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EDITORIAL
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Beautiful death: point of view

Dinaldo Cavalcanti de Oliveira[1], João Victor Batista Cabral[1], Maria Mariana Barros Melo da Silveira[1], Carolina Gomes Cavalcanti de Oliveira[2], Dário Celestino Sobral Filho[3]

INTRODUCTION

Sparta is known as a city whose people were specialized in war, and indeed it was[1]. When people from all over the world think of Sparta, they think of a powerful army and men trained for combat, violence, conquest, victory, defeat, etc. However, one day, that image was supplanted by the value of other cities. I invite you to reflect on whether that city was associated only with war and brutality.

There is a certain historical episode[2], adapted for film, in which 300 Spartan warriors were strategically positioned in one place to "delay" an extremely powerful army so that some Spartan objectives could be achieved. In this description, I do not intend to go into all the details of this battle, but I invite you to put yourself in the place of those 300 warriors.

Certainly, that strategic decision was not instantaneous, some time elapsed between when the decision was made and when the warriors reached the determined place. Let us imagine something: when it was announced that 300 warriors were needed for such a mission, surely there was a group of prequalified warriors, who spent a lifetime preparing for such a task.

However, when the mission arrived, expectation turned to reality. We can wonder if, at some point, those warriors wanted to give up; it would be a great battle, but the probability of defeat and death was immense, practically 100%.

How, then, did the chosen warriors prepare to march toward the place to face the enemy army? Did they feel fear? Fear of death itself? Who would be with the wives and children of those warriors? Who would support their family? Should we take part in a battle that is already lost? Did those Spartans have thought about these and other questions?

Some Greek warriors wished to have a noble death (*kalos thanein*)[3], which was only possible when facing an enemy during a challenging journey that must be accepted consciously with courage and morals.

They were fully aware of the concept of a beautiful death (*kalos thanatos*)[3], and when facing certain death without any possibility of living, one should seek the *kalos thanatos*. Therefore, death is an act of heroic confrontation, worthy of the crown of life.

The search for a glorious death (*euklees thanatos*)[3] was something that existed in some Greek cultures since antiquity, and immortal fame (*kleos aphthiton*) was also an object of desire.

Such cultures put value in ideals of virtue, dignity, a desire to do good, a code of morality, a sense that there was something beyond death (an afterlife), and a heroic inscription. Life is lived within the precepts of virtue, following the moral values people believe, and practicing good. I understand that there is something beyond death, and that we should live our lives in a manner that would leave a legacy for the rest of humanity[4,5].

Based on the concept of *kalos thanatos*, we can understand that, while we are alive, we should immerse ourselves in cultural, economic, and social activities, leading us to take positive actions that impact our lives and the lives of others, and understand that has meaning. We should transform our lives into a journey in which death is a specific point, but not the end by itself.

Thus, if we believe that those 300 Greek warriors developed their faith and took actions based on the concepts described above, we can understand that in no way did they see going into battle against the mighty enemy army as an act of giving up or fear it, even if death were certain.

We must believe that those 300 noble men lived their lives continually preparing for battle, for war. They gave their best during training; sought to evolve, grow, and learn from their mistakes; understood that their role was not that of a lone warrior fighting, but being part of an army in which each warrior had a specific function; and accepted that the final objective, i.e., victory, would be the product of collective efforts.

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The Spartans understood that there was an enemy that could not be defeated, i.e., death. However, they did not believe death was the end, but rather the beginning of a new stage. To be valorous, this new stage should follow a life lived with dignity and moral values, leaving a legacy for those who continue to live[6]. Those 300 men left their legacy.

Aging is a normal human process. Depending on the country of residence, a person can be considered "older" when they are 60 or 65 years old. Not all older people are the same, and we must understand that humans have a biological and functional age[7,8].

Health professionals are in a battle. We try to promote health, avoid diseases, treat or cure them, or offer some comfort. However, we all know that death is certain, sooner or later.

Therefore, we should try to be prepared for death and help our patients to be prepared as well. In this sense, there are many opportunities to be explored by human beings, whether health professionals or not.

A good death comprises a set of characteristics for facing death, including seeking to improve the quality of the dying process in the remaining period of life[9].

A good death may be related to being cared for with love, affection, and dedication; avoiding physical, psychic or emotional suffering; being surrounded by those you love and were loved by; avoiding conflict with your team of health professionals; having some control over your death; reducing or eliminating pain; and having your preferences respected. It also includes resolving unfinished businesses, eliminating false expectations, accessing specialists, receiving palliative care (PC), and so on[9].

When a person dies, they disappear from the society; this rupture can lead to very damaging consequences. To minimize them and to resume the path of life, those who remain alive ritualize the situation with funerals, burials, sharing of goods, tributes, mourning, etc. In this way, society remains alive and strengthened[9].

According to the World Health Organization (WHO), PC comprises assistance provided by a multidisciplinary team with the objective of improving the quality of life of patients and their families in the face of a life-threatening disease. This is achieved through the prevention and relief of suffering, early identification of the illness, impeccable evaluation and treatment of pain, and other physical, social, psychological, and spiritual complaints[10]. The WHO showed that only 14% of patients worldwide needing PC receive this type of attention[11].

Unfortunately, PC training is not often included in the educational curriculum of health professionals. Moreover, the availability of drugs for pain—the most basic topic when talking about minimizing patient suffering—is unfortunately inadequate in most parts of the world[12].

It is necessary to be prepared for death, both as a person and as a society. From the perspective of health professionals, undergraduate curricula need to be adjusted for this purpose. Moreover, there is also a need for continuous learning after joining the workforce. Health professionals should help prepare their patients for a good death, starting from when the patient has a serious illness with the probability of death or even earlier.

As human beings, we must be prepared to face the certainty of death, building a happy life through physical, economic, financial, religious planning, etc. We must live the present but understand that the past existed; we must learn from our mistakes and successes, so that we can live better lives. To ensure happiness in the future, we must understand that we can achieve the things we imagine, desire, and hope for thorough planning and, if necessary, adapt to possible changes, but sometimes we won't be having what we wanted, so we must begin again having a new dream!

