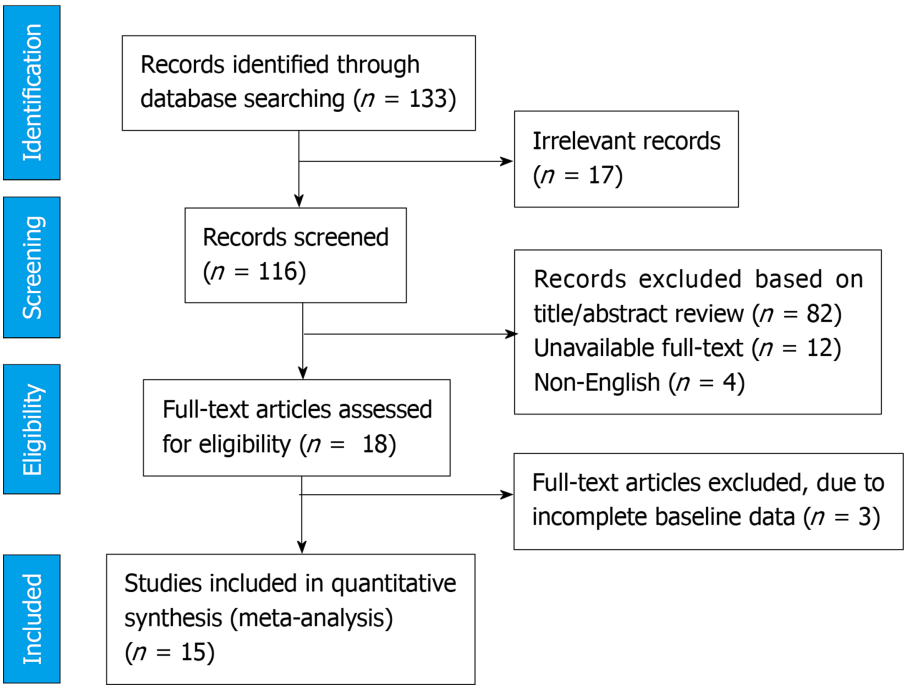

Figure 1 The flowchart of screening the eligible studies.

identifying patients, who are prone to the side effects, helps to minimize the potential harms. By identifying susceptible individuals, it may be possible to use other drug protocols to maintain patient survival. Herein, we summarize the findings about the prevalence and the risk of QTc prolongation in patients treated with hydroxychloroquine ± azithromycin. Also, we discuss the life-threatening conditions in patients taking these medications.

MATERIALS AND METHODS

Search strategy

We performed this review according to established methods and in compliance with PRISMA-P (Preferred Reporting Items for Systematic Review and Meta-Analysis) Protocols. Two investigators searched the manuscript databases including Medline, Web of Knowledge, Google Scholar, Scopus, and Cochrane Central Register of Controlled Trials in The Cochrane Library until October 31, 2020 for all eligible studies under the considered keywords including COVID-19, arrhythmia, QT interval, therapy, azithromycin, and hydroxychloroquine until. The studies were restricted to the English language. We included all randomized controlled trials, including individually randomized and cluster-randomized trials. We reviewed the studies reported as full-text and those published as abstracts. We also conducted a search of ClinicalTrials.gov and the World Health Organization International Clinical Trials Registry Platform Search Portal for ongoing or unpublished trials. The inclusion criterion was a study population of adult patients, who suffered from a definitive diagnosis of COVID-19 and were treated with at least one or a combination of these medications: hydroxychloroquine, chloroquine, and azithromycin. The exclusion criteria were: (1) A lack of clear and reproducible results; (2) Non-English studies; (3) Lack of access to the full texts of the articles; and (4) Case reports, case series, and review papers. If a subset of patients in a study met our inclusion criteria and data from this specific population were missing in the original publication, at first, we tried to contact the corresponding author to get the necessary data. Afterward, if the relevant data was not accessible, we only included the studies when more than 50% of the participants met the inclusion criteria. However, we performed sensitivity analysis and excluded studies in which < 100% of patients met the inclusion criteria.

Data abstraction and validity assessment

Two un-blinded reviewers performed the data abstraction independently in structured

Ref.	Selection				Comparability		Outcome			Total
	1	2	3	4	5	6	7	8	9	
Afsin *et al*[12], Turkey	★	★	★		★	★	★	★	★	8
Bakhshaliyev *et al*[13], Turkey	★	★	★	★	★	★	★	★		8
Bernardini *et al*[14], Italy	★	★	★	★	★	★	★	★		8
Bun *et al*[15], France	★	★	★	★	★	★	★	★	★	9
Chorin *et al*[16], United States	★	★	★		★	★	★	★	★	8
Cipriani *et al*[17], Italy	★	★	★	★	★	★	★	★		8
Hsia *et al*[18], United States	★	★	★	★		★	★	★		7
Maraj *et al*[19], United States	★	★	★		★	★	★	★	★	8
Mercuro *et al*[20], United States	★	★	★	★	★	★	★	★		8
Ramireddy *et al*[21], United States	★	★	★	★	★	★	★	★		8
García-Rodríguez *et al*[22], Spain	★	★	★	★	★	★	★	★	★	9
Saleh *et al*[23], United States	★	★	★		★	★	★	★	★	8
Saleh *et al*[24], United States	★	★	★	★	★	★	★	★		8
Sinkeler *et al*[25], Netherlands	★	★	★	★		★	★	★		7
van den Broek *et al*[26], Netherlands	★	★	★	★	★	★	★	★		8

Figure 2 The quality assessment of the studies according to the nine-star Newcastle-Ottawa Scale scoring system.

collection forms with no divergences in the data collection method. We resolved disagreements by consensus or by involving a third person. One of the authors transferred data into the Review Manager file. We double-checked for correct data entry, comparing the data presented in the systematic review with the data extraction form. The second author spotted-check study characteristics for accuracy against the trial report. The details will be assessed by systematically reviewing the manuscripts are as follows:

The study quality was evaluated based on the following criteria: (1) The systematic review and meta-analysis based on the questions primarily described and formulated; (2) Inclusion and exclusion criteria predefined in the studies as eligibility criteria; (3) Searching the literature performed on a systematic and comprehensive approach; (4) To minimize the bias, two authors reviewed the full texts of the articles; (5) The quality of included studies were rated independently by the reviewers for appraising internal validity; (6) The characteristics and findings of the studies were listed comprehensively; (7) The publication and risk of bias were listed; and (8) Heterogeneity was also assessed. The endpoints were to determine the overall prevalence of QT prolongation and estimate the occurrence of fatal arrhythmia. Along with pooled relative risk for QT prolongation, the year of publishing, the number of patients included, and

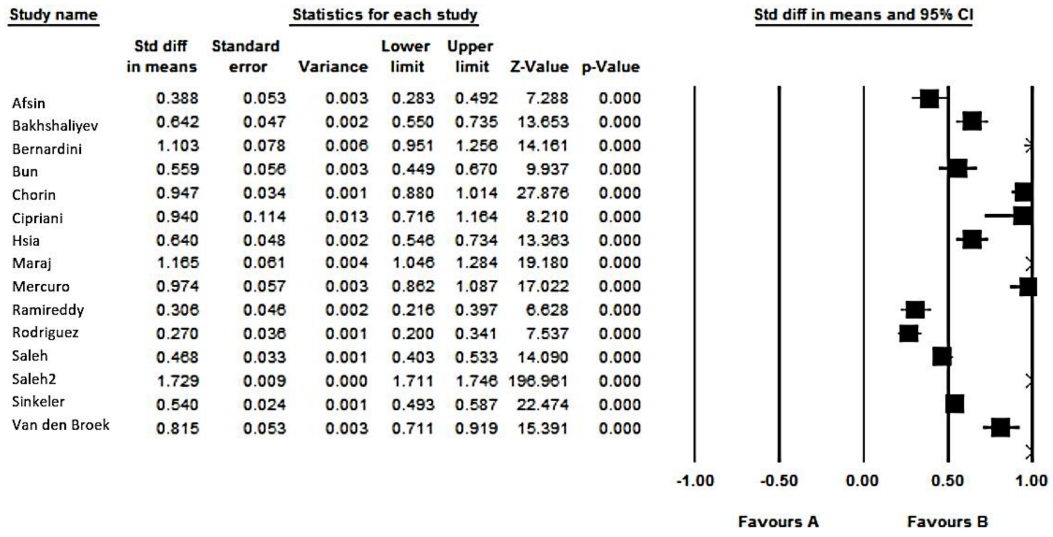

Figure 3 The pooled analysis on the difference in mean corrected QT after treatment compared to the baseline. CI: Confidence interval.

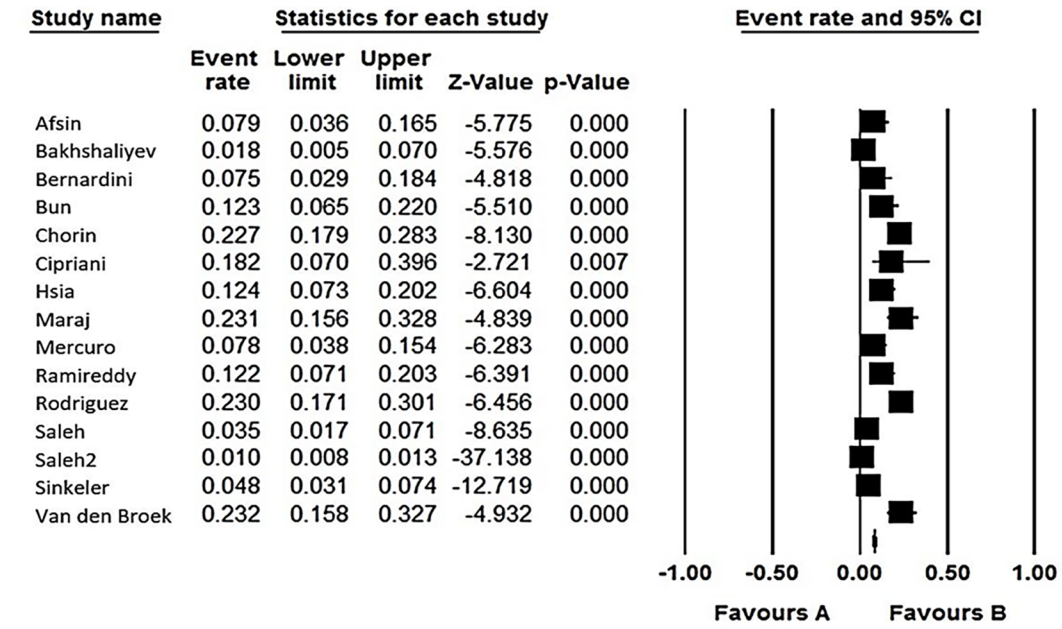

Figure 4 The pooled prevalence of corrected QT prolongation after treatment. CI: Confidence interval.

the method of the design was also pointed.

Statistical analysis

We assessed the risk of bias for each study with the criteria outlined in the Cochrane Handbook for Systematic Reviews of Interventions. Any disagreement was resolved by discussion in the whole study team. We assessed the risk of bias according to the following domains: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, and selective outcome reporting. We judged each potential source of bias as high, low, or unclear and provided a quote from the study report together with a justification for our judgment in the "Risk of bias" table. We summarized the risk of bias judgments across different studies for each of the domains listed. When considering therapeutical effects, we took the risk of bias into account for studies that contribute to that outcome. We contacted investigators or study sponsors to verify key study characteristics and obtain missing numerical outcome data where possible, for example, when a study is identified as abstract only. We used the RevMan calculator (version 5.3) to calculate missing standard deviations from other statistics, such as confidence intervals (CI) or *P*

-values. Where it was not possible, we imputed the missing standard deviations and explored the impact of including such studies in the overall assessment of results by a sensitivity analysis. Dichotomous variables were reported as proportions and percentages, and continuous variables as mean values. Binary outcomes from individual studies were combined with both the Mantel-Hansel fixed-effect model. The risk ratios (RRs) and 95%CI for RR were used as summary statistics to compare dichotomous variables and to determine the likelihood of each adverse event after interventions.

We used Cochran's Q test to estimate the statistical heterogeneity, complemented with the I^2 statistic. It quantifies the proportion of total variation across studies that is due to heterogeneity rather than chance. A value of I^2 of 0%–25% indicates insignificant heterogeneity, 26%–50% low heterogeneity, 51%–75% moderate heterogeneity, and 76%–100% high heterogeneity. Publication bias was assessed by the rank correlation test and also confirmed by the funnel plot analysis. The nine-star Newcastle-Ottawa Scale scoring system was employed to assess the methodological quality of all eligible studies. In this quality assessment technique, each study assessed qualitatively for the three criteria of (1) The selection of the study groups; (2) The comparability of study groups; and (3) The ascertainment of the outcome and is finally scored that the studies awarding 7 stars or over were deemed as high quality. Reported values were two-tailed, and hypothesis testing results were considered statistically significant at $P = 0.05$. Statistical analysis was performed using the Comprehensive Meta-Analysis Software (CMA, version 3.0).

RESULTS

Figure 1 demonstrates the flow diagram of the study selection. Initially, 133 articles were collected by database searching and other sources. After removing duplications, 116 records were primarily under-screened. Based on the titles and abstracts, 98 records were excluded, and the remaining 18 citations were assessed for further eligibility. Of those, 3 were also excluded due to incompleteness of the data and contents. Finally, 15 articles [12-26] were eligible for the final analysis (Figure 1). The studies included were assessed qualitatively by the QUADAS-2 tool. According to our risk of bias assessment, all 15 studies yielded good quality, and none of them had a high risk of bias. Therefore, the pooled results should be persuasive (Figure 2). In total, 15 studies (twelve retrospective and three prospective) were included in our final analysis. The anti-COVID-19 medications focused in the studies were mostly a combination of hydroxychloroquine plus azithromycin[13-24], hydroxychloroquine plus moxifloxacin[12], and hydroxychloroquine alone[25,26]. Overall, 8298 patients suffering from COVID-19 were treated with these regimens. The details of the participants are summarized in Table 1. As shown in Table 2, all studies found a significant increase in the mean QTc compared to the baseline. The weighted standard differences in means are 0.766 (95%CI: 0.394 to 1.137, $P < 0.001$), with significant heterogeneity across the studies relevant to the I^2 value of 99.33% ($P < 0.001$) (Figures 3 and 4). In this regard, the pooled prevalence rate of QT prolongation was estimated to be 9.2% (95%CI: 4.5% to 18.1%) with a significant level of heterogeneity across the studies ($I^2 = 98.10\%$, $P < 0.001$). The Egger test also detected significant publication bias for all assessments.

Across all studies, 132 patients stopped taking the medications due to QTc ≥ 500 ms or an increase of more than 60 ms in QTc. The pooled prevalence was 0.9% (95%CI: 0.6% to 1.1%) with significant level of heterogeneity across the studies ($I^2 = 81.50\%$, $P < 0.001$).

Studies did not report any mortality caused by sudden cardiac death or arrhythmogenic death. However, 4 cases of TdP and 34 cases of ventricular tachycardia/fibrillation were reported with a pooled prevalence of 0.1% < and 0.4% (95%CI: 0.2% to 0.5%), respectively.

DISCUSSION

There are controversies about the effectiveness and safety of medications used to treat COVID-19. In some cases, serious side effects following the use of these drugs may contribute to the morbidity caused by the disease and even may lead to its progression. QTc prolongation is a potential side effect of hydroxychloroquine and, also, one of the most critical complications, leading to some fatal arrhythmias like TdP.

Table 1 The baseline details of studies included in meta-analysis

Ref.	Study design	Population	Mean age	Male/female	HTN	DM	Medication
Afsin *et al*[12], Turkey	Retrospective	76	61 ± 14	32/44	41	26	Hydroxychloroquine plus Moxifloxacin
Bakhshaliyev *et al*[13], Turkey	Retrospective	109	57 ± 14	48/61	49	32	Hydroxychloroquine plus azithromycin
Bernardini *et al*[14], Italy	Retrospective	53	67 ± 12	37/16	21	6	Hydroxychloroquine plus azithromycin
Bun *et al*[15], France	Prospective	73	62 ± 14	49/24	33	19	Hydroxychloroquine plus azithromycin
Chorin *et al*[16], United States	Retrospective	251	64 ± 13	75/176	54	27	Hydroxychloroquine plus azithromycin
Cipriani *et al*[17], Italy	Retrospective	22	64 ± 11	18/4	12	6	Hydroxychloroquine plus azithromycin
Hsia *et al*[18], United States	Retrospective	105	67 ± 15	58/47	51	41	Hydroxychloroquine plus azithromycin
Maraj *et al*[19], United States	Retrospective	91	62 ± 15	51/40	42	26	Hydroxychloroquine plus azithromycin
Mercuro *et al*[20], United States	Prospective	90	60 ± 16	46/44	48	26	Hydroxychloroquine plus azithromycin
Ramireddy *et al*[21], United States	Retrospective	98	62 ± 17	60/38	59	22	Hydroxychloroquine plus azithromycin
Rodrıguez *et al*[22], Spain	Retrospective	161	63 ± 14	103/42	71	25	Hydroxychloroquine plus azithromycin
Saleh *et al*[23], United States	Prospective	201	58 ± 9	115/86	84	65	Hydroxychloroquine plus azithromycin
Saleh *et al*[24], United States	Retrospective	6476	64 ± 15	3980/2496	3184	2161	Hydroxychloroquine plus azithromycin
Sinkeler *et al*[25], Netherlands	Retrospective	397	67 ± 12	262/135	---	---	Hydroxychloroquine
van den Broek *et al*[26], Netherlands	Retrospective	95	65 ± 12	63/32	---	---	Hydroxychloroquine

Interestingly, mechanisms other than drug-related toxicity have been suggested to cause QTc prolongation. In this regard, we can mention cardiac ischemia due to direct viral invasion, electrolyte imbalances, activation of inflammatory cascades, and oxidative stress destroying myocardial tissue[27]. Studies have shown that myocardial ischemia may result in repolarization abnormality leading to cardiac arrhythmias[28]. Also, inflammatory processes, per se, may lead to disruption of cardiomyocyte ion channels by enhancing inward calcium currents and delaying outward potassium currents. It causes prolonged action potential duration and stimulation of cardiomyocytes[29]. Therefore, the occurrence of such arrhythmia may not be primarily related to drug-induced toxicity. However, our study shows a significant increase in QTc after taking hydroxychloroquine/chloroquine. These findings are in line with the previous studies, which suggest that QTc prolongation is due to drug toxicity. Further interventional studies and clinical trials are required to find an explanation for this controversy.