Human beings should accept death as a fact and part of life, and life should be happy, joyful, and pleasant based on moral and cultural precepts.

When a disease is diagnosed by a health professional, it should be understood that many of them will lead to death over the years and that aging increases the chance of death.

Therefore, healthcare professionals should accept the journey of facing death with their patients. However, this should not be a journey of dread, anguish, suffering, and the like. In this context, several actions by healthcare professionals (such as type of communication with the patient and family, attention, showing concern and love, and honesty) have the potential to have a positive impact on people. In addition, spirituality, regardless of religious belief, has the potential benefit. I invite health professionals to dream about these possibilities with us and to make a positive difference in people's lives!

The greatest battle on this journey "takes place in the mind." Obviously, there are other confrontations in the human body that are sometimes painful; however, the battle of thoughts, beliefs, convictions, values, and traditions is fundamental. Therefore, regardless of whether there is an adverse situation or not, the person, patient, or human being should be at peace, comforted, joyful, smiling, feeling good, and supported. Have we never seen a patient having a serious illness with a short-life expectancy but well, happy, and laughing?

Health professionals should not just provide technical assistance or limit themselves to offering patients the best scientific assistance possible, they should instead be part of a multidisciplinary team working toward the greatest goal, i.e., to do good for the patient.

Each team member must fully exercise his/her abilities in the physical and psychological aspects as well. It is necessary to have professionals thriving to have mastery over all areas of human health, leading to a multidisciplinary team offering physical and psychic care to promote health and care for the

patient. Individuality must be left at the door, and health professionals must understand that the collective is what makes a difference in our profession.

We must work as a team in which communication should be effective, actions are undertaken together and aim at a common goal, each member supports the others, moral and ethical values are prioritized, knowledge and experiences are shared, the management is respectful and works in harmony with the rest of the team, and so on.

The time has come for a revolution! It is time for us to reinvent healthcare, promote health from pregnancy and throughout life, understand that health is a continuum, and recognize that death is certain but the journey of life can be pleasant and happy. At this time, we should not only be health professionals but also be in health teams in which potential and ability of each individual emerges and the whole is used for the benefit of the people.

In this way, death will not be seen as something to be feared or as a desperate, distressing event, because life will have meaning: a legacy will be left, there will be an understanding of the natural history of human life and death, and with this, death can be understood as another life event.

In order to facilitate a good death to our patients, we need to work together with them, their families, their friends, and the society to understand the cycle of life, accepting death as an event in this cycle. We need to develop knowledge of this journey through beliefs, cultural values, traditions, etc., and as health professionals, we cannot fall behind. We should share our vision of the life cycle with our patients and with society, but also seek broader knowledge of this process and of the various historical strategies of facing death. Death should not be an unprepared event in people's lives.

Sometimes, death is the solution: freedom. For some people in hospital rooms, in bed, lying down without contact with the outside world, death can be liberating. Let us share with you one case: a patient well-known to Dinaldo Cavalcanti de Oliveira (DCO) was with tracheostomy (cervical region) in the room. The patient's monitor showed a 60 bpm, and beside him was a caregiver. The window was closed, and the room was almost absolutely silent. DCO entered the room and asked the caregiver to leave. DCO was now next to the bed, staring into his eyes that seemed to want to tell something; however, no words came out of his mouth, which insistently remained closed and immovable. In that moment, the childhood flashed through DCO's mind: the jokes and smiles they had together, their trips, family celebrations, his happy way of speaking, his children who were all working professionals, and his frequent family reunions to celebrate happy occasions (the last one two months ago to celebrate his 99th birthday). Now, the man who smiled and played with everyone at his party was lying in that bed immovable. Nobody anticipated the overwhelming stroke that put him in this situation, although they always knew that one day something would happen to end his life. Two months alive in a hospital room; alive but lifeless. The tears came down from eyes, DCO could not help calling out to him "…let's travel, let's go to the beach… I miss you". At that moment, looking into his eyes, DCO saw tears flowing down. DCO was static for about one minute, surprised by the sound of the monitor warning that his heart rate was below 60 bpm. It was decreasing together with the blood pressure; soon after, there was a flat line on the monitor. He was no longer in that room; the journey of his battle had been crowned with memorable victories, unforgettable ones that left marks in the lives of many; marks that are impossible to forget or erase. He lived for two months on a bed, inert, passive, alive without being alive, and now he was no longer there; he had passed on to a glorious phase: death. Death, where is your victory? His life became immortal because those who lived with him are carrying on his legacy, and he continues his journey even though he is no longer in this world!

CONCLUSIONS

Healthcare professionals should accept the journey of facing death with their patients. However, this should not be a journey of dread, anguish, suffering, and so on. In this context, several actions by healthcare professionals have the potential to make a positive impact on people and the patients alike.

The time has come for a revolution! It is time for us to reinvent healthcare, promote health starting from pregnancy and throughout life, understand that health is a continuum, and recognize that death is certain but the journey of life can be pleasant and happy. At this time, we should not only be health professionals but also be in health teams in which the potential and ability of each individual emerges and the whole is used for the benefit of people.

AUTHORS' CONTRIBUTIONS

DCO: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **JVBC:** Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **MMBMS:** Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **CGCO:** Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **DCSF:** Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Software, Supervision, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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LETTER TO THE EDITOR
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Emphasis on the novel age cutoff, 55 years, for postsurgical adjuvant radioiodine as consideration for American Thyroid Association ¾ low-intermediate risk differentiated thyroid carcinoma

Ilker Sengul[1,2], Demet Sengul[3]

Dear Editor,

We have read with a great deal of respect the research article entitled 'Older patients with differentiated thyroid cancer exhibit more aggressive pathological characteristics than younger patients'[1]. This beneficial research seems to be demanded out of determining any alteration in the clinical and/or pathological characteristics of differentiated thyroid cancer (DTC) without microcarcinomas, extrathyroidal extension (ETE); tumor size ≥2 cm, an emphasized size cutoff for the thyroid nodules, particularly with indeterminate cytology[2-5]; completeness of resection; multifocality; angioinvasion; and regional/distant metastasis, with regard to revised age cut-point, 55 years.

They stated that the younger cases differed significantly from the older ones, in terms of ETE, tumor size ≥2 cm, completeness of resection, except multifocality, angioinvasion, and regional/distant metastasis. Interestingly, only ETE was recognized as significant regarding an alternate cut-point, 45 years[1]. In 2015, management guidelines from the American Thyroid Association (ATA) for adult patients with thyroid nodules and differentiated thyroid cancer[2] reported characteristics in accordance with the ATA Risk Stratification System and the American Joint Committee on Cancer (AJCC)/TNM Staging System that may impact Postoperative Radioiodine Decision-Making (Recommendation 51, Table 14). They recommended postsurgical radioactive iodine, radioiodine (RAI) to be considered in four different categories of ATA low-intermediate risk. Engrossingly, they proposed the use of 55 years as a more appropriate prognostic age cutoff for the relevant classification systems, rather than 45 years, particularly for women, considering the suggestion of recent data from the National Thyroid Cancer Treatment Cooperative Study Group (NTCTCSG) for three of four categories of ATA low-intermediate risk [(i) T3, N0, Nx, M0, Mx; (iii) T1-3, N1a, M0, Mx; and (iv) T1-3, N1b, M0, Mx]. Interestingly, only one category of ATA low-intermediate risk [(ii) T3, N0, Nx, M0, Mx] includes "microscopic ETE, at any tumor size" for which the NTCTCSG and also the 2015 ATA management guidelines did not recommend to reckon 55 years as a more convenient prognostic age cutoff for the associated classification systems. Johar and colleagues[1] also reported solely ETE as being a revealed difference while the age cutoff described was 45 years. As we evaluate and comment from the other side, the NTCTCSG and also the 2015 ATA management guidelines recommended the use of postsurgical RAI treatment for advanced age patients for whom (with a latter age cutoff; i.e, >55 years) that may favor the use in the following three conditions: tumor size >4 cm, central compartment neck lymph node metastases, and lateral neck or mediastinal lymph node metastases [(i), (iii), and (iv),

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respectively], which were aggressive pathologic characteristics, Johar et al.[1] expressed.

Consequently, postsurgical adjuvant RAI treatment is recommended in compliance with the ATA low-intermediate risk. The NTCTCSG and also the latest ATA management guidelines indicate its use considering the age cutoff of 55 years, instead of 45 years, for three of four categories of ATA low-intermediate risk, those consisting of more aggressive pathological characteristics. Due to that fact, this issue merits further investigation. We thank Johar et al.[1] for their valuable research.

AUTHORS' CONTRIBUTIONS

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LETTER TO THE EDITOR
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Quality of sleep and use of computers and cellphones among university students

Rui Wang[1], Baohong Xue[2]*

Dear Editor,

We read the research of Diogo von Gaevernitz Lima et al.[1] with great interest. Their study found higher rates of poor sleep quality among medical students who used a computer and a mobile phone during the night and before going to sleep, and among dentistry students who longer time of computer use before going to sleep [sic]. They believed that the use of a mobile phone and a computer can influence poor sleep quality among medical and dentistry students during the night and immediately before going to sleep. In my opinion, it is necessary to investigate and analyze to reach conclusions.

First, the authors surveyed respondents who use a mobile phone and a computer over the past month. These data relied on the personal subjective impression to fill in and the real situation is quite different, especially the time of use of the mobile phone in 24 hours. The data should be recorded by pre-installed software. In addition, the author fails to analyze the reasons for using computers and mobile phones. Possibly medical and dentistry students study or play using computers and mobile phones. If high academic pressure leads to losing sleep[2], so it is not related to the use of computers and mobile phones. The author should pay attention to the importance of this problem.

There are several risk factors for poor sleep quality among medical and dentistry students. If you want to know whether the use of computers and mobile phones influences sleep quality, several factors need to be eliminated, such as academic pressure, student work, domestic violence[3], and possibly other factors that contribute to poor sleep quality among medical and dentistry student. Therefore, it is evident that further research needs to be done to prove the influence of the use of computers and mobile phones on poor sleep quality.

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LETTER TO THE EDITOR
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Individualized masks and respirators for COVID-19 patients and involved medical staff

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Dear Editor,

Since the declaration of COVID-19 as a pandemic by the World Health Organization on March 11, 2020, healthcare providers and medical staff all over the world have been increasingly facing limited availability of personal protective equipment (PPE). In the management of COVID-19 patients, the availability of PPE is crucial to protect the frontline medical staff[1].

Adherence to PPE is a crucial point regarding its efficacy. Different studies showed that the adherence of healthcare workers is not high enough due to several reasons. One of them is a gap of knowledge about the disease and transmission method, but numerous studies have suggested that education alone does not improve PPE adherence[2]. Thus, identifying an effective education methodology is a crucial subsequent step for improving PPE adherence. The other important factors are time pressure, heavy workload, and unsureness about the quality and the safety.

Personalized medicine could adapt the therapeutic strategies through the appropriate response and highest safety context to ensure providing the best care. Regarding prevention and PPE, masks/respirators can be an interesting topic in COVID-19. 3D printing translates computer-designed virtual 3D models into physical objects. 3D printing is used in the manufacturing industry, medical and pharmaceutical research, drug production, and clinical medicine and dentistry with implications for precision and personalized medicine[3].

Among these, masks/respirators are of pronounced importance, since they should be appropriately worn as they fit around the nose, mouth, chin, and face. Although ensuring proper sealing seems to be difficult, it can be optimized by the personalized 3D printed masks/respirators, specially designed for each healthcare worker. These masks/respirators can be made of poly-lactic acid nanoparticles (PLA-NP), commonly used in 3D printers, in a short time. Furthermore, 3D printing plans for customizable masks/respirators can be shared publicly through social media and be accessible for everyone. Designing disposable face shields is another vital requirement that aims to help increase PPE availability through the global emergency.