Besides, there is strong evidence that using azithromycin can induce QTc prolongation. According to case-control studies, azithromycin has increased the risk of QTc prolongation up to 1.5 times[30]. Of course, it seems that pre-existing cardiovascular conditions or concomitant use of other QT-prolonging drugs are the conditions for the effectiveness of this drug are in inducing arrhythmia[31].

Overall, a significant proportion of patients with COVID-19 have experienced QTc prolongation. According to our meta-analysis, 4.5% to 18.1% of COVID-19 patients have episodes of QTc prolongation, regardless of the drugs they are taking. Despite this, based on the studies, mortality from the disease does not appear to be due to arrhythmogenic events. However, providing reliable guidelines is essential for managing patients who develop QTc prolongation during treatment. A conventional cut-off for discontinuing treatment with QT-prolonging drugs is a QTc ≥ 500 ms or a rise in QTc more than 60 ms. This strategy is widely popular among the studies that we reviewed, and it led to 4 episodes of TdP and 34 episodes of ventricular tachycardia/fibrillation among 8298 participants. The authors suggest that patients with other risk factors for QTc prolongation should have continuous cardiac monitoring. Furthermore, if the patient had any indications and the clinical status deteriorated, clinicians should discontinue the medications to protect the patient from the potentially fatal arrhythmias.

Table 2 The change in QTc interval and pooled prevalence of QT prolongation following medication

Ref.	Mean QTc (base)	Mean QTc (treatment)	QT prolongation	Overall mortality	Torsades de pointes	Other ventricular arrhythmias	Sudden cardiac death	Stopped medication	Arrhythmo-genic death	Special considerations
Afsin *et al* [12], Turkey	424 ± 28	442 ± 42	6	5	0	0	0	0	0	N/A
Bakhshaliyev *et al*[13], Turkey	435 ± 32	459 ± 38	2	0	0	0	0	0	0	N/A
Bernardini *et al*[14], Italy	424 ± 24	452 ± 26	4	20	0	0	0	0	0	Atrial tachyarrhythmia: *n* = 9 (8%); Premature atrial or ventricular ectopies: *n* = 17 (15.2%); First-degree AV block: *n* = 3 (2.6%)
Bun *et al*[15], France	436 ± 44	460 ± 39	9	0	0	0	0	1	0	Counterclockwise atrial flutter: *n* = 1 (1.3%)
Chorin *et al* [16], United States	439 ± 29	473 ± 36	57	20	1	0	0	8	0	N/A
Cipriani *et al* [17], Italy	426 ± 26	450 ± 22	4	0	0	Non-sustained VT, *n* = 1	0	0	0	N/A
Hsia *et al* [18], United States	439 ± 28	459 ± 32	13	29	0	VT, *n* = 1	0	21	0	N/A
Maraj *et al* [19], United States	437 ± 25	473 ± 31	21	8	1	VF, *n* = 1	0	0	6	Bradyarrhythmia, *n* = 9 (10%)
Mercuro *et al* [20], United States	442 ± 26	473 ± 32	7	4	1	0	0	10	0	N/A
Ramireddy *et al*[21], United States	448 ± 29	459 ± 36	12	0	0	0	0	0	0	N/A
Rodrıguez *et al*[22], Spain	435 ± 25	443 ± 30	37	7	0	0	0	0	0	N/A
Saleh *et al* [23], United States	439 ± 24	463 ± 42	7	0	0	Non-sustained monomorphic VT, *n* = 1; Sustained monomorphic VT, *n* = 1	0	7	0	Atrial fibrillation: *n* = 17 (8.4%)
Saleh *et al* [24], United States	473 ± 35	532 ± 31	67	0	1	Non-sustained monomorphic VT, *n* = 18; Sustained monomorphic VT, *n* = 5; VF, *n* = 4 Sustained polymorphic VT, *n* = 1	0	58	0	N/A
Sinkeler *et al* [25], Netherlands	448 ± 34	468 ± 38	19	0	0	Non-sustained monomorphic VT, *n* = 1	0	27	0	N/A
van den Broek *et al* [26],	444 ± 32	479 ± 42	22	0	0	0	0	0	0	N/A

CONCLUSION

In conclusion, according to our systematic review and meta-analysis, a significant change in QTc interval following the use of hydroxychloroquine alone or in combination with azithromycin is highly expected that may be life-threatening. However, it should be noted that these changes may not be solely due to the toxicity of drugs. Interventional studies are required to confirm this hypothesis.

ARTICLE HIGHLIGHTS

Research background

Current evidence suggests that using hydroxychloroquine and azithromycin for coronavirus disease 2019 (COVID-19) increases the risk of cardiac arrhythmias. Previous studies reported that these drugs caused corrected QT (QTc) prolongation, leading to life-threatening conditions like torsades de pointes and sudden cardiac death. Although both *in vivo* and *in vitro* studies recommended the combination therapy of azithromycin and hydroxychloroquine, even as the first-line approach in preventing disease, it has also led to QTc prolongation.

Research motivation

In addition to cardiac monitoring, identifying patients, who are prone to side effects, helps to minimize the potential harms. By identifying susceptible individuals, it may be possible to use other drug protocols to maintain patient survival.

Research objectives

We summarize the findings about the prevalence and the risk of QTc prolongation in patients treated with hydroxychloroquine ± azithromycin. Also, we discuss the life-threatening conditions in patients taking these medications.

Research methods

We comprehensively searched Medline, Web of Knowledge, Google Scholar, Scopus, and Cochrane Central Register of Controlled Trials databases until October 31, 2020 for all eligible studies under the considered keywords COVID-19, arrhythmia, QT interval, therapy, azithromycin, and hydroxychloroquine until. The study protocols were established in compliance with PRISMA-P guidelines. Outcome measures were QTc prolongation, cardiac arrhythmias, or sudden cardiac death.

Research results

Fifteen studies enrolling 8298 patients with targeted COVID-19 therapeutic regimes were included. The eligible studies found a significant increase in the mean QTc interval following treatment with the described medications compared to baseline QTc with weighted standard differences in means of 0.766. The pooled prevalence rate of QTc prolongation was estimated to be 9.2% (95%CI: 4.5% to 18.1%).

Research conclusions

Hydroxychloroquine ± azithromycin regimen can significantly increase the risk of developing QTc prolongation.

Research perspectives

According to our systematic review and meta-analysis, a significant change in QTc interval following the use of hydroxychloroquine alone or in combination with azithromycin is highly expected that may be life-threatening. However, it should be noted that these changes may not be solely due to the toxicity of drugs. Interventional studies are required to confirm this hypothesis.

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META-ANALYSIS

MicroRNAs as prognostic biomarkers for survival outcome in osteosarcoma: A meta-analysis

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Abstract

BACKGROUND

Osteosarcoma was considered to be one of the most prevalent malignant bone tumors in adolescents.

AIM

To explore the prognostic significance of microRNA (miRNA) in osteosarcoma.

METHODS

The literature was selected by searching online in PubMed, Embase, Web of Science, Cochrane Library, China National Knowledge Infrastructure, and Wanfang Database until July 1, 2021. The pooled hazard ratio (HR) with corresponding 95% confidence interval (CI) for the outcomes of overall survival (OS), disease-free survival (DFS), progression-free survival (PFS) and recurrence-free survival were calculated. Subgroup analyses were carried out to identify potential sources of heterogeneity. Publication bias was assessed by Egger's bias indicator test.

RESULTS

A total of 60 studies from 54 articles with 5824 osteosarcoma patients were included for this meta-analysis. The pooled HR for OS, DFS, PFS were 2.92 (95%CI: 2.43-3.41, P = 0.000), 3.70 (95%CI: 2.80-4.61, P = 0.000), and 3.57 (95%CI: 1.60-5.54, P = 0.000), respectively. The high miR-21 expression levels were related to poor OS in osteosarcoma (HR = 2.86, 95%CI: 1.20-4.53, P = 0.001). Subgroup analysis demonstrated that a high expression level of miRNA correlated with worse OS (HR: 3.56, 95%CI: 2.59-4.54, P = 0.000). In addition, miRNA from tissue (HR: 3.20, 95%CI: 2.16-4.23, P = 0.000) may be a stronger prognostic biomarker in comparison with that from serum and plasma.

CONCLUSION

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

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miRNA (especially miR-21) could be served as a potential prognostic biomarker for osteosarcoma. A high expression level of miRNA in tumor tissue correlated with worse OS of osteosarcoma.

Key Words: Osteosarcoma; MicroRNA; Prognosis; Biomarker; Meta-analysis

Core Tip: Osteosarcoma is a common primary malignant bone tumor with a high degree of malignancy and poor prognosis. MicroRNA can be used as a valuable biomarker for the prognosis of osteosarcoma.

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INTRODUCTION

Osteosarcoma was considered to be one of the most prevalent malignant bone tumors in adolescents. These kind of tumors have an insidious onset, rapid progress, and a high degree of malignancy[1]. The global incidence of osteosarcoma is 4.4 cases per million people[2]. The most common locations of this tumor were the distal femur and proximal tibia, they may also involve the shoulders, mandible, skull and pelvis[3]. The main treatment therapies currently for patients with osteosarcoma are extensive tumor resection, neoadjuvant chemotherapy, and radiotherapy. However, it is well known that patients with osteosarcoma are prone to local relapse and distant metastasis (the most common being lung metastasis), and so the prognosis of osteosarcoma is still unfavorable[4]. The 5-year survival rate of patients with osteosarcoma without metastatic disease is 70%, but for patients with metastasis or relapse it is only 27.4% [5]. At present it is difficult to determine the early stage of treatable osteosarcoma, as a result, it is urgent to determine potential biomarkers that can be used to accurately assess the prognosis of osteosarcoma.

MicroRNA (miRNA) is a group of small non-coding single-stranded RNA that can interfere with the translation of many proteins after gene transcription[6]. MiRNAs participates in diverse biological processes[7], and can act as tumor suppressor genes as well as oncogenes[8]. Therefore, miRNAs may be capable of offering a sensitive method for the diagnosis, monitoring and prognosis of osteosarcoma. Previous studies have shown that miRNAs have been proposed as a valuable biomarkers for the diagnosis[9] and prognosis of osteosarcoma[10]. Although numerous studies have reported the correlation between the prognosis of osteosarcoma and miRNA expression, the results were inconsistent[11-14].

MATERIALS AND METHODS

Literature search strategy

This meta-analysis was performed according to the guidelines of the Meta-analysis of Observational Studies in Epidemiology[15]. To identify relevant studies, two investigators independently and systematically searched online in PubMed, Embase, Web of Science, Cochrane Library, China National Knowledge Infrastructure, and Wanfang Database until July 1, 2021. The combination of keywords used were ("osteosarcoma" or "osteosarcoma tumor") and ("microRNA" or "miRNA" or "miR") and ("prognosis" or "prognostic" or "survival" or "outcome"). Although this online search had no language restrictions, it was limited to publications with human subjects.

Inclusion and exclusion criteria

Included studies met the following criteria: (1) Studies that investigated the prognostic

value of miRNAs in osteosarcoma; (2) Assayed type either blood or tissue samples; (3) Studies that presented sufficient data to allow calculation of hazard ratio (HR) and 95% confidence interval (CI); and (4) Studies that clearly defined the cut-off and described the miRNA measurement method. Studies were excluded if they had one or more of the following criteria: (1) Duplicated or retracted studies; (2) Case reports, comments, letters, review articles or meta-analysis; (3) The obtained miRNAs were from animals or cell lines; and (4) Studies that did not have survival outcomes report or where the data presented was not sufficient to estimate HR.

Data extraction and quality assessment

All data were extracted independently by two investigators. The extracted data were as follows: first author, publication year, country, miRNA type, miRNA expression with poor prognosis (high or low), sample size, specimen type (serum, plasma, or tissue), cut off value, follow-up time (basic unit: month), clinical outcomes, detection method, survival analysis methods, and HR values of miRNAs for predicting overall survival (OS), disease-free survival (DFS), progression-free survival (PFS), and recurrence-free survival (RFS), as well as their 95%CI and *P* values. For studies that did not directly report HR values and 95%CI, we calculated those from the given Kaplan-Meier (K-M) curves by using Engauge Digitizer[16,17]. In case of different publications that investigated the same cohort patients, we selected the most complete research. Also, when univariate and multivariate analysis were carried out at the same time, we chose the last as the more precise result. The quality of all the selected studies was assessed independently by two investigators according to the guidelines of the Newcastle-Ottawa scale (NOS)[18]. The total score of NOS ranged from 0 to 9, studies with a score ≥ 6 were considered as high quality.

Statistical analysis

All statistical analyses were carried out using STATA package version 13.0. The pooled HR with 95%CI was calculated to evaluate the correlation between miRNA expression and the clinical outcome (OS, DFS, PFS, and RFS) of osteosarcoma patients. A pooled HR < 1 implied a more favorable prognosis in patients with aberrant miRNA, and a pooled HR > 1 implied a poor prognosis. Heterogeneity among the studies was assessed using Cochran's *Q* test and Higgins's I^2 statistics. A random effect model was applied if heterogeneity was observed ($P < 0.10$ or $I^2 > 50\%$), while a fixed effect model was applied in the absence of heterogeneity ($P \geq 0.10$ or $I^2 \leq 50\%$). Subsequently, subgroup analyses for OS were carried out to identify potential sources of heterogeneity and to assess the prognostic value of different subgroups. In addition, sensitivity analysis was carried out to explore the impact of one single study on the pooled HR. Publication bias was evaluated using the Egger's bias plot[19], where $P < 0.05$ indicates significant publication bias.

RESULTS

Literature search results

The detailed literature search results are presented in Figure 1. The primary search identified 6327 articles. After excluding 2486 duplicate studies, 3841 articles remained. Secondly, 3698 studies were excluded after screening titles and abstracts, among which 2674 irrelevant research, 736 not human research, and 288 case report, letters or reviews. Subsequently, through careful reading and screening of the full text of 143 studies, 89 studies were excluded due to insufficient data. Finally, 60 studies from 54 articles were included in our meta-analysis.