COVID-19 patients are the other overlooked, yet important, part of this equilibrium. Fundamentally, non-invasive ventilation techniques (NIV) are commonly used in non-severe forms of respiratory failure. However, if the patient's condition does not improve or even deteriorates within a short period (1–2 hours), mechanical ventilation should be considered. Nevertheless, as coronavirus or any other pathogens can be spread through NIV, this ventilation mode is not recommended in these patients. Airway devices providing 6 L/min or more of oxygen are considered as high-flow systems, and their use is discouraged if an airborne infection isolation room is unavailable[4].

We believe that designing the masks that fit the person's face (personalized masks) can eliminate the leakage and make non-invasive ventilation an appropriate option for the treatment of COVID-19 patients[5]. The personalized masks also help to apply gentle manual breathing with minimum leakage during bag-mask ventilation for airway management without the spreading of the virus. This not only leads to more effective and safe airway management for the patients but also helps to protect the healthcare workers from virus spreading.

AUTHORS' CONTRIBUTIONS

SS: Conceptualization, Data curation, Writing – original draft.
AM: Conceptualization, Data curation, Writing – original draft.
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POINT OF VIEW
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Equipoise, placebo in clinical trials and Brazilian Code of Medical Ethics

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INTRODUCTION

Physicians must act to their patients' best interests and thus they are always expected to offer them what they believe is the best treatment for their diseases. Owing to this moral commitment, when physicians are researchers conducting randomized controlled trials (RCTs), a dilemma may arise if they suspect that one trial arm is more effective and/or safer than the other(s). If this is the case, professional ethics dictates that they should recommend their patients not to enroll in the trial and to take the therapy the physician thinks is the best for the patient's illness. As a scientist, however, they know that by doing so the insight on the clinical superiority of one trial arm (treatment) over the other(s) may remain unproven. Moreover, the physician is also aware that clinical studies, if scientifically valid and soundly designed, are beneficial for the society. For instance, the practice of evidence-based medicine requires good-quality empiric evidence as that provided by RCTs.

This ethical issue was made explicit by Shaw and Chalmers[1] when addressing the ethics of cooperative clinical trials. According to them, a clinical research must not be initiated if the physician–researcher "knows or has a good reason to believe that one arm of the trial is even slightly better than the other." It was Charles Fried[2] in "Medical Experimentation: Personal Integrity and Social Policy," however, who first used the term "equipoise" (an equilibrium state or situation in which things are perfectly balanced) for an ethical principle according to which the physician/researcher must be in a state of genuine uncertainty about the relative efficacy (and/or safety) of the therapeutic alternatives being tested to render an ethically acceptable RCT. Some doctors embraced the equipoise concept, whereas others, including Freedman[3], claimed that it was flawed and unworkable, and what is worse, its fragility would render many good-quality and relevant RCTs unethical. To tackle this issue, Freedman[3] proposed to replace the notion of equipoise by that of clinical equipoise, or a situation in which there would be "no consensus among the expert clinical community about the comparative merits of the alternatives to be tested," or in other words, there would exist a collective professional uncertainty about treatment alternatives. Nonetheless, some physicians and researchers argue that Freedman's reformulation of equipoise concept in reality did not overcome a major objection to this principle, namely, that it is ambiguous and that, if strictly interpreted, it would also render unethical too many RCTs. So far, the controversy on whether equipoise — or clinical equipoise—is a prerequisite to make a RCT ethical has remained unresolved. Many clinicians and researchers endorse the point of view that equipoise principle, even if it eventually requires amendments, is an important ethical standard for RCTs whereas others maintain that it is inherently flawed and must be abandoned[6-12].

ETHICAL ISSUES ON THE USE OF PLACEBO IN RANDOMIZED CONTROLLED TRIALS

Another debatable issue regarding the ethics of RCTs is the use of a placebo-controlled arm when there is an effective treatment for the disease or condition being investigated. Whenever we assume that there are effective treatments, the use of a placebo-controlled arm in clinical trials clashes with doctors' moral commitment to their patients, even if physicians are genuinely uncertain whether the particular treatment under investigation would be in fact superior to placebo. In principle, therefore, to use or not to use a placebo when it seems to be scientifically justified apparently opposes science to ethics. One should have in mind, however, that, according to the Hippocratic oath and medical codes of ethics, such as the World Medical Association's (WMA) Declaration of Helsinki (DoH), physicians are bound

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Table 1. Updates of the statement on the use of placebo in clinical trials in successive revisions of the Declaration of Helsinki by the World Medical Association (WMA) between 1964 and 2013.

Declaration of Helsinki (year)	Statement
Original version of DOH (Helsinki, 1964)	Use of placebo is not directly addressed. Statement II.2 says: "The doctor can combine clinical research with professional care, the objective being the acquisition of new medical knowledge, only to the extent that clinical research is justified by its therapeutic value for the patient."
1st revision of DOH (Tokyo, 1975)	Use of placebo—indirectly—forbidden if there is proven treatment: Statement II.3 says: "In any medical study, every patient—including those of a control group, if any—should be assured of the best proven diagnostic and therapeutic method."
2nd revision of DOH (Venice, 1983)	Previous statement wording unaltered.
3rd revision of DOH (Hong Kong, 1989)	Previous statement wording unaltered.
4th revision of DOH (Somerset-West, South Africa, 1996)	Statement II.3 explicitly forbade the use of placebo whenever there is a proven effective therapy. "In any medical study, every patient—including those of a control group, if any—should be assured of the best proven diagnostic and therapeutic method. This does not exclude the use of inert placebo in studies where no proven diagnostic or therapeutic method exists."
5th revision of DOH (Edinburgh, 2000)	Statement wording slightly modified, again it (C 29) explicitly forbade the use of placebo if there is effective therapy: The benefits, risks, burdens, and effectiveness of a new method should be tested against those of the best current prophylactic, diagnostic, and therapeutic methods. This does not exclude the use of placebo, or no treatment, in studies where no proven prophylactic, diagnostic, or therapeutic method exists.
6th revision of DOH (Tokyo, 2004)	Previous statement wording unaltered.
7th revision of DOH (Seoul, 2008)	The statement was modified to allow the use of placebo, even when there is effective treatment, in some particular cases: • Use of Placebo. 33. The benefits, risks, burdens, and effectiveness of a new intervention must be tested against those of the best current proven intervention, except in the following circumstances: • The use https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/of placebo, or no treatment, is acceptable in studies where no current proven intervention exists or • Where for compelling and scientifically sound methodological reasons the use of placebo is necessary to determine the efficacy or safety of an intervention and the patients who receive placebo or no treatment will not be subject to any risk of serious or irreversible harm. • Extreme care must be taken to avoid abuse of this option[#]
Current version of DOH (Fortaleza, 2013)	Use of Placebo (statement 33) remains allowed under certain circumstances. Previous statement wording unaltered.

[#]Portuguese translation (by AMB—Brazilian Medical Association): "Uso de Placebo. Os benefícios, riscos, ônus e efetividade de uma nova intervenção devem ser testados contra aqueles da(s) melhor(es) intervenção(ões) comprovada(s), exceto nas seguintes circunstâncias: Quando não existe intervenção comprovada , o uso de placebo, ou não intervenção, é aceitável; ou Quando por razões metodológicas e cientificamente sólidas, o uso de qualquer intervenção menos efetiva que a melhor comprovada, o uso de placebo, ou não intervenção, é necessário para determinar a eficácia ou segurança de uma intervenção e os pacientes que recebem qualquer intervenção menos efetiva que a melhor comprovada,placebo, ou não intervenção, não estarão sujeitos a riscos adicionais de danos graves ou irreversíveis como resultado de não receber a melhor intervenção comprovada. Extremo cuidado deve ser tomado para evitar abuso desta opção." Current and previous DoHs and translation into Portuguese are available on WMA website: https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/

Table 2. Statements on clinical research and placebo use in the Brazilian Code of Medical Ethics (CME) from 1945 to 2018.

Codes of Medical Ethics Updates (year)	Statements about clinical research and placebo use		Comment
	Portuguese version	English translation[+]	
Code of Medical Deontology. Federal decree-law 7.955, September 13, 1945	Nenhuma referência à pesquisa médica.	No statement about medical research.	In 1947, 2 years after the publication of the Code of Medical Deontology, the Nuremberg code laid down a set of 10 standards to which physicians must conform when conducting human experimentation[6].
Code of Medical Ethics. Union Official Journal. DOU sect. I, part II, pp. 95–99; January 11, 1965	Art. 58. As experiências in anima nobili só poderão ser permitidas para fins de tratamento ou diagnóstico, sempre precedidas de consentimento do paciente, quando em perfeita higidez mental, ou de seus responsáveis, devidamente informados das possíveis consequências.	Art. 58. Experiments in anima nobili are allowed only if intended to treat or to make a diagnosis, and must always be preceded by patients' consent, when they are fully mentally competent, or by their guardians' consent, after had been duly informed of the possible consequences.	One year earlier, the Declaration of Helsinki (a set of ethical principles for medical research involving human subjects) had been adopted by the 18th WMA General Assembly, held in Helsinki, Finland, in June 1964[5]. The 1965 CE statement is in line with the first version of the declaration. The emphasis was on research aims and on the Informed consent.
Code of Medical Ethics. Resolution CFM 1246/1988	É vedado ao médico: Art. 129. Executar ou participar de pesquisa médica em que haja necessidade de suspender ou deixar de usar terapêutica consagrada e, com isso, prejudicar o paciente.	It is forbidden for physicians: Art. 129. To conduct or take part in medical studies that require to discontinue or not to use established therapies and by doing this to cause harm to the patient.	Although not explicitly using the term placebo, the 1988 CE stated that, for not causing harm, investigators must treat (all) patients involved in clinical with "established therapies." This statement is aligned with the 1975 DH update: "every patient—including those of a control group, if any—should be assured of the best proven diagnostic and therapeutic method"[4].
Code of Medical Ethics. Resolution CFM 1931/2009	É vedado ao médico: Art. 106. Manter vínculo de qualquer natureza com pesquisas médicas, envolvendo seres humanos, que usem placebo em seus experimentos, quando houver tratamento eficaz e efetivo para doença pesquisada.	It is forbidden for physicians: Art. 106. To maintain any type of connection with clinical studies, conducted in humans, using a placebo in the trial, whenever there is an effective treatment for the researched disease.	The term placebo first appeared in ethical standards statements for Brazilian physicians. This statement of 2009 CE was very stringent and forbade any use of placebo in clinical trials unless there is no effective treatment for the medical condition. This statement strictly prohibits any use of placebo when there is effective treatment.
Code of Medical Ethics. Resolution CFM 2.217/2018 (the latest version)	É vedado ao médico: Art. 106. Manter vínculo de qualquer natureza com pesquisas médicas em seres humanos que usem placebo de maneira isolada em experimentos, quando houver método profilático ou terapêutico eficaz.	It is forbidden for physicians: Art. 106. To maintain any type of connection with clinical studies in humans using a placebo *as the sole medical intervention*, whenever there is an effective prophylactic or therapeutic measure available.	It is unclear what is meant by "as the sole medical intervention."

[+]Translation into English by the author. DH, Declaration of Helsinki. The versions of Brazilian CME are available on "Conselho Federal de Medicina" website: https://portal.cfm.org.br/index.php?option=com_content&view=article&id=41&Itemid=124

to their solemn oath ("The health of my patient will be my first consideration") and so their acts as healers must always take precedence over their duties as scientists[13].