Characteristics of studies

In Table 1 and Table 2, we summarized the basic information of included studies. A total of 5824 osteosarcoma patients across the 60 studies were included in this meta-analysis, among them 58 studies were conducted in China, 1 in United States and 1 in Japan. These studies have reported 51 miRNAs with different prognostic values. Among the articles included in this meta-analysis, 27 studies showed high miRNA expression and 33 studies showed low miRNA expression, indicating poor prognosis of osteosarcoma. The expression levels of miRNAs from 28 serum specimens, 6 plasmas specimens, and 26 tissue specimens were analyzed using PCR. In most of the studies, the mean/median were considered as the critical cut-off values. The follow-up time of all the studies ranged from 30 mo to 150 mo, of which 40 studies had a follow-

Table 1 Characteristics of eligible studies in this meta-analysis

Ref.	Country	MicroRNA	MicroRNA expression[1]	Sample size			Specimen	Cut off value	Follow-up (mo)	Outcome	Detection method	Survival analysis	Source of HR	NOS score
				Total	Low	High								
Yuan *et al*[20], 2012	China	miR-21	High	65	NR	NR	Serum	Median	46	OS	RT-PCR	U/M	Reported	6
Ji *et al*[21], 2013	China	miR-133a	Low	92	46	46	Tissue	Median	60	OS	RT-PCR	U	K-M curve	6
Cai *et al*[22], 2013	China	miR-210	High	92	44	48	Tissue	Median	100	OS/PFS	RT-PCR	U	K-M curve	6
Yang *et al*[23], 2013	China	miR-132	Low	166	90	76	Tissue	Median	100	OS/DFS	RT-PCR	U/M	K-M curve	6
Li *et al*[24], 2014	China	miR-17-92	High	117	45	72	Tissue	Median	60	OS/RFS	RT-PCR	U/M	Reported	6
Xu *et al*[25], 2014	China	miR-9	High	79	39	40	Tissue	Median	60	OS	RT-PCR	M	Reported	6
Zhang *et al*[26], 2014	China	miR-133b/ 206	Low	100	NR	NR	Serum	2.7/2.8	40	OS/DFS	RT-PCR	M	K-M curve	8
Cai *et al*[27], 2014	China	miR-340	Low	92	NR	NR	Tissue	Median	100	OS/PFS	RT-PCR	M	K-M curve	7
Fei *et al*[28], 2014	China	miR-9	High	118	NR	NR	Serum	Median	80	OS	RT-PCR	U	K-M curve	6
Zhang *et al*[13], 2014	China	miR-196a/b	High	100	NR	NR	Serum	4.9/5.5	40	OS/DFS	RT-PCR	M	K-M curve	8
Ma *et al*[29], 2014	China	miR-148a	High	148	NR	NR	Plasma	Median	100	OS/DFS	RT-PCR	U/M	K-M curve	7
Lin *et al*[30], 2014	China	miR-17-92	High	63	25	38	Tissue	Median	60	OS	RT-PCR	U	K-M curve	6
Song *et al*[31], 2014	China	miR-26a	Low	144	63	81	Tissue	Median	150	OS/DFS	RT-PCR	M	K-M curve	7
Wang *et al*[32], 2014	China	miR-214	High	82	33	49	Tissue	Median	60	OS/FFS	RT-PCR	U	K-M curve	7
Cai *et al*[33], 2015	China	miR-195	Low	166	88	78	Serum	1.44	100	OS/DFS	RT-PCR	M	K-M curve	8
Liu *et al*[34], 2015	China	miR-126	Low	122	60	62	Tissue	Median	60	OS	RT-PCR	M	Reported	7
Sun *et al*[35], 2015	China	miR-217	Low	168	84	84	Tissue	Median	80	OS	RT-PCR	U/M	Reported	6
Wang, *et al*[36], 2015	China	miR-152	Low	80	42	38	Serum	Median	60	OS	RT-PCR	M	Reported	6
Yang *et al*[37], 2015	China	miR-221	High	108	55	53	Serum	Median	60	OS/RFS	RT-PCR	M	Reported	7
Tang *et al*[38], 2015	China	miR-27a	High	166	80	86	Serum	Median	100	OS/DFS	RT-PCR	U/M	K-M curve	8
Wang *et al*[39], 2015	China	miR-191	High	100	42	58	Serum	3.56	60	OS/DFS	RT-PCR	M	K-M curve	8
Cao *et al*[40], 2016	China	miR-326	Low	60	25	35	Serum	Median	60	OS	RT-PCR	M	Reported	6
Dong *et al*[41], 2016	China	miR-223	Low	112	53	59	Serum	Median	60	OS	RT-PCR	M	Reported	6
Liu *et al*[42], 2016	China	miR-300	High	114	46	68	Serum	Median	60	OS/DFS	RT-PCR	U/M	Reported	6
Luo *et al*[14], 2016	China	miR-125b	Low	56	NR	NR	Plasma	Median	30	OS	RT-PCR	U/M	Reported	7

Niu *et al*[43], 2016	China	miR-95-3p	Low	133	97	36	Serum	Median	60	OS	RT-PCR	M	Reported	7
Pang *et al*[44], 2016	China	miR-497	Low	185	91	94	Serum	Median	70	OS	RT-PCR	U/M	Reported	7
Li *et al*[45], 2016	China	miR-145	low	39	19	20	Tissue	Median	60	OS	RT-PCR	U	K-M curve	7
Zhang and Wang [46], 2016	China	miR-222	High	57	28	29	Serum	Median	80	OS/DFS	RT-PCR	U/M	K-M curve	7
Ren *et al*[47], 2016	China	miR-21	High	84	33	51	Tissue	Median	100	OS/DFS	RT-PCR	U/M	K-M curve	6
Wang *et al*[48], 2017	China	miR-34a	low	120	NR	NR	Serum	4.16	60	OS	RT-PCR	M	Reported	7
Yang *et al*[49], 2017	China	miR-203	Low	103	52	51	Tissue	Median	60	OS/DFS	RT-PCR	U/M	Reported	7
Zhu *et al*[11], 2017	China	miR-17-5p	High	62	NR	NR	Plasma	Median	70	OS/PFS	RT-PCR	U/M	Reported	6
Nakka *et al*[50], 2017	United States	miR-221	Low	32	NR	NR	Plasma	Median	150	OS	RT-PCR	M	K-M curve	6
Zou *et al*[51], 2017	China	miR-19a	High	166	80	86	Tissue	Median	100	OS/DFS	RT-PCR	U/M	Reported	7
Hu *et al*[52], 2017	China	miR-19a	High	32	NR	NR	Tissue	Median	50	OS	RT-PCR	U/M	Reported	6
Wang *et al*[53], 2017	China	miR-491	Low	102	58	44	Serum	Median	60	OS	RT-PCR	U/M	Reported	7
Wang *et al*[54], 2017	China	miR-491-5p	Low	72	36	36	Tissue/ Serum	Median	60	OS/DFS	RT-PCR	M	Reported	6
Tang *et al*[55], 2017	China	miR-490-3p	Low	148	76	72	Tissue	Median	70	OS	RT-PCR	M	K-M curve	6
Gao *et al*[56], 2018	China	miR-564	Low	217	107	110	Tissue	Median	60	OS/DFS	RT-PCR	M	K-M curve	7
Yao *et al*[57], 2018	China	miR-101	Low	152	82	70	Serum	Median	60	OS/RFS	RT-PCR	U/M	K-M curve	6
Cong *et al*[58], 2018	China	miR-124	Low	114	NR	NR	Serum	Median	60	OS/DFS	RT-PCR	M	Reported	6
Liu *et al*[59], 2018	China	miR-375	Low	95	47	48	Serum	Median	60	OS	RT-PCR	M	Reported	6
Zhong *et al*[60], 2018	China	miR-1270	High	143	69	74	Tissue	Median	70	OS	RT-PCR	U/M	Reported	7
Li *et al*[61], 2018	China	miR-542-3p	High	76	NR	NR	Serum	0.87	70	OS/DFS	RT-PCR	U/M	Reported	8
Zhou *et al*[62], 2018	China	miR-139-5p	Low	98	53	45	Serum	Median	60	OS	RT-PCR	U/M	Reported	7
Heishima *et al*[63], 2019	Japan	miR-214	High	106	NR	NR	Plasma	Median	72	OS/DFS	RT-PCR	U/M	Reported	7
Liang *et al*[12], 2019	China	miR-765	Low	33	NR	NR	Tissue	Median	72	OS	RT-PCR	U/M	Reported	6
Xu, *et al*[64], 2019	China	miR-106b	High	134	64	70	Tissue	Median	60	OS	RT-PCR	M	Reported	7
Diao *et al*[65], 2020	China	miR-22	Low	120	60	60	Plasma	Median	60	OS	RT-PCR	U/M	Reported	6
Li *et al*[66], 2020	China	miR-1826	Low	122	69	53	Tissue	Median	60	OS	RT-PCR	U/M	Reported	7
Li *et al*[67], 2020	China	miR-629	High	110	45	65	Tissue	Median	60	OS	RT-PCR	M	Reported	7

Yang *et al*[68], 2020	China	miR-21/ 214	High	63	NR	NR	Tissue	Median	60	OS	RT-PCR	M	Reported	7
Shi *et al*[69], 2020	China	miR-194	Low	124	69	55	Serum	Median	60	OS/DFS	RT-PCR	M	Reported	7

[1]MicroRNA expression with poor prognosis.

High: High expression; Low: Low expression; OS: Overall survival; PFS: Progression-free survival; DFS: Disease-free survival; RFS: Recurrence-free survival; NR: Not reported; M: Multivariate analysis; U: Univariate analysis; NOS: Newcastle-Ottawa scale; RT-PCR: Real-time reverse transcription-PCR.

up time of less than 5 years, and 20 studies had more than 5 years of follow-up. All of the articles were analyzed to evaluate the correlation between miRNAs and survival outcomes: 60 studies for OS, 25 studies for DFS, 4 studies for PFS, and 2 studies for RFS. The HRs and 95%CIs were directly reported in 35 studies, and the values of HRs and 95%CI in the remaining 25 studies were extracted by K-M curve. Furthermore, we assessed the quality for each study that was included in this meta-analysis and found that the NOS score varied from 6 to 8, which means the quality of each study was high.

Meta-analysis in clinical outcome of osteosarcoma

This meta-analysis was conducted based on the survival endpoint used by each study. There were 60 studies evaluated OS of osteosarcoma, the pooled HR for OS was 2.92 (95%CI: 2.43-3.41, P = 0.000). For the 25 studies that evaluated DFS of patients, the pooled HR was 3.70 (95%CI: 2.80-4.61, P = 0.000). For the studies that tested PFS (n = 4) and RFS (n = 2) as their outcome assessments, the pooled HR were 3.57 (95%CI: 1.60-5.54, P = 0.000) and 3.71 (95%CI: 0.09-7.51, P = 0.056), respectively. The pooled HR of OS studies as well as that of DFS and PFS (P < 0.05) were found to be statistically significant; however, the effect of RFS studies did not reach the level of statistical significance. The forest plots of OS are presented in Figure 2A, and the forest plots of DFS, PFS and RFS are presented in Figure 2B.

Prognostic value of miR-21 in osteosarcoma

In our meta-analysis, there were three studies that assessed miR-21 as a predictor of OS in osteosarcoma. We calculated the pooled HRs for these studies by using the random-effects model. The results suggested that high expression levels of miR-21 are correlated with poor OS (HR = 2.86, 95%CI: 1.20-4.53, P = 0.001). In addition, there was only one study that accessed the correlation between miR-21 expression and DFS, which indicates that the high expression levels of miR-21 are correlated with poor DFS. Therefore, miR-21 may be used as a biomarker of poor prognosis in osteosarcoma.

Subgroup analyses

As shown in Table 3, six subgroup analyses of OS in osteosarcoma patients were done depending on miRNA expression, specimen, survival analysis, source of HR, follow-up, and country of publication. To analyze the prognostic value of miRNA expression in osteosarcoma, we assessed the subgroups for high expression and low expression

Table 2 Hazard ratios and 95% confidence intervals of survival outcome extracted from included studies

Ref.	MicroRNA	OS		PFS		DFS		RFS	*P*
		HR (95%CI)	*P*	HR (95%CI)	*P*	HR (95%CI)	*P*	HR (95%CI)	
Yuan *et al*[20], 2012	miR-21	2.33 (1.11-4.89)	0.026						
Ji *et al*[21], 2013	miR-133a	1.87 (1.12–3.31)	0.015	2.6 (0.8–7.2)	0.02				
Cai *et al*[22], 2013	miR-210	3.3 (1.0–8.1)	0.01						
Yang *et al*[23], 2013	miR-132	4.9 (1.0–10.2)	0.001			4.1 (0.8–9.7)	0.006		
Li *et al*[24], 2014	miR-17-92	2.94 (1.51-5.75)	0.002					2.50 (1.77-6.92)	0.001
Xu *et al*[25], 2014	miR-9	4.77 (2.86–5.91)	0.002						
Zhang *et al*[26], 2014	miR-133b	5.36 (1.26-11.03)	0.02			5.69 (1.33-11.26)	0.02		
Zhang *et al*[26], 2014	miR-206	5.42 (1.31-11.28)	0.02			5.88 (1.56-12.08)	0.02		
Zhang *et al*[26], 2014	miR-133b/206	9.28 (2.69-20.79)	0.001			9.69 (2.80-21.82)	0.001		
Cai *et al*[27], 2014	miR-340	6.2 (1.4–13.9)	0.006	4.5 (1.0–9.2)	0.01				
Fei *et al*[28], 2014	miR-9	3.18 (1.35-4.42)	0.002						
Zhang *et al*[13], 2014	miR-196a	6.28 (1.62–13.39)	0.01			6.95 (1.63–14.61)	0.01		
Zhang *et al*[13], 2014	miR-196b	6.33 (1.61–13.48)	0.01			6.98 (1.65–14.82)	0.01		
Zhang *et al*[13], 2014	miR-196a/196b	9.89 (2.66–20.98)	0.001			10.09 (2.82–21.99)	0.001		
Ma *et al*[29], 2014	miR-148a	2.70 (1.41-5.17)	0.003			2.53 (1.28-5.01)	0.008		
Lin *et al*[30], 2014	miR-17-92	2.93 (1.25-6.88)	0.007						
Song *et al*[31], 2014	miR-26a	5.72 (1.01-10.94)	0.007			3.97 (1.19-9.87)	0.014		
Wang *et al*[32], 2014	miR-214	5.6 (1.2–12.9)	0.008	3.5 (1.0–8.2)	0.01				
Cai *et al*[33], 2015	miR-195	5.16 (1.92-11.88)	0.002			3.62 (1.83-9.09)	0.01		
Liu *et al*[34], 2015	miR-126	3.10 (1.11–9.02)	0.018						
Sun *et al*[35], 2015	miR-217	2.83 (1.13–7.05)	0.026						
Wang *et al*[36], 2015	miR-152	0.13 (0.02–0.70)	0.004						
Yang *et al*[37], 2015	miR-221	7.66 (1.83–15.92)	0. 01					6.82 (1.33–13.69)	0.01
Tang *et al*[38], 2015	miR-27a	2.17 (1.30-3.62)	0. 01			1.39 (0.46-4.22)	0. 01		
Wang, *et al*[39], 2015	miR-191	3.56 (1.62–7.88)	0.01			2.63 (1.28–6.06)	0.02		
Cao *et al*[40], 2016	miR-326	3.90 (1.13-12.53)	0.001						
Dong *et al*[41], 2016	miR-223	4.59 (1.84–11.45)	0.001						
Liu *et al*[41], 2016	miR-300	5.96 (2.35-10.70)	0.009			5.11 (2.14-9.48)	0.019		
Luo *et al*[14], 2016	miR-125b	0.49 (0.24-0.10)	0.049						
Niu *et al*[43], 2016	miR-95-3p	4.22 (2.31–8.07)	0.014						
Pang *et al*[44], 2016	miR-497	3.79 (1.99-8.57)	0.004						
Li *et al*[45], 2016	miR-145	2.07 (1.17-3.66)	0.0124						
Zhang and Wang[46], 2016	miR-222	1.19 (1.05-1.36)	0.008			1.23 (1.07-1.40)	0.003		
Ren *et al*[47], 2016	miR-21	6.3 (1.8–12.4)	0.001			5.8 (1.7–13.5)	0.001		
Wang *et al*[48], 2017	miR-34a	3.18 (2.87-9.56)	0.001						
Yang *et al*[49], 2017	miR-203	2.73 (1.69-8.91)	0.01			4.19 (2.91-10.12)	0.005		
Zhu *et al*[11], 2017	miR-17-5p	10.47 (2.29-47.87)	0.002	5.53 (2.02-15.16)	0.001				
Nakka *et al*[50], 2017	miR-221	0.73 (0.49-1.11)	0.139						
Zou *et al*[51], 2017	miR-19a	5.06 (1.12–11.62)	0.001			4.66 (0.88–10.97)	0.006		
Hu *et al*[52], 2017	miR-19a	2.62 (2.12-6.15)	0.037						

Wang *et al*[53], 2017	miR-491	2.96 (1.09-8.07)	0.03		
Wang *et al*[54], 2017	miR-491-5p	2.55 (1.28-4.64)	0.039	2.44 (1.21-4.51)	0.03
Wang *et al*[54], 2017	miR-491-5p	2.72 (1.30-4.92)	0.028	2.64 (1.27-5.02)	0.022
Tang *et al*[55], 2017	miR-490-3p	2.13 (1.33-7.76)	0.001		
Gao *et al*[56], 2018	miR-564	4.25 (1.66-8.63)	0.001	4.63 (1.88-9.38)	0.001
Yao *et al*[57], 2018	miR-101	3.82 (1.34-6.52)	0.014	4.23 (1.45-7.22)	0.008
Cong *et al*[58], 2018	miR-124	3.54 (1.38–5.64)	0.019	3.92 (1.57–6.39)	0.012
Liu *et al*[59], 2018	miR-375	3.27 (1.36-7.85)	0.008		
Zhong *et al*[60], 2018	miR-1270	4.34 (4.08–4.79)	0.018		
Li *et al*[61], 2018	miR-542-3p	5.98 (3.26–18.44)	0.01	6.89 (2.83–9.67)	0.018
Zhou *et al*[62], 2018	miR-139-5p	3.24 (1.81–4.76)	0.007		
Heishima *et al*[63], 2019	miR-214	2.95 (1.35-7.77)	0.005	3.51 (1.68-8.55)	0.0004
Liang *et al*[12], 2019	miR-765	0.39 (0.20-0.77)	0.007		
Xu, *et al*[64], 2019	miR-106b	2.77 (1.37-5.60)	0.005		
Diao *et al*[65], 2020	miR-22	3.31 (1.41-7.77)	0.006		
Li *et al*[66], 2020	miR-1826	2.45 (1.25-4.37)	0.014		
Li *et al*[67], 2020	miR-629	2.89 (1.13-7.42)	0.027		
Yang *et al*[68], 2020	miR-21	3.46 (2.17-11.52)	0.013		
Yang *et al*[68], 2020	miR-214	3.14 (2.01-10.26)	0.017		
Shi *et al*[69], 2020	miR-194	3.65 (1.58-5.89)	0.014	4.39 (1.90-6.91)	0.005

HR: Hazard ratio; CI: Confidence interval; OS: Overall survival; PFS: Progression-free survival; DFS: Disease-free survival; RFS: Recurrence-free survival.

levels of miRNA for OS. The high expression levels of miRNA showed a pooled HR of 3.56 (95%CI: 2.59-4.54, P = 0.000), while the low expression levels of miRNA showed a pooled HR of 2.23 (95%CI:1.74-2.72, P = 0.000) (Figure 3A). The results were found to be statistically significant for both high expression and low expression levels of miRNA; however, the high expression levels of miRNA were significantly correlated with poor prognosis in osteosarcoma. The pooled HR of different specimen of miRNAs are as follows: tissue, 3.20 (95%CI: 2.16-4.23, P = 0.000); serum, 3.04 (95%CI: 2.37-3.71, P = 0.000); plasma, 0.94 (95%CI: 0.36-1.51, P = 0.001) (Figure 3B). The result show that pooled HR for univariate analysis was 2.36 (95%CI: 1.67-3.04, P = 0.000) and for multivariate analysis was 3.29 (95%CI: 2.52-4.07, P = 0.000), and the pooled HR for univariate analysis and multivariate analysis was 2.73 (95%CI: 1.95-3.50, P = 0.000) (Figure 3C). As shown in Figure 3D, the pooled HR calculated directly from the original reported studies was 3.00 (95%CI: 2.21-3.79, P = 0.000), and the pooled HR calculated from K-M curve was 2.48 (95%CI: 1.92-3.04, P = 0.000). In addition, we found that miRNAs contributed to a different OS when the follow-up time for patients was different, the pooled HR for less than 5 years of follow-up time was 3.08 (95%CI: 2.44-3.71, P = 0.000), and the pooled HR for more than 5 years of follow-up time was 2.75 (95%CI: 1.88-3.63, P = 0.000) (Figure 3E). Furthermore, the pooled HR for OS was 3.05 in China (95%CI: 2.52-3.57, P = 0.000) (Figure 3F).

Sensitivity analysis and publication bias

We conducted a sensitivity analysis using pooled HR and by omitting single studies, and none of these studies exceeded the outliers, which indicates that the pooled results of this meta-analysis are credible. The publication bias of included studies was assessed using Egger's graphical tests, and the P values of Egger's tests were 0.987, which indicates that there was no publication bias in our meta-analysis (Figure 4).

Table 3 Subgroup analyses of the relationship between microRNA expression and overall survival.

Subgroup	Number of datasets	Heterogeneity		Pooled HR (95%CI)	*P* value
		I^2 (%)	*P* value		
Overall	60	89.0%	0.000	2.92 (2.43–3.41)	0.000
Expression					
High	27	91.4%	0.000	3.56 (2. 59–4.54)	0.000
Low	33	74.5%	0.000	2.23 (1. 74–2.72)	0.000
Specimen					
Tissue	26	92.1%	0.000	3.20 (2.16–4.23)	0.000
Serum	28	77.1%	0.000	3.04 (2.37–3.71)	0.000
Plasma	6	53.7%	0.055	0.94 (0.36–1.51)	0.001
Survival analysis					
U	6	0.0%	0.569	2.36 (1.67–3.04)	0.000
M	30	76.9%	0.000	3.29 (2.52–4.07)	0.000
U/M	24	93.9%	0.000	2.73 (1.95–3.50)	0.000
Source of HR					
Reported	35	92.6%	0.000	3.00 (2.21–3.79)	0.000
K-M curve	25	65.6%	0.000	2.48 (1.92–3.04)	0.000
Follow-up					
≤ 5 years	40	76.2%	0.000	3.08 (2.44–3.71)	0.000
> 5 years	20	94.8%	0.000	2.75 (1.88–3.63)	0.000
Country					
China	58	89.0%	0.000	3.05 (2.52–3.57)	0.000
Non-China	2	44.9%	0.178	1.24 (0.59–3.07)	0.183

OS: Overall survival; M: Multivariate analysis; U: Univariate analysis; HR: Hazard ratio; CI: Confidence interval.

DISCUSSION

Osteosarcoma is a malignant bone tumor occurring in teenagers. It is a mesenchymal tumor with histological features such as the presence of malignant mesenchymal cells and the production of bone stroma[70]. As one of the most destructive malignant tumors, osteosarcoma has a 5-year survival rate of less than 60% due to its high aggressiveness and the tendency to metastasize early[71]. It is well known that the key to the successful treatment of osteosarcoma is its accurate and timely diagnosis, and is also an important guarantee for improving prognosis and survival. This motivated us to explore more effective and/or more accurate biological biomarkers for clinical applications of osteosarcoma. MiRNAs have been used as biomarkers for the diagnosis, metastasis, and prognosis of many cancers. A large number of studies have reported the prognostic value of miRNA for osteosarcoma. However, the results are still controversial and no consensus has been reached. Therefore, we performed this meta-analysis to explore the prognostic function of miRNA in patients with osteosarcoma.

We collected all the literature on the prognostic value of miRNAs in osteosarcoma. First, we calculated the pooled HR according to the survival endpoint used in each study: the pooled HR for overall OS was 2.92 (95%CI: 2.43-3.41, $P = 0.000$), for DFS was 3.70 (95%CI: 2.80-4.61, $P = 0.000$), for PFS was 3.57 (95%CI: 1.60-5.54, $P = 0.000$), and for RFS was 3.71 (95%CI: 0.09-7.51, $P = 0.056$). It was found that the pooled HR were statistically related to the clinical outcome (OS, DFS, and PFS) of osteosarcoma ($P < 0.05$), but the impact of RFS did not reach a statistically significant level. We also evaluated the prognostic value of miR-21 in osteosarcoma. The result showed that high expression levels of miR-21 were associated with poor OS in osteosarcoma (HR =

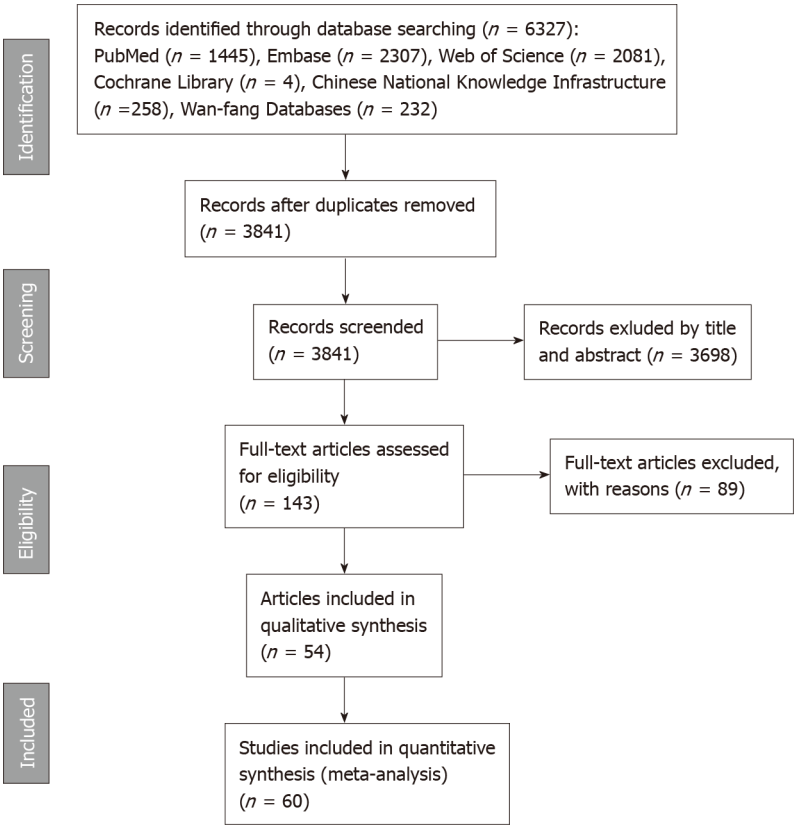

Figure 1 The flow chart of this meta-analysis to identify inclusion studies.

2.86, 95%CI: 1.20-4.53, P = 0.001), which may be used as a marker of poor prognosis in patients with osteosarcoma. MiR-21 has been demonstrated to be a tumor oncogene and plays an important role in the progression of various types of tumors[72]. A recent study suggested that, LncRNA neuroblastoma-associated transcript 1 suppresses the expression of miR-21 and also targets the miR-21-associated genes in osteosarcoma [73]. MiR-21 modulates cell invasion and migration by directly targeting reversion-inducing-cysteine-rich protein with kazal motifs and phosphatase and tensin homologue gene in osteosarcoma[74,75], it is also reduces the anti-tumor effect of cisplatin in osteosarcoma by regulating Bcl-2 expression[76].

Subsequently, due to heterogeneity of studies, we conducted a subgroup analysis of OS in patients with osteosarcoma on the basis of miRNA expression, specimens, survival rate analysis, HR source, follow-up status, and publishing country. The high expression level of miRNA shows that the combined HR is 3.56 (95%CI: 2.59-4.54, P = 0.000), while the low expression level of miRNA shows that the combined HR is 2.23 (95%CI: 1.74-2.72, P = 0.000). This indicates that the altered miRNA is related to the poor prognosis of osteosarcoma, and the high expression level of miRNA indicates a higher risk of poor prognosis of osteosarcoma. Different miRNA specimen sources may lead to different clinical outcomes. We found that miRNA from tissues (HR: 3.20, 95%CI: 2.16-4.23, P = 0.000) may be a stronger prognostic biomarker for patients with osteosarcoma. As regards the source of HR, there is no big difference between the HR directly reported in the original article and the HR extracted in the K-M survival curve, which indicates that the data extracted from the K-M survival curve according to the previous methods were reliable[17]. The length of follow-up time has different prognosis in osteosarcoma, and we found that patients with less than 5 years of follow-up have a higher risk of poor prognosis than those with more than 5 years of follow-up. This may be related to tumor aggressiveness and metastasis of osteosarcoma. The survival time of orthotopic osteosarcoma is significantly longer than that of patients with metastasis[71]. Moreover, the country of publication has no significant impact on the prognosis of patients with osteosarcoma. Finally, we conducted sensitivity analysis and publication bias and found that the combined results of the study are credible without publication bias.

This is a comprehensive meta-analysis to evaluate the prognostic function of miRNAs for osteosarcoma. Two investigators independently conducted literature screening and data extraction based on strict inclusion and exclusion criteria. We make

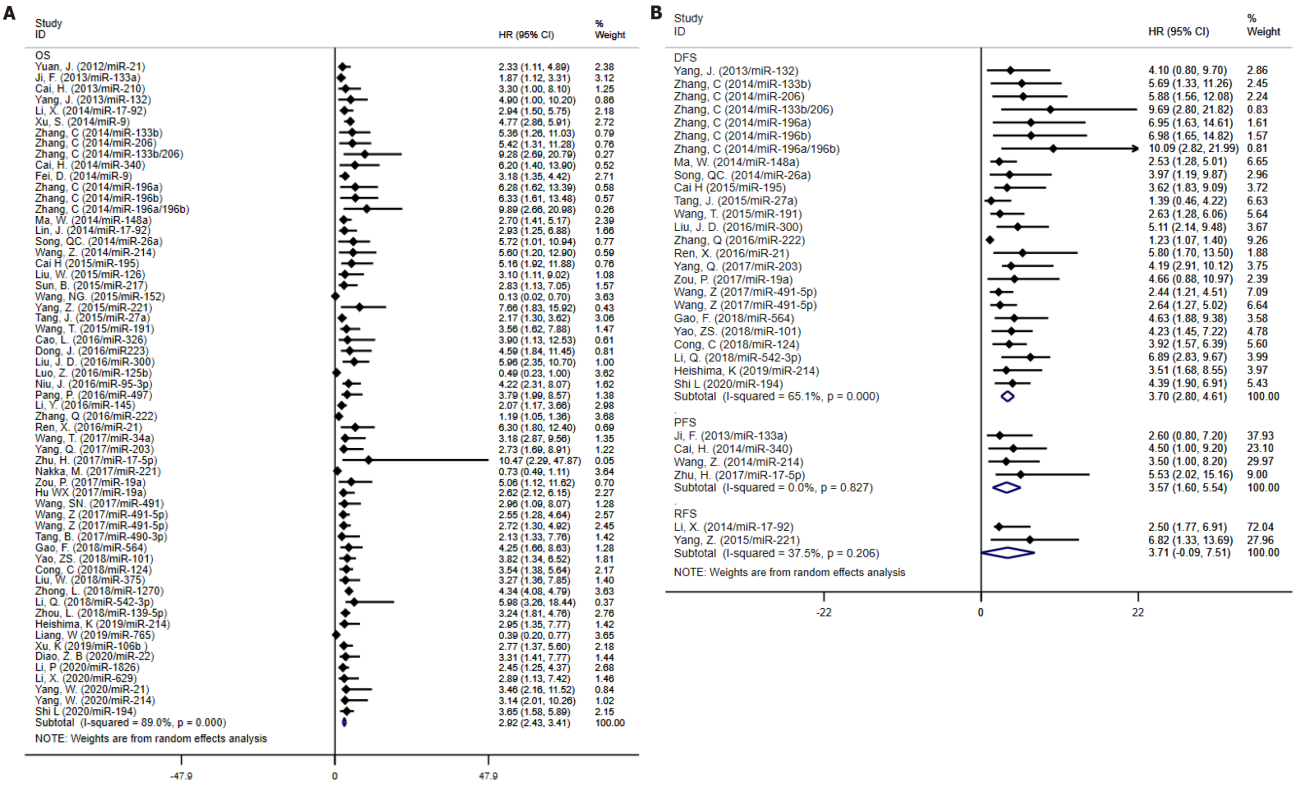

Figure 2 Forest plots for the microRNAs associated with clinical outcome. A: Forest plots for the microRNA (miRNA) associated with overall survival; B: Forest plots for the miRNAs associated with disease-free survival, progression-free survival and recurrence-free survival.

every effort to avoid publishing biases, but we acknowledge that this meta-analysis still has some limitations. First, we excluded a small number of studies that lacked original data or could not calculate HR, which may relatively reduce the convincing power of the results. Secondly, some HR data are extracted from the survival curve, which may bring small errors. Future research should be based on data obtained directly from the original article if possible. Third, although there is no obvious publication bias in this meta-analysis, positive results are more likely to be published than negative results, which may lead to an overestimation of the association between miRNAs and poor prognosis of osteosarcoma. Fourth, almost all of the 54 articles included are from China, except for one from the United States and one from Japan. Therefore, the applicability of miRNA as a prognostic indicator of osteosarcoma in other countries and regions is unclear, and our results need to be proved by more studies from other regions.

CONCLUSION

In summary, miRNA (especially miR-21), could be used as a potential prognostic biomarker for osteosarcoma. A high expression level of miRNA in tumor tissue correlated with worse OS of osteosarcoma. However, we should also focus on the low expression level of several special miRNAs. Furthermore, a large number of samples, particularly multi-center, multi-country prospective studies, will be required in the future to enhance the persuasiveness of the prognostic role of miRNA in osteosarcoma.