Whether placebo use in RCTs is scientifically justifiable and ethical, if there are approved treatments, has been debated over the last 6 decades or so[14]. At the time when WMA issued the first version of DoH, Bradford Hill, a pioneer in the use of randomization in clinical trials, expressed his view that "If there is such an orthodox treatment the question hardly arises, for the doctor will wish to know whether a new treatment is more, or less, effective than the old, not that it is more effective than nothing"[14]. Hill's opinion is consistent with WMA's ethical guidance (DoHs from 1975 to 2008) stating that: "every patient — including those of a control group, if any — should be assured of the best proven diagnostic and therapeutic method" (Table 1). In 1996s, DoH revision, a sentence ("This does not exclude the use of inert placebo in studies where no proven diagnostic or therapeutic method exists") was added to emphasize that placebo use is forbidden whenever there are proven effective treatments. This emphasis was found necessary in the aftermath of a questionable placebo-controlled RCT conducted to investigate whether a specific AZT regimen ("076 Regimen") would reduce mother–child HIV-1 transmission in Uganda and Thailand. In 1994, when the trial began, it was already known that AZT was effective against HIV, and thus treating HIV-infected women with placebo was considered morally outrageous. Moreover, ethical double standard for trials in developed and developing countries was considered unacceptable[16].

A subsequent DoH (2008) revision tempered the ban on placebo use wherever there exist proven therapies ("where for sound methodological reasons the use of placebo is necessary … and the patients who receive placebo or no treatment will not be subject to any risk of serious or irreversible harm"). This exception was maintained unaltered in 2013s revision.

PLACEBO ADVANTAGES OVER ACTIVE COMPARATORS

Depending on the RCT, placebos may have advantages over proven effective comparators. Active comparators show whether efficacy and safety of test treatments (drugs) are better, noninferior (noninferiority trials), or worse than those of approved drugs whereas a placebo control indicates whether they are effective and safe in absolute terms. As commented by Hill, as far as effectiveness is concerned, comparison with standard ("orthodox") therapy is what generally matters. A different picture may arise, however, when the primary focus is safety.

Studies on the safety of rofecoxib (Vioxx®), a COX-2-selective NSAID, illustrate how comparisons with approved drugs may mislead the interpretation of findings if modes of action are distinct. A premarketing (VIGOR — Vioxx® Gastrointestinal Outcomes Research) RCT of rofecoxib versus naproxen, a nonselective COX inhibitor, suggested that it increased cardiovascular risks, a finding then attributed to heart protective effects of the active comparator. A postmarketing placebo-controlled trial (APPROVe — Adenomatous Polyp PRevention On Vioxx), however, confirmed that rofecoxib increased (twofold) risks of serious cardiovascular events, a conclusion that led to its withdrawn from the market[17]. Vioxx® RCTs exemplify how using active instead of inactive comparators may bias the evaluation of safety.

It is of note that lack of a placebo-control arm in RCTs may downplay effectiveness evaluation as well. Placebos are by no means "inactive" interventions. Placebo effects are useful in the management of anxiety, depression, pain, and some other illnesses or psychosomatic conditions with strong emotional components[18]. For instance, meta-analyses of antidepressant RCTs have brought to light not only a strong placebo response but also the statistically significant differences of response between ADs and placebos may not be clinically meaningful. The real magnitude of antidepressant and anxiolytic response to pharmacological interventions is likely to remain blunted if placebo arms are missing in RCTs. The same holds true for anti-obesity drugs[19]. Although there is a set of weight-loss medications in the market, anti-obesity RCTs, as a rule, use placebo controls. If active comparators instead of placebos had been used in these trials, the absolute efficacy and safety of tested new anti-obesity drugs would have remained unclear.

Although not supplying data on relative efficacy/tolerability, placebos are better than active comparators to estimate assay sensitivity and thus the study internal validity and to fully unveil its clinical relevance. Additional advantages are that inactive comparators generally require smaller sample sizes and are less costly.

COUNTERBALANCING ETHICAL ARGUMENTS

The use of placebo in clinical practice is unacceptable because by doing so, physicians consciously deceive their patients, even if, presumably, they do it for their benefit. In soundly designed RCTs, however, patients are informed about trial details, benefits, and risks and are expected to freely give their consent to participate. If participants are fully aware about the health consequences of receiving a placebo instead of the test drug, then the burden of the decision-making is transferred to them. Patients' informed consent is a *prima facie* obligation for RCTs and patients' right to self-determination must always take precedence. Nonetheless, full autonomy in decision-making, to

provide the relevant information in an understandable way and check whether patients in fact understood it, is necessary to obtain a valid consent[20].

The social value of the study, one of the requirements to render it ethical, and the scientific justification for using inactive comparators, must be fairly and clearly explained to obtain an informed consent. Patients (who do not have limitations to consent) must have the right to self-determination and to freely decide to take part in placebo RCTs not only because of expected individual health benefits but also for altruistic reasons. In this regard, a parallel can be drawn on placebo RCTs and the enrollment of healthy volunteers in early clinical phases of drug research and development. In the absence of foreseeable benefits to healthy volunteers or patients, an ethical consensus has been reached that individual risks have to be proportional to the social (collective) value of the clinical trial[21–23]. That is, even if a trial is scientifically and socially valid, the use of inactive comparator arm is unethical whenever it implies in exposing some participants to a risk of "serious or irreversible harm" that can be treated with drugs of proven effectiveness. The anticipated risks of serious harm associated with no treatment, therefore, are insurmountable obstacles to replace active with inactive (placebo) comparators in RCTs.

PLACEBO IN THE BRAZILIAN CODE OF MEDICAL ETHICS

The Code of Medical Ethics (CME) laid down in 1965 to replace the Code of Medical Deontology of 1945, for the first time established (Art. 58) the general conditions under which a medical research is ethical. A further revision of CME in 1988, although not using the term placebo, prohibited physicians (Art. 129) "to discontinue or not to use established therapies and by doing this to cause harm to the patient." This strict prohibition on "not to use established therapies" is in line with the 1975-DoH statement that all participants should be assured of the best-proven therapeutic method. The term placebo was introduced in the 2009-CME that strictly forbade its use "whenever there is an effective treatment for the researched disease." In this new CME, the prohibition to "take part in" was extended "to maintain any type of connection with." The most recent CME (2018) maintained the prohibition but replaced "placebo" with "placebo as the sole medical intervention." It is not entirely clear what is meant by "the sole medical intervention" (Are placebo combined to "standard of care" and/or "any adjuvant therapy" allowed?), and so it opens a room for divergent interpretations by local Ethical Committees on Clinical Research. It is noteworthy that, since 2008, WMA code (DoH) contains a straightforward statement on the conditions under which placebo use could be accepted in RCTs, even if proven effective interventions exist. A Portuguese translation of DoH by AMB is available on WMA's website.