A
Study ID
HR (95% CI)
% Weight
High
Yuan, J. (2012/miR-21) 2.33 (1.11, 4.89) 2.38
Cai, H. (2013/miR-210) 3.30 (1.00, 8.10) 1.25
Li, X. (2014/miR-17-92) 2.94 (1.50, 5.75) 2.18
Xu, S. (2014/miR-9) 4.77 (2.86, 5.91) 2.72
Fei, D. (2014/miR-9) 3.18 (1.35, 4.42) 2.71
Zhang, C (2014/miR-196a) 6.28 (1.62, 13.39) 0.58
Zhang, C (2014/miR-196b) 6.33 (1.61, 13.48) 0.57
Zhang, C (2014/miR-196a/196b) 9.89 (2.66, 20.98) 0.26
Ma, W. (2014/miR-148a) 2.70 (1.41, 5.17) 2.39
Lin, J. (2014/miR-17-92) 2.93 (1.25, 6.88) 1.66
Wang, Z. (2014/miR-214) 5.60 (1.20, 12.90) 0.59
Yang, Z. (2015/miR-221) 7.66 (1.83, 15.92) 0.43
Tang, J. (2015/miR-27a) 2.17 (1.30, 3.62) 3.06
Wang, T. (2015/miR-191) 3.56 (1.62, 7.88) 1.47
Liu, J. D. (2016/miR-300) 5.96 (2.35, 10.70) 1.00
Zhang, Q. (2016/miR-222) 1.19 (1.05, 1.36) 3.68
Ren, X. (2016/miR-21) 6.30 (1.80, 12.40) 0.69
Zhu, H. (2017/miR-17-5p) 10.47 (2.29, 47.87) 0.05
Zou, P. (2017/miR-19a) 5.06 (1.12, 11.62) 0.70
Hu WX (2017/miR-19a) 2.62 (2.12, 6.15) 2.27
Zhong, L. (2018/miR-1270) 4.34 (4.08, 4.79) 3.63
Li, Q. (2018/miR-542-3p) 5.98 (3.26, 18.44) 0.37
Heishima, K (2019/miR-214) 2.95 (1.35, 7.77) 1.42
Xu, K (2019/miR-106b) 2.77 (1.37, 5.60) 2.18
Li, X. (2020/miR-629) 2.89 (1.13, 7.42) 1.46
Yang, W. (2020/miR-21) 3.46 (2.16, 11.52) 0.84
Yang, W. (2020/miR-214) 3.14 (2.01, 10.26) 1.02
Subtotal (I-squared = 91.4%, p = 0.000) 3.56 (2.59, 4.54) 41.55
Low
Ji, F. (2013/miR-133a) 1.87 (1.12, 3.31) 3.12
Yang, J. (2013/miR-132) 4.90 (1.00, 10.20) 0.86
Zhang, C (2014/miR-133b) 5.36 (1.26, 11.03) 0.79
Zhang, C (2014/miR-206) 5.42 (1.31, 11.28) 0.76
Zhang, C (2014/miR-133b/206) 9.28 (2.69, 20.79) 0.27
Cai, H. (2014/miR-340) 6.20 (1.40, 13.90) 0.52
Song, QC. (2014/miR-26a) 5.72 (1.01, 10.94) 0.77
Cai H (2015/miR-195) 5.16 (1.92, 11.88) 0.76
Liu, W. (2015/miR-126) 3.10 (1.11, 9.02) 1.08
Sun, B. (2015/miR-217) 2.83 (1.13, 7.06) 1.57
Wang, NG. (2015/miR-152) 0.13 (0.02, 0.70) 3.63
Cao, L. (2016/miR-326) 3.90 (1.13, 12.53) 0.61
Dong, J. (2016/miR-223) 4.59 (1.84, 11.45) 0.81
Luo, Z. (2016/miR-125b) 0.49 (0.23, 1.00) 3.62
Niu, J. (2016/miR-95-3p) 4.22 (2.31, 8.07) 1.62
Pang, P. (2016/miR-497) 3.79 (1.99, 8.57) 1.38
Li, Y. (2016/miR-145) 2.07 (1.17, 3.66) 2.98
Wang, T. (2017/miR-34a) 3.18 (2.87, 9.56) 1.35
Yang, Q. (2017/miR-203) 2.73 (1.69, 8.91) 1.22
Nakka, M. (2017/miR-221) 0.73 (0.49, 1.11) 3.64
Wang, SN. (2017/miR-491) 2.96 (1.09, 8.07) 1.28
Wang, Z (2017/miR-491-5p) 2.55 (1.28, 4.64) 2.57
Wang, Z (2017/miR-491-5p) 2.72 (1.30, 4.92) 2.45
Tang, B. (2017/miR-490-3p) 2.13 (1.33, 7.76) 1.42
Gao, F. (2018/miR-564) 4.25 (1.66, 8.63) 1.28
Yao, ZS. (2018/miR-101) 3.82 (1.34, 6.52) 1.81
Cong, C (2018/miR-124) 3.54 (1.38, 5.64) 2.17
Liu, W. (2018/miR-375) 3.27 (1.36, 7.85) 1.40
Zhou, L. (2018/miR-139-5p) 3.24 (1.81, 4.76) 2.76
Liang, W (2019/miR-765) 0.39 (0.20, 0.77) 3.65
Diao, Z. B (2020/miR-22) 3.31 (1.41, 7.77) 1.44
Li, P (2020/miR-1826) 2.45 (1.25, 4.37) 2.68
Shi L (2020/miR-194) 3.65 (1.58, 5.89) 2.15
Subtotal (I-squared = 74.5%, p = 0.000) 2.23 (1.74, 2.72) 58.45
Overall (I-squared = 89.0%, p = 0.000) 2.92 (2.43, 3.41) 100.00
NOTE: Weights are from random effects analysis
-47.9
0
47.9
B
Study ID
HR (95% CI)
% Weight
DFS
Yang, J. (2013/miR-132) 4.10 (0.80, 9.70) 2.86
Zhang, C (2014/miR-133b) 5.69 (1.33, 11.26) 2.45
Zhang, C (2014/miR-206) 5.88 (1.56, 12.08) 2.24
Zhang, C (2014/miR-133b/206) 9.69 (2.80, 21.82) 0.83
Zhang, C (2014/miR-196a) 6.95 (1.63, 14.61) 1.61
Zhang, C (2014/miR-196b) 6.98 (1.65, 14.82) 1.57
Zhang, C (2014/miR-196a/196b) 10.09 (2.82, 21.99) 0.81
Ma, W. (2014/miR-148a) 2.53 (1.28, 5.01) 6.65
Song, QC. (2014/miR-26a) 3.97 (1.19, 9.87) 2.96
Cai H (2015/miR-195) 3.62 (1.83, 9.09) 3.72
Tang, J. (2015/miR-27a) 1.39 (0.46, 4.22) 6.63
Wang, T. (2015/miR-191) 2.63 (1.28, 6.06) 5.64
Liu, J. D. (2016/miR-300) 5.11 (2.14, 9.48) 3.67
Zhang, Q (2016/miR-222) 1.23 (1.07, 1.40) 9.26
Ren, X. (2016/miR-21) 5.80 (1.70, 13.50) 1.88
Yang, Q. (2017/miR-203) 4.19 (2.91, 10.12) 3.75
Zou, P. (2017/miR-19a) 4.66 (0.88, 10.97) 2.39
Wang, Z (2017/miR-491-5p) 2.44 (1.21, 4.51) 7.09
Wang, Z (2017/miR-491-5p) 2.64 (1.27, 5.02) 6.64
Gao, F. (2018/miR-564) 4.63 (1.88, 9.38) 3.58
Yao, ZS. (2018/miR-101) 4.23 (1.45, 7.22) 4.78
Cong, C (2018/miR-124) 3.92 (1.57, 6.39) 5.60
Li, Q. (2018/miR-542-3p) 6.89 (2.83, 9.67) 3.99
Heishima, K (2019/miR-214) 3.51 (1.68, 8.55) 3.97
Shi L (2020/miR-194) 4.39 (1.90, 6.91) 5.43
Subtotal (I-squared = 65.1%, p = 0.000) 3.70 (2.80, 4.61) 100.00
PFS
Ji, F. (2013/miR-133a) 2.60 (0.80, 7.20) 37.93
Cai, H. (2014/miR-340) 4.50 (1.00, 9.20) 23.10
Wang, Z. (2014/miR-214) 3.50 (1.00, 8.20) 29.97
Zhu, H. (2017/miR-17-5p) 5.53 (2.02, 15.16) 9.00
Subtotal (I-squared = 0.0%, p = 0.827) 3.57 (1.60, 5.54) 100.00
RFS
Li, X. (2014/miR-17-92) 2.50 (1.77, 6.91) 72.04
Yang, Z. (2015/miR-221) 6.82 (1.33, 13.69) 27.96
Subtotal (I-squared = 37.5%, p = 0.206) 3.71 (-0.09, 7.51) 100.00
NOTE: Weights are from random effects analysis
-22
0
22
C
Study ID
HR (95% CI)
% Weight
U
Ji, F. (2013/miR-133a) 1.87 (1.12, 3.31) 3.12
Cai, H. (2013/miR-210) 3.30 (1.00, 8.10) 1.25
Fei, D. (2014/miR-9) 3.18 (1.35, 4.42) 2.71
Lin, J. (2014/miR-17-92) 2.93 (1.25, 6.88) 1.66
Wang, Z. (2014/miR-214) 5.60 (1.20, 12.90) 0.59
Li, Y. (2016/miR-145) 2.07 (1.17, 3.66) 2.98
Subtotal (I-squared = 0.0%, p = 0.596) 2.36 (1.67, 3.04) 12.31
M
Xu, S. (2014/miR-9) 4.77 (2.86, 5.91) 2.72
Zhang, C (2014/miR-133b) 5.36 (1.26, 11.03) 0.79
Zhang, C (2014/miR-206) 5.42 (1.31, 11.28) 0.76
Zhang, C (2014/miR-133b/206) 9.28 (2.69, 20.79) 0.27
Cai, H. (2014/miR-340) 6.20 (1.40, 13.90) 0.52
Zhang, C (2014/miR-196a) 6.28 (1.62, 13.39) 0.58
Zhang, C (2014/miR-196b) 6.33 (1.61, 13.48) 0.57
Zhang, C (2014/miR-196a/196b) 9.89 (2.66, 20.98) 0.26
Song, QC. (2014/miR-26a) 5.72 (1.01, 10.94) 0.77
Cai H (2015/miR-195) 5.16 (1.92, 11.88) 0.76
Liu, W. (2015/miR-126) 3.10 (1.11, 9.02) 1.08
Wang, NG. (2015/miR-152) 0.13 (0.02, 0.70) 3.63
Yang, Z. (2015/miR-221) 7.66 (1.83, 15.92) 0.43
Wang, T. (2015/miR-191) 3.56 (1.62, 7.88) 1.47
Cao, L. (2016/miR-326) 3.90 (1.13, 12.53) 0.61
Dong, J. (2016/miR-223) 4.59 (1.84, 11.45) 0.81
Niu, J. (2016/miR-95-3p) 4.22 (2.31, 8.07) 1.62
Wang, T. (2017/miR-34a) 3.18 (2.87, 9.56) 1.35
Nakka, M. (2017/miR-221) 0.73 (0.49, 1.11) 3.64
Wang, Z (2017/miR-491-5p) 2.55 (1.28, 4.64) 2.57
Wang, Z (2017/miR-491-5p) 2.72 (1.30, 4.92) 2.45
Tang, B. (2017/miR-490-3p) 2.13 (1.33, 7.76) 1.42
Gao, F. (2018/miR-564) 4.25 (1.66, 8.63) 1.28
Cong, C (2018/miR-124) 3.54 (1.38, 5.64) 2.17
Liu, W. (2018/miR-375) 3.27 (1.36, 7.85) 1.40
Xu, K (2019/miR-106b) 2.77 (1.37, 5.60) 2.18
Li, X. (2020/miR-629) 2.89 (1.13, 7.42) 1.46
Yang, W. (2020/miR-21) 3.46 (2.16, 11.52) 0.84
Yang, W. (2020/miR-214) 3.14 (2.01, 10.26) 1.02
Shi L (2020/miR-194) 3.65 (1.58, 5.89) 2.15
Subtotal (I-squared = 76.9%, p = 0.000) 3.29 (2.52, 4.07) 41.60
U/M
Yuan, J. (2012/miR-21) 2.33 (1.11, 4.89) 2.38
Yang, J. (2013/miR-132) 4.90 (1.00, 10.20) 0.86
Li, X. (2014/miR-17-92) 2.94 (1.50, 5.75) 2.18
Ma, W. (2014/miR-148a) 2.70 (1.41, 5.17) 2.39
Sun, B. (2015/miR-217) 2.83 (1.13, 7.06) 1.57
Tang, J. (2015/miR-27a) 2.17 (1.30, 3.62) 3.06
Liu, J. D. (2016/miR-300) 5.96 (2.35, 10.70) 1.00
Luo, Z. (2016/miR-125b) 0.49 (0.23, 1.00) 3.62
Pang, P. (2016/miR-497) 3.79 (1.99, 8.57) 1.38
Zhang, Q (2016/miR-222) 1.19 (1.05, 1.36) 3.68
Ren, X. (2016/miR-21) 6.30 (1.80, 12.40) 0.69
Yang, Q. (2017/miR-203) 2.73 (1.69, 8.91) 1.22
Zhu, H. (2017/miR-17-5p) 10.47 (2.29, 47.87) 0.05
Zou, P. (2017/miR-19a) 5.06 (1.12, 11.62) 0.70
Hu WX (2017/miR-19a) 2.62 (2.12, 6.15) 2.27
Wang, SN. (2017/miR-491) 2.96 (1.09, 8.07) 1.28
Yao, ZS. (2018/miR-101) 3.82 (1.34, 6.52) 1.81
Zhong, L. (2018/miR-1270) 4.34 (4.08, 4.79) 3.63
Li, Q. (2018/miR-542-3p) 5.98 (3.26, 18.44) 0.37
Zhou, L. (2018/miR-139-5p) 3.24 (1.81, 4.76) 2.76
Heishima, K (2019/miR-214) 2.95 (1.35, 7.77) 1.42
Liang, W (2019/miR-765) 0.39 (0.20, 0.77) 3.65
Diao, Z. B (2020/miR-22) 3.31 (1.41, 7.77) 1.44
Li, P (2020/miR-1826) 2.45 (1.25, 4.37) 2.68
Subtotal (I-squared = 93.9%, p = 0.000) 2.73 (1.95, 3.50) 46.10
Overall (I-squared = 89.0%, p = 0.000) 2.92 (2.43, 3.41) 100.00
NOTE: Weights are from random effects analysis
-47.9
0
47.9
D
Study ID
HR (95% CI)
% Weight
Reported
Yuan, J. (2012/miR-21) 2.33 (1.11, 4.89) 2.38
Li, X. (2014/miR-17-92) 2.94 (1.50, 5.75) 2.18
Xu, S. (2014/miR-9) 4.77 (2.86, 5.91) 2.72
Liu, W. (2015/miR-126) 3.10 (1.11, 9.02) 1.08
Sun, B. (2015/miR-217) 2.83 (1.13, 7.06) 1.57
Wang, NG. (2015/miR-152) 0.13 (0.02, 0.70) 3.63
Yang, Z. (2015/miR-221) 7.66 (1.83, 15.92) 0.43
Cao, L. (2016/miR-326) 3.90 (1.13, 12.53) 0.61
Dong, J. (2016/miR-223) 4.59 (1.84, 11.45) 0.81
Liu, J. D. (2016/miR-300) 5.96 (2.35, 10.70) 1.00
Luo, Z. (2016/miR-125b) 0.49 (0.23, 1.00) 3.62
Niu, J. (2016/miR-95-3p) 4.22 (2.31, 8.07) 1.62
Pang, P. (2016/miR-497) 3.79 (1.99, 8.57) 1.38
Wang, T. (2017/miR-34a) 3.18 (2.87, 9.56) 1.35
Yang, Q. (2017/miR-203) 2.73 (1.69, 8.91) 1.22
Zhu, H. (2017/miR-17-5p) 10.47 (2.29, 47.87) 0.05
Zou, P. (2017/miR-19a) 5.06 (1.12, 11.62) 0.70
Hu WX (2017/miR-19a) 2.62 (2.12, 6.15) 2.27
Wang, SN. (2017/miR-491) 2.96 (1.09, 8.07) 1.28
Wang, Z (2017/miR-491-5p) 2.55 (1.28, 4.64) 2.57
Wang, Z (2017/miR-491-5p) 2.72 (1.30, 4.92) 2.45
Cong, C (2018/miR-124) 3.54 (1.38, 5.64) 2.17
Liu, W. (2018/miR-375) 3.27 (1.36, 7.85) 1.40
Zhong, L. (2018/miR-1270) 4.34 (4.08, 4.79) 3.63
Li, Q. (2018/miR-542-3p) 5.98 (3.26, 18.44) 0.37
Zhou, L. (2018/miR-139-5p) 3.24 (1.81, 4.76) 2.76
Heishima, K (2019/miR-214) 2.95 (1.35, 7.77) 1.42
Liang, W (2019/miR-765) 0.39 (0.20, 0.77) 3.65
Xu, K (2019/miR-106b) 2.77 (1.37, 5.60) 2.18
Diao, Z. B (2020/miR-22) 3.31 (1.41, 7.77) 1.44
Li, P (2020/miR-1826) 2.45 (1.25, 4.37) 2.68
Li, X. (2020/miR-629) 2.89 (1.13, 7.42) 1.46
Yang, W. (2020/miR-21) 3.46 (2.16, 11.52) 0.84
Yang, W. (2020/miR-214) 3.14 (2.01, 10.26) 1.02
Shi L (2020/miR-194) 3.65 (1.58, 5.89) 2.15
Subtotal (I-squared = 92.6%, p = 0.000) 3.00 (2.21, 3.79) 62.09
K-M curve
Ji, F. (2013/miR-133a) 1.87 (1.12, 3.31) 3.12
Cai, H. (2013/miR-210) 3.30 (1.00, 8.10) 1.25
Yang, J. (2013/miR-132) 4.90 (1.00, 10.20) 0.86
Zhang, C (2014/miR-133b) 5.36 (1.26, 11.03) 0.79
Zhang, C (2014/miR-206) 5.42 (1.31, 11.28) 0.76
Zhang, C (2014/miR-133b/206) 9.28 (2.69, 20.79) 0.27
Cai, H. (2014/miR-340) 6.20 (1.40, 13.90) 0.52
Fei, D. (2014/miR-9) 3.18 (1.35, 4.42) 2.71
Zhang, C (2014/miR-196a) 6.28 (1.62, 13.39) 0.58
Zhang, C (2014/miR-196b) 6.33 (1.61, 13.48) 0.57
Zhang, C (2014/miR-196a/196b) 9.89 (2.66, 20.98) 0.26
Ma, W. (2014/miR-148a) 2.70 (1.41, 5.17) 2.39
Lin, J. (2014/miR-17-92) 2.93 (1.25, 6.88) 1.66
Song, QC. (2014/miR-26a) 5.72 (1.01, 10.94) 0.77
Wang, Z. (2014/miR-214) 5.60 (1.20, 12.90) 0.59
Cai H (2015/miR-195) 5.16 (1.92, 11.88) 0.76
Tang, J. (2015/miR-27a) 2.17 (1.30, 3.62) 3.06
Wang, T. (2015/miR-191) 3.56 (1.62, 7.88) 1.47
Li, Y. (2016/miR-145) 2.07 (1.17, 3.66) 2.98
Zhang, Q. (2016/miR-222) 1.19 (1.05, 1.36) 3.68
Ren, X. (2016/miR-21) 6.30 (1.80, 12.40) 0.69
Nakka, M. (2017/miR-221) 0.73 (0.49, 1.11) 3.64
Tang, B. (2017/miR-490-3p) 2.13 (1.33, 7.76) 1.42
Gao, F. (2018/miR-564) 4.25 (1.66, 8.63) 1.28
Yao, ZS. (2018/miR-101) 3.82 (1.34, 6.52) 1.81
Subtotal (I-squared = 65.6%, p = 0.000) 2.48 (1.91, 3.04) 37.91
Overall (I-squared = 89.0%, p = 0.000) 2.92 (2.43, 3.41) 100.00
NOTE: Weights are from random effects analysis
-47.9
0
47.9