CONCLUDING REMARKS

Equipoise is an ethical concept that, if taken literally, would render unethical many scientifically relevant RCTs. Whether it should be adopted as originally proposed, amended, or abandoned remains an unresolved issue. Possibly because it is controversial, equipoise requirement for RCTs is not referred to in DoH nor is it mentioned in CME.

In contrast with omission regarding equipoise, placebo use in RCTs, when proven treatment exists, is explicitly or implicitly forbidden by DoH (as of 1974) and CME (as of 1988). Since 2008, DoH allowed use of placebo instead of active comparators in some particular cases, namely when it is demonstrated that it is scientifically needed and does not result in risk of serious and/or permanent harm to patients. In 2008, CME eased the ban by replacing "placebo" with "placebo as the sole medical intervention." What "sole medical intervention" exactly refers to (standard of care?) is unclear. A rewording of CME Art. 106 to make it a straightforward statement aligned with current DoH is strongly recommended.

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SHORT COMMUNICATION
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Cognitive impairment and metabolic syndrome in a population of Brazilian oldest-old

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INTRODUCTION

The global oldest-old population is projected to increase 351% between 2010 and 2050. In Brazil, approximately 4.3 million people are aged over 80 years, making it the fastest-growing stratum of the country's population. The total number of people with dementia worldwide is set to reach 82 million in 2030 and 152 million in 2050. Approximately 60% of people with dementia live in low- and middle-income countries. The prevalence of dementia doubles for every 5-year increase in age; therefore, dementia is a major health issue among those who aged above 80 years[1,2].

Metabolic syndrome (MS) is a common clinical condition in the older population. It consists of a set of cardiovascular risk factors and is associated with an increased risk of developing diseases, including cerebrovascular disease. Vascular risk factors are associated with cognitive impairment no dementia (CIND) and dementia, particularly vascular dementia[3,4].

Recently, the World Health Organization published a document containing key topics related to dementia prevention including management of weight, hypertension, diabetes, and dyslipidemia, all of which are MS criteria[5].

Cognitive impairment no dementia denotes individuals whose cognitive functioning falls below normal, but who do not meet the criteria for dementia. The follow-up of older people with CIND is important in order to identify those who will progress to dementia[6].

The literature is conflicting regarding the association between MS and CIND or dementia, with some studies confirming this association and others not, particularly in the oldest-old[7].

The aim of this study was to determine the association between MS and CIND in a population of functionally independent oldest-old.

METHODS

A population of functionally independent community-dwelling subjects who aged above 80 years participated in this cross-sectional study. The population was part of a cohort ("Projeto Longevos") routinely followed at an outpatient clinic of a university hospital in São Paulo, Brazil. Data were obtained from the first assessment of the individuals at the clinic and included sociodemographic information, cognitive screening results, and presence of clinical factors related to MS.

The clinical and laboratory data used for MS diagnosis were as follows: waist circumference ≥102 cm (male) and ≥88 cm (female); triglycerides ≥150 mg/dL or use of medication for dyslipidemia, high-density lipoprotein (HDL) cholesterol <40 mg/dL (male) and <50 mg/dL (female) or use of lipid-lowering drugs; systemic arterial hypertension reported by the participant or use of antihypertensive drugs; and fasting glycemia ≥100 mg/dL or use of medication for diabetes. In accordance with the National Cholesterol Education Program-Adult Treatment Panel Guide III (NCEP/ATP III) criteria[8], three out of these five items needed to be present in order to establish an MS diagnosis.

The instruments used for cognitive screening were the Mini-Mental State Exam[9], the Clock Drawing Test[10], and Verbal Fluency[11]. Scores on two of the three instruments had to be below the expected level for this population in order to consider the participant as having CIND.

The study was approved by the Ethics Committee of UNIFESP (1532/09).

Statistical analysis

Categorical variables were expressed as absolute and relative values, whereas continuous variables were expressed as central tendency measures. The association of categorical variables was

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assessed using the chi-square test and the comparison of continuous variables between two groups was assessed using the Student's *t* test. Logistic regression was used in the multivariate analysis. The level of statistical significance adopted was 0.05.

RESULTS

The total number of oldest-old in the cohort was 323, of which 68 were excluded due to missing items of information collected for this study. Data from 255 participants were analyzed. There were 171 (67%) women and 84 (33%) men. Overall, 55% had MS in the female group versus 39% in the male group.

The mean age of the total sample was 85.4 (±4.4) years and the mean schooling was 4.8 (±4.5) years. Considering the groups "with and without metabolic syndrome," there was no statistically significant difference for age (p=0.84) and for schooling (p=0.09).

The mean age of the group with CIND was statistically and significantly higher than the group without CIND (p=0.02). No statistically significant difference was observed between the groups with and without CIND considering schooling (p=0.17).

As shown in Table 1, no association was found between CIND and MS (p=0.35).

As shown in Table 2, no association was found between any of the components of MS and CIND.

Simple logistic regression was then performed using independent variables with p<0.25 in the bivariate analysis which in this case were age and schooling. "With CIND" was the dependent variable. Only the difference in age was associated with "having CIND" (odds ratio [OR] 1.07; p=0.02; and 95% confidence interval [CI] 1.008–1.13), which means that for each increased year there is 7.07% more chance to have CIND.

DISCUSSION

The sample population was predominantly women, consistent with the global demographic profile showing feminization of old age. In Brazil, 55.7% of its 20 million older people are women. As expected, there was a statistically significant association between older age and CIND[2].

There was no association between CIND and MS in the cohort of oldest-old studied. This is in line with the literature, where previous studies have failed to find this association among the oldest-old[7]. An analysis of relevant scientific publications revealed that the association between MS and cognitive impairment depends on the age group of the population studied. In younger older adults, most studies suggest an association between MS and cognitive impairment[12-17], whereas in the oldest-old, the results fail to confirm this association[18-22].