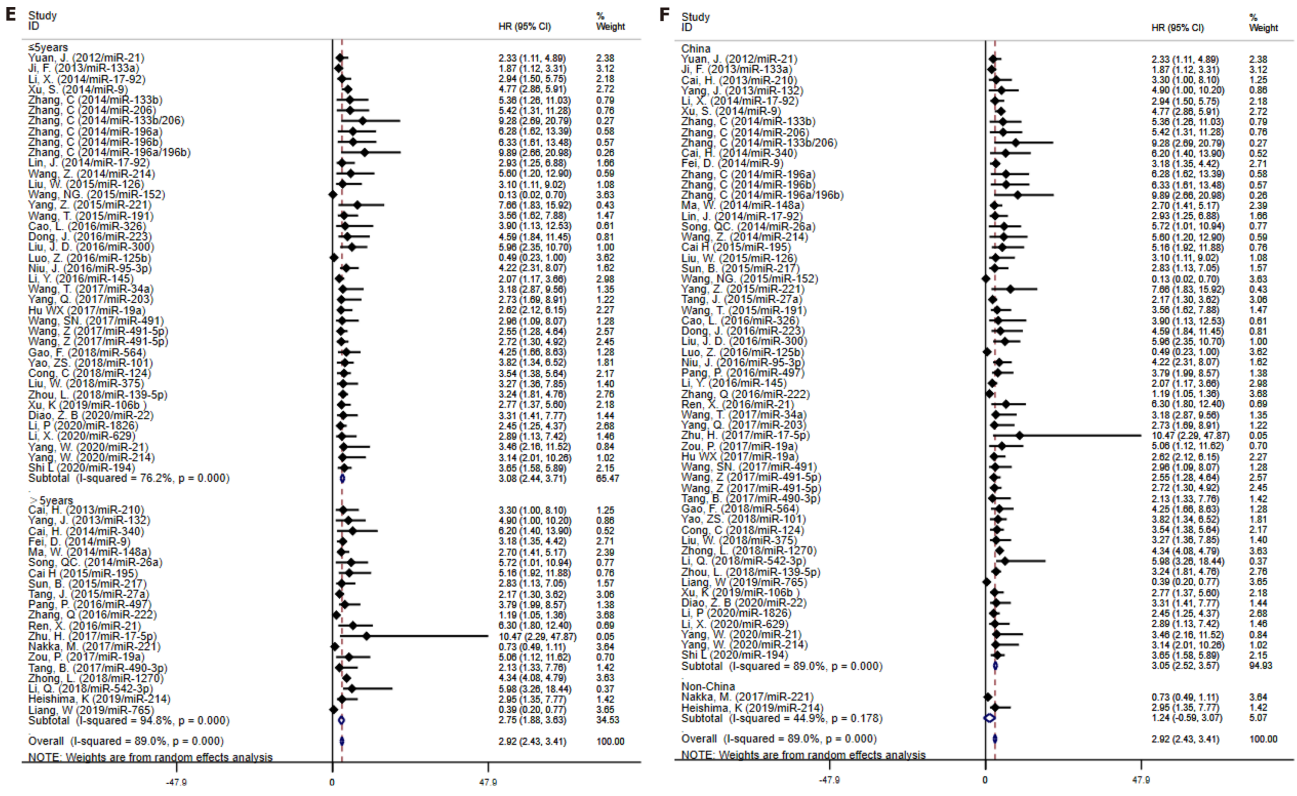

Figure 3 Forest plot of subgroup analysis. A: Subgroup analysis based on microRNA expression; B: Subgroup analysis based on specimen type; C: Subgroup analysis based on survival analysis; D: Subgroup analysis based on source of hazard ratio; E: Subgroup analysis based on follow-up; F: Subgroup analysis based on country of publication.

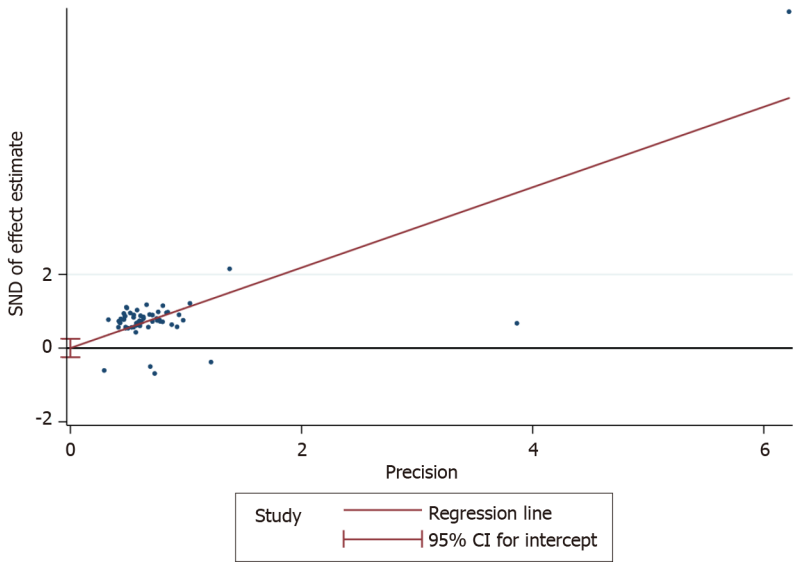

Figure 4 Egger's test of publication bias.

ARTICLE HIGHLIGHTS

Research background

Osteosarcoma is one of the most common primary malignant bone tumors in children, with rapid progress and poor prognosis. microRNA (miRNA) has been proposed as a valuable biomarker for the prognosis of osteosarcoma, but the results are not consistent.

Research motivation

We wished to gain a detailed insight into the effect of circulating miRNA on the prognosis of patients with osteosarcoma.

Research objectives

The aim of this meta-analysis was to study the prognostic value of miRNA expression in patients with osteosarcoma.

Research methods

A literature search was conducted to identify studies published through July 1, 2021. Random-effects meta-analyses were conducted for combinations of the outcomes of overall survival (OS), disease-free survival (DFS), progression-free survival (PFS), and recurrence-free survival in osteosarcoma patients. Subgroup analyses were conducted to identify sources of heterogeneity. Publication bias was assessed by Egger's bias indicator test.

Research results

A total of 60 studies were included for this meta-analysis. The pooled HR for OS, DFS, PFS were 2.92 (95%CI: 2.43-3.41, P = 0.000), 3.70 (95%CI: 2.80-4.61, P = 0.000), and 3.57 (95%CI: 1.60-5.54, P = 0.000), respectively. The high expression levels of miR-21 were associated with poor OS in osteosarcoma (HR = 2.86, 95%CI: 1.20-4.53, P = 0.001).

Research conclusions

Circulating miRNA could be used as a potential prognostic biomarker for osteosarcoma.

Research perspectives

Evidence from later studies could consolidate these conclusions. A large number of samples prospective studies are needed in the future to enhance the persuasiveness of our conclusion.

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META-ANALYSIS

Efficacy and safety of fingolimod in stroke: A systemic review and meta-analysis

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Author contributions: Zhang Q and Zhao K conceived the idea and designed the study; Zhao K and Guo Y screened the studies and extracted the data independently; Yang MF and Guo Y analysed and interpreted the data; Zhao K and Guo Y wrote the first draft of the manuscript; Zhang Q proofread the manuscript before submission; all authors reviewed the manuscript and approved the final version.

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PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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Abstract

BACKGROUND

Brain tissue injury in stroke patients involves inflammation around the infarction lesion or hematoma, which is an important reason for disease deterioration and can result in a poor prognosis. The meta-analysis of animal experiments has concluded that fingolimod could treat stroke in animal models by effectively reducing lymphocyte infiltration. However, no evidence-based efficacy and safety evaluation of fingolimod in stroke patients is currently available.

AIM

To determine whether fingolimod could promote reduction of infarction lesion or hematoma and improve neurological prognosis in stroke patients.

METHODS

Data extracted for treatment effect included count of T-lymphocytes with cluster of differentiation 8 expression ($CD8^+$ T cells, $\times 10^6$/mL), lesion volume (infarction or hematoma, mL), and modified Barthel indexes. Data extracted for safety was risk ratio (RR). Overall standard mean difference (SMD) with its 95% confidence interval (95%CI) and pooled effect with its 95%CI were calculated with a fixed-effects model. *I*-square (I^2) was used to test the heterogeneity. Funnel plot symmetry and Egger's regression were used to evaluate publication bias.

RESULTS

Four high-quality randomized controlled trials were included. There was a significant difference in $CD8^+$ T cell count ($I^2 = 0$, overall SMD = -3.59, 95%CI: -4.37-2.80, $P = 0.737$) and modified Barthel index ($I^2 = 0$, overall SMD = 2.42, 95%CI: 1.63-3.21, $P = 0.290$) between the fingolimod and control groups. However, there was no significant difference in lesion volume ($I^2 = 10.6\%$, overall SMD = -0.17, 95%CI: -0.75-0.42, $P = 0.917$), fever (pooled RR = 0.93, 95%CI: 0.97-2.32, $P = 0.864$),

Country/Territory of origin: China

Specialty type: Clinical neurology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
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Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): D, D
Grade E (Poor): 0

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suspected lung infection (pooled RR = 0.90, 95%CI: 0.33-2.43, P = 0.876), or any adverse events occurring at least once (pooled RR = 0.82, 95%CI: 0.36-1.87, P = 0.995) between the fingolimod and control groups. There was no publication bias. All of the results were stable as revealed by sensitivity analysis.

CONCLUSION
Fingolimod improves neurological function in stroke patients without promotion of lesion absorption. Taking fingolimod orally (0.5 mg/d, 3 consecutive days) is safe except for patients with rare severe adverse events.

Key Words: Acute ischemic stroke; Intracerebral hemorrhage; Fingolimod; Meta-analysis; Treatment

Core Tip: Brain tissue injury in stroke patients involves inflammation around the infarction lesion or hematoma, which is an important reason for disease deterioration and can result in a poor prognosis. Our systemic review and meta-analysis of recent randomized controlled trials found that fingolimod might improve neurological function in stroke patients by reducing lymphocyte infiltration in the brain effectively; however, we did not find the evidence that fingolimod could promote infarction lesion or hematoma absorption. In general, oral fingolimod (0.5 mg/d, 3 consecutive days) was safe in stroke patients except for some rare severe adverse events.

Citation: Zhao K, Guo Y, Yang MF, Zhang Q. Efficacy and safety of fingolimod in stroke: A systemic review and meta-analysis. *World J Meta-Anal* 2021; 9(6): 585-597
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INTRODUCTION

Stroke, which mainly consists of acute ischemic stroke (AIS) with cerebral infarction and hemorrhagic stroke with intracerebral hematoma (ICH), is a common neurological disease with a high mortality and disability rate. Especially, during the coronavirus disease 2019 pandemic, related coagulopathy might be associated with ischemic stroke, cerebral hemorrhage, and cerebral venous thrombosis, which aggravated the financial burden in the world[1,2]. For severe stroke patients whose condition has deteriorated rapidly, surgical intervention should be performed, which is considered the best way to save lives[3]. Thus, to reduce the risk of disease deterioration and promote the recovery of nervous system function, it is important to perform timely intervention at the early stage of stroke.

Fingolimod is a newly approved drug for the treatment of multiple sclerosis. It can be metabolized to the active metabolite of fingolimod phosphate *via* sphingosine kinase. Fingolimod phosphate has a high affinity for sphingosine-1-phosphate receptors 1, 3, 4, and 5[4]. Sphingosine-1-phosphate and its receptors can regulate lymphocyte migration *via* upstream cell signaling pathways. Thus, fingolimod phosphate can block the ability of lymphocytes to enter or exit the lymph nodes, which can reduce the number of lymphocytes in peripheral blood[5]. Although fingolimod can be used for the treatment of multiple sclerosis with unknown mechanism, its role as an immunomodulator might involve the reduction of the migration of lymphocytes to central nervous system[6].

Brain tissue injury in stroke patients involves inflammation around the infarction lesion or hematoma, which is an important reason for disease deterioration and can result in a poor prognosis[7]. The meta-analysis of animal experiments has concluded that fingolimod could treat stroke in animal models by effectively reducing lymphocyte infiltration[8]. However, evidence-based efficacy and safety evaluation of fingolimod in stroke patients is currently not available. We hypothesized that fingolimod could effectively and safely promote reduction of infarction lesion or hematoma and improve neurological prognosis in AIS or ICH patients by reducing

lymphocyte infiltration. In this study, we performed a systemic review and meta-analysis of recent randomized controlled trials (RCTs) to confirm this hypothesis.

MATERIALS AND METHODS

Protocol used

This systemic review and meta-analysis were performed referring to the protocol published in the database of International Platform of Registered Systematic Review and Meta-analysis Protocols (INPLASY, https://inplasy.com/).

Literature search

A literature search was performed in the three public electronic databases: PubMed, Embase, and Cochrane. The strategy of literature search in PubMed was as follows: ("Fingolimod Hydrochloride"[Mesh]) OR (((((2-Amino-2-(2-(4-octylphenyl)ethyl)-1,3-propanediol hydrochloride[Title/Abstract]) OR (FTY?720[Title/Abstract])) OR (Gilenya[Title/Abstract])) OR (Gilenia[Title/Abstract])) OR (Fingolimod [Title/Abstract])) AND (("Stroke"[Mesh]) OR ((((((((((((((((((Stroke*[Title/Abstract]) OR (Cerebrovascular Accident*[Title/Abstract])) OR (CVA* [Title/Abstract])) OR (Cerebrovascular Apoplexy[Title/Abstract])) OR (Apoplexy, Cerebrovascular [Title/Abstract])) OR (Vascular Accident, Brain[Title/Abstract])) OR (Brain Vascular Accident*[Title/Abstract])) OR (Vascular Accidents, Brain[Title/Abstract])) OR (Cerebrovascular Stroke*[Title/Abstract])) OR (Stroke*, Cerebrovascular[Title/Abstract])) OR (Apoplexy[Title/Abstract])) OR (Cerebral Stroke*[Title/Abstract])) OR (Stroke*, Cerebral[Title/Abstract])) OR (Stroke*, Acute[Title/Abstract])) OR (Acute Stroke*[Title/Abstract])) OR (Cerebrovascular Accident*, Acute[Title/Abstract])) OR (Acute Cerebrovascular Accident*[Title/Abstract]))). The strategy of literature search in Embase was as follows: (exp Stroke or Stroke$:ab,ti or 'Cerebrovascular Accident$':ab,ti or CVA$:ab,ti or 'Cerebrovascular Apoplexy':ab,ti or 'Apoplexy, Cerebrovascular':ab,ti or 'Vascular Accident, Brain':ab,ti or 'Brain Vascular Accident$':ab,ti or 'Vascular Accidents, Brain':ab,ti or 'Cerebrovascular Stroke$':ab,ti or 'Stroke$, Cerebrovascular':ab,ti or Apoplexy:ab,ti or 'Cerebral Stroke$':ab,ti or 'Stroke$, Cerebral':ab,ti or 'Stroke$, Acute':ab,ti or 'Acute Stroke$':ab,ti or 'Cerebrovascular Accident$, Acute':ab,ti or 'Acute Cerebrovascular Accident$':ab,ti) or (exp 'Cerebral Hemorrhage' or 'Hemorrhage$, Cerebrum':ab,ti or 'Cerebrum Hemorrhage$':ab,ti or 'Cerebral Parenchymal Hemorrhage$':ab,ti or 'Hemorrhage$, Cerebral Parenchymal':ab,ti or 'Parenchymal Hemorrhage$, Cerebral':ab,ti or 'Intracerebral Hemorrhage$':ab,ti or 'Hemorrhage$, Intracerebral':ab,ti or 'Hemorrhage$, Cerebral':ab,ti or 'Cerebral Hemorrhage$':ab,ti or 'Brain Hemorrhage$, Cerebral':ab,ti or 'Cerebral Brain Hemorrhage$':ab,ti or 'Hemorrhage$, Cerebral Brain':ab,ti).

Study selection

The articles involving the efficacy and safety of fingolimod in the treatment of stroke patients were screened. The inclusion criteria were: (1) Language and regions were not restricted; (2) Date of publication was up to December 31, 2020; (3) RCTs; (4) Patients suffered from AIS or ICH and the onset time was less than 6 h; (5) Fingolimod was used as the clinical intervention and the basic treatment methods were standard treatment according to the current American Heart Association guidelines; and (6) Outcomes involved assessment of immune cells, lesion volume, neurological function, and adverse events. The exclusion criteria were: (1) Duplicated publications; (2) Reviews, comments, letters, case reports, protocols of clinical trials, and conference papers; (3) Animal experiments; and (4) Articles with data irrelevant to this meta-analysis.

Data extraction

In consideration of the pharmacological action of fingolimod, absolute immunological cell counts ($\times 10^6$/mL) in blood might be the most intuitive index. Especially, T-lymphocytes with cluster of differentiation 8 expression ($CD8^+$ T cells) play a key role in damaging the nervous system due to its cell killing effect, which could result in inflammatory edema[9]. The edema around the infarction or hematoma could result in serious neurologic impairment in stroke patients[10,11]. Thus, a rapid reduction of absolute infarction or hematoma volume might be the key of treatment in stroke patients. Lesion volume (mL) was defined as absolute infarction volume in AIS patients or absolute hematoma in ICH patients. The National Institute of Health stroke

scale (NIHSS) is the specialized assessment to measure recovery of neurological function[12]. The Modified Barthel index (mBI) is one of the earliest and the most commonly used methods for assessing ADL (activity of daily living) in rehabilitation medicine, which has high validity and reliability in assessment of stroke[13]. Although risk ratio (RR) is often used as an effect size in cohort studies, it might be significant to assess the probability of adverse events like the number of patients to be harmed in clinical trials[14]. In this study, the data extracted included $CD8^+$ T cell count, lesion volume (infarction or hematoma), NIHSS, mBI, and RR of safety. The data at time points near baseline, which might reflect condition of stroke at early treatment, were extracted. In addition, some confounders, which might result in errors, were adjusted, including characteristics of participation, period of treatment, and other factors. If the original data could not be acquired, other data were also extracted such as the difference between data at baseline and those at other time point.

Risk of bias

The quality assessment of included articles was performed *via* the Cochrane Collaboration's Tool of Assessing Risk of Bias using Review Manager 5.3 before data extraction.

Statistical analysis

We made the definition that the counts of studies selected or those with data extracted were positive events (+). The others were negative events (-). Kappa was calculated to test the agreement between Kai Zhao and Yu Guo. Kappa > 0.6 meant a high agreement. Zhang Q would make the final decision. Continuous variables with a normal distribution are expressed as the mean ± SD. Statistical difference of data before treatment in the intervention and control groups was tested by one-way analysis of variance (ANOVA) using SigmaStat version 4.0. If there was no statistical difference, data after treatment were directly used for meta-analysis. Relative numbers and their 95% confidence intervals (95%CI) are used to describe count data. Meta-analysis was performed using corresponding modules in Software for Statistics and Data Science (Stata, version 15.1; College Station, Texas 77845, United States). The overall standard mean difference (SMD) with its 95%CI and pooled effect with its 95%CI were calculated with a fixed-effects model. *I*-square (I^2) was used to test the heterogeneity. Sensitivity analysis was performed to evaluate the stability of overall results by recalculating the overall SMD or pooled effect of the remaining studies after omitting the study with the highest quality or the fixed-effects model was switched to a random-effects model. Funnel plot symmetry and Egger's regression were used to evaluate publication bias. The methods to reduce heterogeneity and enhance sensitivity were the deletion of studies with publication bias or studies with the lowest quality and subgroup analysis. All *P* values were two-sided with a significant level at 0.05.

RESULTS

Study selection and characteristics

Totally, 185 articles were retrieved from three databases according to the strategy. After screening according to the inclusion and exclusion criteria, four articles[15-18] reporting RCTs were enrolled ultimately (Figure 1). The Kappa of agreement of studies included by Kai Zhao and Yu Guo was 0.601 ($P < 0.001$; Table 1). One study emphasized the changes of inflammatory factors in patients with ICH after taking fingolimod orally[19]. One study emphasized the role of rehabilitation nursing in AIS patients after taking fingolimod orally[20]. The assessment of article quality *via* Cochrane Collaboration's Tool of Assessing Risk of Bias is shown in Figure 2. Three studies (Ying Fu *et al*[15], Ying Fu *et al*[16], and Zilong Zhu *et al*[17]) were evaluated as having insignificant risk bias in allocation concealment except for low risk in one study (De-Cai Tian *et al*[18]). There was no bias in performance, detection, attrition, or reporting. Other risk biases were insignificant in all studies. The articles finally included were published in 2014 to 2018 (Table 2). There were 67 stroke patients, including 56 AIS patients and 11 ICH patients, whose age ranged from 49 to 74 years. The period of oral fingolimod were 3 consecutive days after hospitalization. The oral dose was 0.5 mg/d. Conclusion with the same tendency was that fingolimod could effectively and safely treat AIS or ICH.

Table 1 Agreement of studies included by Zhao K and Guo Y

Zhao K	Guo Y		Total
	+	-	
+	4	3	7
-	2	176	179
Total	6	179	185

Kappa = 0.601, $P < 0.001$.

Table 2 Characteristics of studies used to perform the meta-analysis

Ref.	Publication	Fingolimod group	Types of stroke	Age (mean ± SD)	Sex (male %)	Dose of fingolimod	Conclusion
Fu *et al*[15]	2014	11	AIS	62.3 ± 8.0	73	0.5 mg/d orally, 3 consecutive days after hospitalization	It could safely limit secondary tissue injury, decrease microvascular permeability, attenuate neurological deficits, and promote recovery.
Fu *et al*[16]	2014	11	ICH	60.7 ± 12.3	36	0.5 mg/d orally, 3 consecutive days after hospitalization	It could safely reduce PHE, attenuate neurologic deficits, and promote recovery.
Zhu *et al* [17]	2015	22	AIS	60.0 ± 2.5	59	0.5 mg/d orally, 3 consecutive days after hospitalization	In this pilot study, combination therapy of fingolimod and alteplase was well tolerated, which attenuated reperfusion injury and improved clinical outcomes in AIS patients.
Tian *et al* [18]	2018	23	AIS	67 ± 6.7	39	0.5 mg/d orally, 3 consecutive days after hospitalization	Fingolimod may enhance the efficacy of alteplase administration in the 4.5- to 6-h time window in patients with a proximal cerebral arterial occlusion and salvageable penumbral tissue by promoting both anterograde reperfusion and retrograde collateral flow.

SD: Standard deviation.

Meta-analysis

Due to that some data were expressed *via* only one figure, some original data could not be acquired directly, which may result in missing of some data. The Kappa of agreement of data extraction between Zhao K and Guo Y was 0.634 ($P < 0.001$; Table 3). To acquire a more accurate result of meta-analysis, we tested the statistical difference of $CD8^+$ T cell counts at baseline and 1 day after taking fingolimod orally, lesion volume at baseline and 7 d after taking fingolimod orally, and mBI scores at baseline and 90 d after taking fingolimod orally between studies *via* ANOVA. Unfortunately, there were significant statistical differences ($P < 0.05$) in all of continuous variables. Finally, we calculated difference between data at different time points manually to perform meta-analysis (Table 4). RRs and their 95%CIs of different adverse events, which consisted of fever, suspected lung infection, all adverse events occurring at least once, and other serious events involving hemorrhage of the digestive tract, cerebral hernia, bradycardia, atrial flutter, and thrombocytopenia, were calculated directly by referring to the count of adverse events occurring in patients of the fingolimod groups and control groups. Since no patients developed bradycardia, atrial flutter, or thrombocytopenia in the control groups, RRs of these adverse events were not calculated (Table 5).

Analysis with a fixed-effects model showed that there was no heterogeneity ($I^2 = 0$, Figure 3) in the two studies used for comparing overall SMD difference in $CD8^+$ T cell counts and lesion volume except for two articles used for comparing overall SMD difference in mBI scores ($I^2 = 10.6\%$, Figure 3). There was a significant difference in $CD8^+$ T cell counts (overall SMD = -3.59, 95%CI: -4.37--2.80, $P = 0.737$, Figure 3) and mBI (overall SMD = 2.42, 95%CI: 1.63-3.21, $P = 0.290$, Figure 3) between the fingolimod and control groups. However, there was no significant difference in lesion volume between the fingolimod and control groups (overall SMD = -0.17, 95%CI: -0.75-0.42, $P = 0.917$, Figure 3). Pooled RRs and their 95%CIs were calculated with a fixed-effects model. The RRs of fever ($I^2 = 0$, pooled RR = 0.93, 95%CI: 0.97-2.32, $P = 0.864$, Figure 4),

Table 3 Agreement of data extraction between Zhao K and Guo Y

Zhao K	Guo Y		Total
	+	-	
+	16	5	21
-	3	20	23
Total	19	25	44

Kappa = 0.634, $P < 0.001$.

Table 4 Changes of T-lymphocytes with cluster of differentiation 8 expression/infarction or hematoma volume/modified Barthel index scores in the fingolimod and control groups

Ref.	Size of fingolimod group	Fingolimod group	Size of control group	Control group
$CD8^+$ T cell count (mean ± SD, × 10^6 /mL)[1]				
Fu *et al*[15]	11	-0.16 ± 0.04	11	0.08 ± 0.08
Tian *et al*[18]	23	-0.09 ± 0.02	23	-0.02 ± 0.02
Lesion Volume (mean ± SD, mL)[2]				
Fu *et al*[15]	11	9.91 ± 79.62	11	25.45 ± 75.45
Fu *et al*[16]	11	-1.97 ± 6.25	12	-1.35 ± 1.77
mBI score (mean ± SD)[3]				
Fu *et al*[15]	11	72.7 ± 7.5	11	45.3 ± 10.9
Fu *et al*[16]	11	75.2 ± 4.7	12	65.4 ± 4.8

[1]The outcome equaled $CD8^+$ T cell count at 1 d after taking fingolimod orally minus $CD8^+$ T cell count at baseline.
[2]Lesion volume consisted of infarct volume of AIS and hematoma volume of ICH. The outcome is lesion volume at 7 d after taking fingolimod orally minus lesion volume at baseline.
[3]The outcome is mBI score at 90 days after fingolimod taken orally minus mBI score at baseline.
$CD8^+$ T cell: T-lymphocytes with cluster of differentiation 8 expression. Lesion volume: Infarction or hematoma volume; mBI score: Modified Barthel index score.

suspected lung infection ($I^2 = 0$, pooled RR = 0.90, 95%CI: 0.33-2.43, $P = 0.876$, Figure 4), and all adverse events occurring at least once ($I^2 = 0$, pooled RR = 0.82, 95%CI: 0.36-1.87, $P = 0.995$, Figure 4) showed no significant difference between the fingolimod and control groups. Meta-analysis was not performed in RRs of other serious events because there was only one study reporting hemorrhage of the digestive tract or cerebral hernia and RRs of bradycardia, atrial flutter, and thrombocytopenia could not be calculated.

Publication bias and influence analysis

There was a symmetrical distribution in funnel plots of continuous variables (Figure 4A) and RRs (Figure 4B). Because only two studies were used to perform meta-analysis of treatment effect, we recalculated overall SMD of $CD8^+$ T cell count ($I^2 = 0$, overall SMD = -3.59, 95%CI: -4.37--2.80, $P = 0.737$, Table 6), lesion volume ($I^2 = 0$, overall SMD = -0.17, 95%CI: -0.75-0.42, $P = 0.917$, Table 6), and mBI scores ($I^2 = 10.6\%$, overall SMD = 2.43, 95%CI: 1.59-3.26, $P = 0.290$, Table 6) after the fixed-effects model was switched to a random-effects model. Pooled RRs and their 95%CIs of fever ($I^2 = 0$, pooled RR = 0.93, 95%CI: 0.37-2.32, $P = 0.723$, Table 6), suspected lung infection ($I^2 = 0$, pooled RR = 0.90, 95%CI: 0.33-2.43, $P = 0.723$, Table 6), and all adverse events occurring at least once ($I^2 = 0$, pooled RR = 0.82, 95%CI: 0.36-1.87, $P = 0.969$, Table 6) were analyzed using the same method as above. In addition, for sensitivity analysis, the pooled effect of the remaining studies was recalculated after omitting the study with the highest quality because more than two studies were used to perform meta-analysis of treatment safety. We considered that the study might be assessed to have higher quality for its larger number of included patients in studies with the same

Table 5 Complications and adverse events in the fingolimod and control groups

Ref.	Size of fingolimod group	Events of fingolimod group	Size of control group	Events of control group	Risk ratio (95%CI)
Fever					
Fu *et al*[15]	11	3	11	3	1 (0.16, 6.08)
Fu *et al*[16]	11	3	12	5	0.64 (0.12, 3.34)
Zhu *et al*[17]	22	5	25	5	1.15 (0.29, 4.51)
Suspected lung infection					
Fu *et al*[15]	11	3	11	3	1 (0.16, 6.08)
Fu *et al*[16]	11	3	12	5	0.64 (0.12, 3.34)
Zhu *et al*[17]	22	3	25	3	1.17 (0.21, 6.39)
Adverse events occurring at least once					
Fu *et al*[15]	11	3	11	3	1 (0.16, 6.08)
Fu *et al*[16]	11	4	12	6	0.72 (0.12, 4.38)
Zhu *et al*[17]	22	6	25	8	0.84 (0.25, 2.81)
Tian *et al*[18]	23	3	23	4	0.76 (0.15, 3.81)
Other serious events					
Hemorrhage of digestive tract					
Zhu *et al*[17]	22	1	25	3	0.42 (0.04, 4.30)
Cerebral hernia					
Tian *et al*[18]	23	2	23	6	0.35 (0.06, 1.90)
Bradycardia					
Fu *et al*[16]	11	1	12	0	N/A
Atrial flutter					
Tian *et al*[18]	23	2	23	0	N/A
Thrombocytopenia					
Tian *et al*[18]	23	2	23	0	N/A

assessment in Cochrane Collaboration's Tool of Assessing Risk of Bias. Finally, after omitting the study with the highest quality, we recalculated pool RRs and their 95%CIs of fever (Zhu *et al*[17] omitted, $I^2 = 0$, pooled RR = 0.78, 95%CI: 0.23-2.68, $P = 0.723$, Table 6), suspected lung infection (Zhu *et al*[17] omitted, $I^2 = 0$, pooled RR = 0.78, 95%CI: 0.23-2.68, $P = 0.723$, Table 6), and all adverse events occurring at least once ($I^2 = 0$, pooled RR = 0.84, 95%CI: 0.33-2.18, $P = 0.969$, Table 6).

DISCUSSION

There were many clinical application of fingolimod due to its original medicine indication of reducing the migration of lymphocytes to the central nervous system, such as Alzheimer's disease and Susac syndrome[21,22]. Although the mechanisms of action of fingolimod in the treatment of AIS or ICH might be different, absorption of infarction lesion in AIS or hematoma in ICH both involved cytophagy *via* microglia in brain tissue or immunocyte in peripheral blood across injured brain-blood barrier[23,24]. Edema around lesion has probable results of microcirculation obstruction, injured brain-blood barrier, and inflammatory response caused by lymphocyte infiltration in stroke patients, which may strongly affect recovery of neurological function and prognosis due to its space occupying and toxic effect. Thus, fingolimod might reduce secondary damage after stroke *via* its pharmacological action. In our meta-analysis of RCTs, although we did not find that fingolimod could accelerate reduction of lesion

Table 6 Overall standard mean difference or pooled risk ratio *via* random-effects model and pooled risk ratio of remaining studies after the study with the highest quality is omitted

Method	Overall SMD or RR (95%CI)	Test of heterogeneity	
		I^2 (%)	*P*
Random-effects model			
$CD8^+$ T cells	-3.59 (-4.37, -2.80)	0.0	0.737
Lesion volume	-0.17 (-0.75, 0.42)	0.0	0.917
mBI score	2.43 (1.59, 3.26)	10.6	0.290
RR of fever	0.93 (0.37, 2.32)	0.0	0.864
RR of suspected lung infection	0.90 (0.33, 2.43)	0.0	0.876
RR of adverse events occurring at least once	0.82 (0.36, 1.87)	0.0	0.995
Article deleted			
Zhu *et al*[17] (RR of fever)	0.78 (0.23, 2.68)	0.0	0.723
Zhu *et al*[17] (RR of suspected lung infection)	0.78 (0.23, 2.68)	0.0	0.723
Tian *et al*[18] (RR of adverse events occurring at least once)	0.84 (0.33, 2.18)	0.0	0.969

SMD: Standard mean difference; RR: Risk ratio; $CD8^+$ T cell: T-lymphocytes with cluster of differentiation 8 expression; Lesion volume: Infarction or hematoma volume; mBI score: Modified Barthel index score.

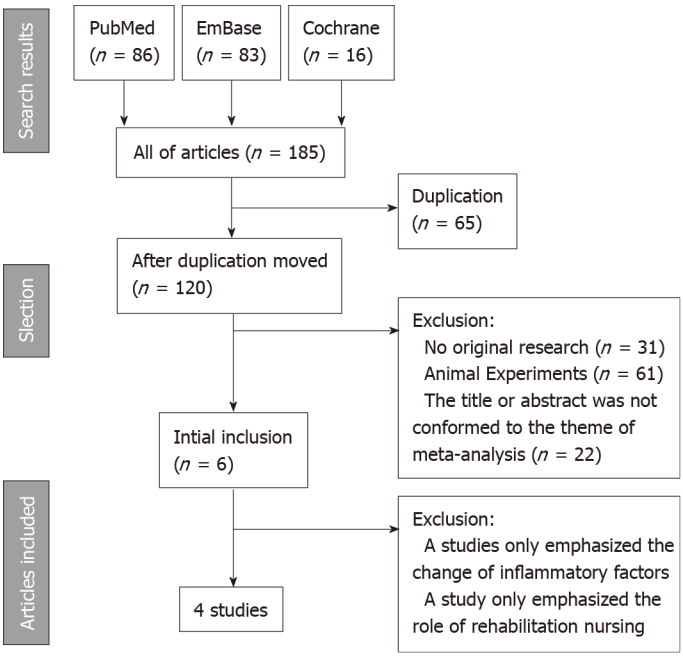

Figure 1 Process of study screening.

volume (I^2 = 0, overall SMD = -0.17, 95%CI: -0.75-0.42, *P* = 0.917, Figure 3) *via* reducing lymphocyte counts in peripheral blood (I^2 = 0, overall SMD = -3.59, 95%CI: -4.37--2.80, *P* = 0.737, Figure 3), there was an effective improvement in ADL in stroke patients (I^2 = 10.6%, overall SMD = 2.42, 95%CI: 1.63-3.21, *P* = 0.290, Figure 3), which might be the evidence that fingolimod might cause reduction of secondary damage *via* reducing lymphocyte infiltration in the brain effectively.

With regard to safety of oral fingolimod in stroke patients, infectious diseases might be the main adverse events. Especially, patients with hemiplegic paralysis may be aggravated to severe hypostatic pneumonia. In our meta-analysis, fever (I^2 = 0, pooled RR = 0.93, 95%CI: 0.97-2.32, *P* = 0.864, Figure 4) and suspected lung infection (I^2 = 0, pooled RR = 0.90, 95%CI: 0.33-2.43, *P* = 0.876, Figure 4) were both considered as infectious diseases. We did not find that fingolimod might increase the risk of

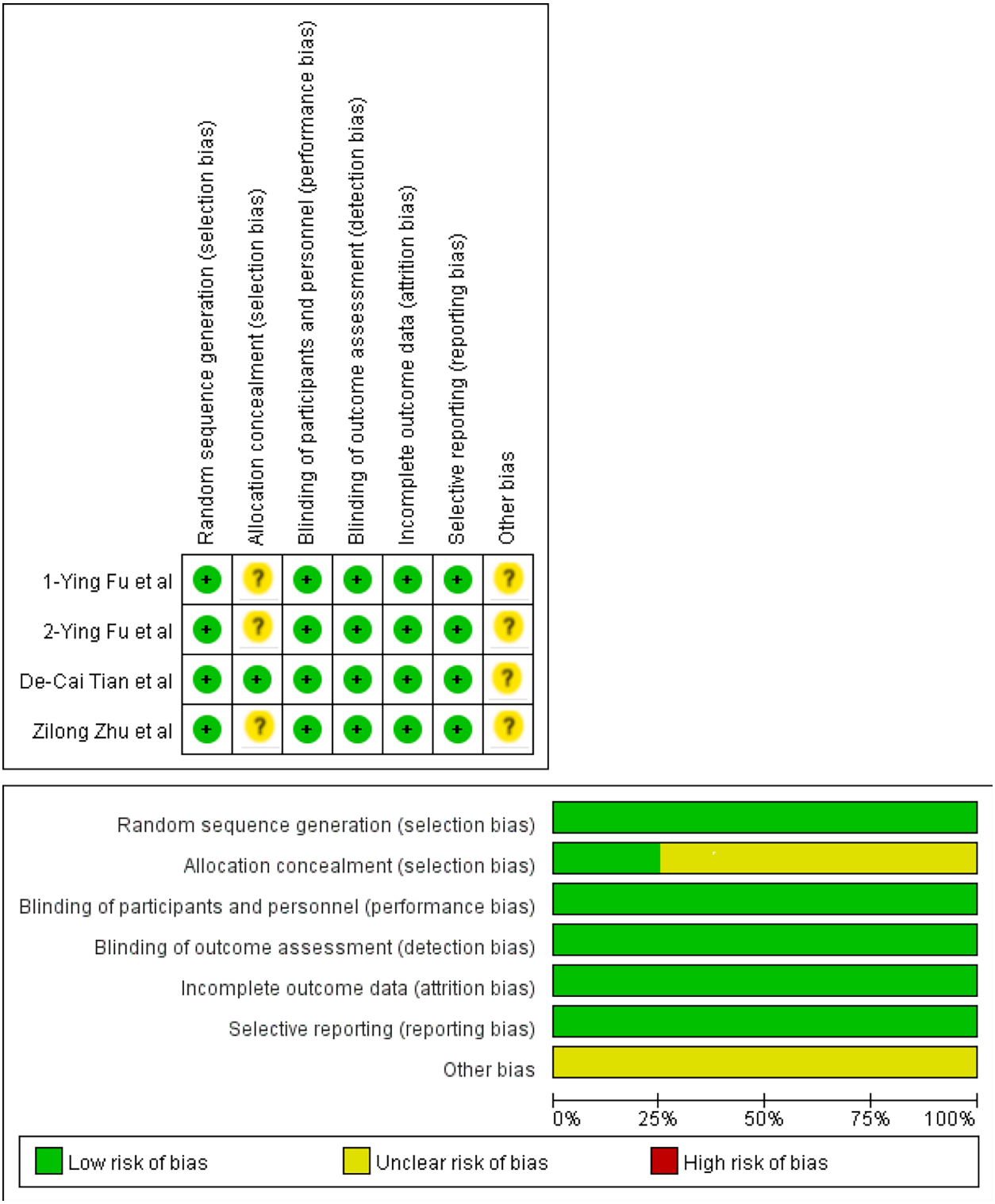

Figure 2 Quality assessment for studies included. 1-Ying Fu *et al*"[15] and 2-Ying Fu *et al*[16] represent two different references.

infection. Regarding other adverse events, there were some rare severe adverse events in the fingolimod group. Risk of cerebral hernia was not increased after the medication (RR = 0.35, 95%CI: 0.06-1.90, Table 3). The reason might be the large lesion volume at baseline. In the clinical trial by Tian *et al*[18], fingolimod was applied based on delayed alteplase administration. Hemorrhage of the digestive tract is definitely the potential risk during thrombolytic therapy. However, we found that the risk of digestive tract hemorrhage was not increased in the fingolimod group (RR = 0.42, 95%CI: 0.04-4.30, Table 3). Sphingosine-1-phosphate can regulate calcium ion movement in cells, which can affect myocardial contractility[25]. Therefore, fingolimod-phosphate can bind to sphingosine-1-phosphate receptors, which might result in arhythmia, such as bradycardia and atrial flutter. Likewise, sphingosine-1-phosphate can be produced

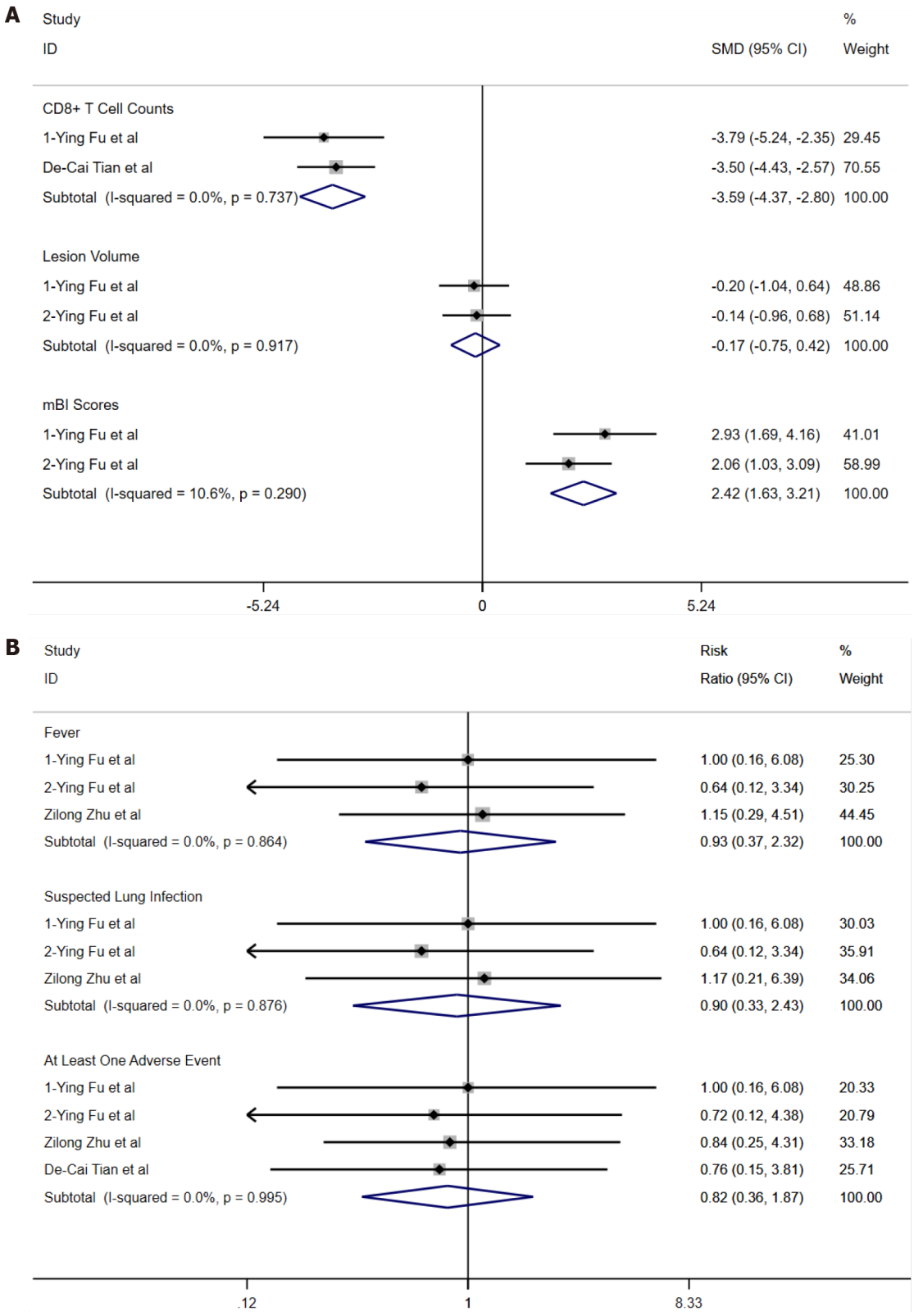

Figure 3 Forest plots of overall standard mean difference and pooled risk ratio. A: Overall standard mean difference of T-lymphocytes with cluster of differentiation 8 expression/infarction or hematoma volume/modified Barthel index scores; B: Pooled risk ratio of fever/suspected lung infection/adverse events occurring at least once. 1-Ying Fu *et al*"[15] and 2-Ying Fu *et al*[16] represent two different references. mBI scores: Modified Barthel index scores. RR: Risk ratio; CD8+ T cell: T-lymphocytes with cluster of differentiation 8 expression; Lesion volume: Infarction or hematoma volume; SMD: Standard mean difference.

from platelets and participate in platelet activation, which might result in thrombocytopenia in the fingolimod group[26]. Therefore, the application of fingolimod should be cautious in patients with a history of cardiac disease and platelet disorder. In general, according to pooled RR of adverse events occurring at least once (I^2 = 0, pooled RR =

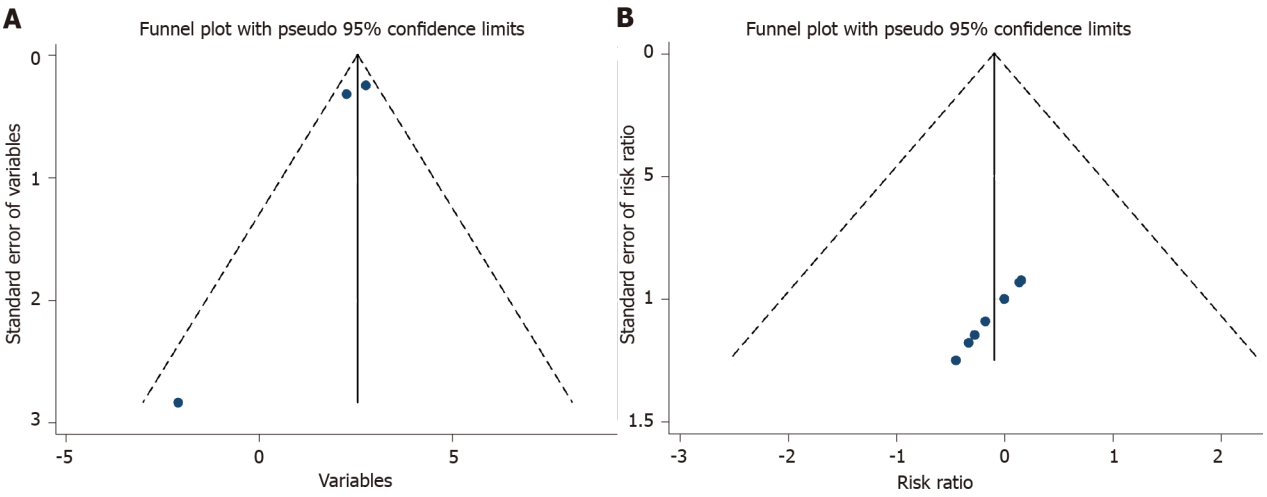

Figure 4 Funnel plots of publication bias. A: Funnel plot of variables used to acquire overall standard mean difference; B: Funnel diagram of risk ratio.

0.82, 95%CI: 0.36-1.87, P = 0.995, Figure 4), fingolimod might be safe for stroke patients.

Only four RCTs were included in the meta-analysis finally. Furthermore, two of them were completed by the team led by the same professor. The studies included was insufficient to perform a meta-analysis with high quality due to an insufficient number of patients. Although publication bias was not found, the outcome might be influenced by tendentiousness of articles with the same first authors. As some results were only shown in figures in some articles, these results were not extracted, which may lead to missing data at other time points and some variables, such as counts of other types of lymphocytes, edema around lesion, and NIHSS scores. In addition, there was potential influence of non-original data because data of continuous variables used in meta-analysis were processed *via* mathematical transformation owing to statistical difference before meta-analysis. Although a few stroke patients used fingolimod as a therapeutic medication, we believe that our research would bring about a new direction of stroke medication.

CONCLUSION

Fingolimod might improve neurological function in stroke patients by reducing lymphocyte infiltration in the brain effectively; however, we did not find the evidence that it could promote infarction lesion or hematoma absorption. In general, oral fingolimod (0.5 mg/d, 3 consecutive days) is safe in stroke patients except for some rare severe adverse events.

ARTICLE HIGHLIGHTS

Research background

Brain tissue injury in stroke patients involves inflammation around the infarction lesion or hematoma, which is an important reason for disease deterioration and can result in a poor prognosis. The meta-analysis of animal experiments has concluded that fingolimod could treat stroke in animal models by effectively reducing lymphocyte infiltration.

Research motivation

The evidence-based of efficacy and safety evaluation of fingolimod in stroke patients is currently unavailable.

Research objectives

We hypothesized that fingolimod could effectively and safely promote reduction of infarction lesion or hematoma and improve neurological prognosis in AIS or ICH

patients by reducing lymphocyte infiltration.

Research methods

In this study, we performed a systemic review and meta-analysis of recent randomized controlled trials to confirm the above hypothesis.

Research results

There was a significant difference in $CD8^+$ T cell count and modified Barthel index between the fingolimod and control groups. However, there was no significant difference in lesion volume, fever, suspected lung infection (pooled RR = 0.90, 95%CI: 0.33-2.43, P = 0.876), and all adverse events occurring at least once between the fingolimod and control groups.

Research conclusions

Fingolimod might improve neurological function in stroke patients by reducing lymphocyte infiltration in the brain effectively. Fingolimod might not promote infarction lesion or hematoma absorption. Oral fingolimod (0.5 mg/d, 3 consecutive days) is safe in stroke patients except for some rare severe adverse events.

Research perspectives

More high-quality randomized controlled studies involving more patients are needed to provide more clinical research evidence.

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