Table 1. Association between cognitive impairment no dementia and metabolic syndrome.

CIND	Metabolic syndrome						p
	No		Yes		Total		
	n	%	n	%	n	%	
No	78	67	101	73	179	70	0.35
Yes	38	33	38	27	76	30	
Total	116	45	139	55	255	100	

CIND: cognitive impairment no dementia.

Table 2. Association between each component of metabolic syndrome and CIND.

CIND		No		Yes		Total		p
		n	%	n	%	n	%	
High waist circumference	No	85	47	38	50	123	48	0.71
	Yes	94	53	38	50	132	52	
Low HDL-cholesterol or use of lipid-lowering medication	No	64	36	29	38	93	36	0.72
	Yes	115	64	47	62	162	64	
Hyperglycemia or use of diabetes medication	No	97	54	46	61	143	56	0.35
	Yes	82	46	30	39	112	44	
Reported hypertension or use of medication	No	38	21	15	20	53	21	0.79
	Yes	141	79	61	80	202	79	
Hypertriglyceridemia	No	142	79	57	75	199	78	0.45
	Yes	37	21	19	25	56	22	

CIND: cognitive impairment no dementia.

The existence of this difference raises questions. One explanation may lie in the MS cutoffs — do they apply to the oldest-old as they do to other populations? Another interesting point is the "survival effect," indicating that the oldest-old reach old age despite cardiovascular risks[18]. The antagonistic pleiotropy hypothesis could partially explain the greater deleterious effect of MS in younger ages compared to oldest-old[23].

The main limitation of this study was its cross-sectional design. However, this is the first study conducted in Brazil investigating the association of CIND with MS in the oldest-old.

AUTHORS' CONTRIBUTIONS

VH: Conceptualization, Data Curation, Writing – Review and Editing. AFJ: Formal Analysis, Writing – Original Draft. **LMQA:** Writing – Review and Editing. **MSC:** Writing – Review and Editing. **CMAF:** Conceptualization, Formal Analysis, Writing – Review and Editing.

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SHORT COMMUNICATION
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Test-retest reliability of the Health Literacy Questionnaire (HLQ-Br) in Brazilian carers of older people

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INTRODUCTION

The rapid aging of the Brazilian population is seeing people living longer but with comorbidities more common in older people and higher dependence in activities of daily living. Due to these factors, support from formal and informal carers is needed more frequently. Many informal carers are family members who manage the health of the older person they are caring for, including accompanying them to medical appointments and advocating for them when they are hospitalized[1,2]. As such, carers of older people often have a key role in accessing, understanding and supporting the implementation of health-related recommendations for the older person they provide care for.

According to the U.S. Department of Health and Human Services, health literacy is defined as "the degree to which individuals can obtain, process, and understand basic health information needed to make appropriate health decisions"[3]. Carers with low levels of health literacy may fail to implement the recommendations of health professionals, thus compromising care[4]. Data on health literacy in Brazilian carers of older people is scarce. In a previous study on health literacy in carers of older people in Brazil, one-third of the carers of older patients assessed in an outpatient clinic had low health literacy[5].

The Health Literacy Questionnaire (HLQ) is an Australian developed instrument used to assess health literacy[6]. The HLQ assesses nine independent health literacy domains to evaluate the experiences of people related to understanding and using health information[6]. It has been translated and validated in Brazil[7] and is internationally recognized for its clinical applicability. However, the retest reliability of the Brazilian version (HLQ-Br) has not been tested. This study aimed to determine the reliability of the HLQ-Br with a sample of Brazilian carers of older people.

METHODS

This was a test-re-test reliability study.

From May to December 2019, carers of older people who were discharged from inpatient wards at a university hospital were invited to participate. Inclusion criteria were to be 18+ years and be an informal family carer (as described above) of an older person aged 60+ years discharged from

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an inpatient ward of the participating hospital. A sociodemographic questionnaire was also applied during the baseline assessment.

The COSMIN guidelines[8] recommend a minimum sample of 50 completed data sets to provide accurate estimates of measurement stability over time. Therefore, we aimed to recruit 50 carers for this study.

The HLQ-Br[7] was administered face-to-face at baseline (pre-discharge from the hospital of the older person) and approximately two weeks later, at time point 2, the HLQ-Br was applied by telephone. The HLQ-Br[7] is a 44-item tool comprising nine domains structured as Likert scales. The first five domains use a 4-point scale to assess participants' agreement with a series of statements (responses range from "strongly disagree" to "agree"). The other four domains require participants to self-report the ease with which they feel they can complete certain health literacy-related tasks (responses range from "cannot do or always difficult" to "always easy". All HLQ scales (Australian original version), confirmed by factor analysis, have good internal consistency reliability (Cronbach's alpha ranging from 0.80-0.89)[6]. A recent reliability study by our team investigating retest reliability of the HLQ among carers of older people being discharged from hospitals in Australia identified moderate to high levels of retest reliability[9].

The study was approved by the Ethics Committee of the institution where the study was conducted (blinded for review), and all informal carers signed a Term of Consent.

Analyses were undertaken using SPSS v25. The categorical variables were expressed as crude and relative values and associations of these variables among groups were determined using the Chi-Square test. Continuous variables were expressed as means and standard deviations (for normal distribution) and compared through Student's t-test. The nine HLQ-Br scale totals were analyzed separately, given that an overall total score for the instrument is not recommended by the authors[6]. Correlations were evaluated between baseline and time point 2 scores of the HLQ-Br scales using the intraclass correlation coefficient (ICC) (two-way mixed effects, absolute agreement, 95% confidence interval (95%CI)). ICC ratings were classified as poor <0.5; 0.5–0.74 moderate; 0.75–0.9 good and values greater than 0.9 excellent[10].

RESULTS

Fifty-one carers completed the initial assessment session, with a mean age of 52.5 (±14.0) years and 41 (80.4%) were female. Almost two-thirds (62.7%) of the carers gave care daily and 54.9% lived with the person they cared for, as presented in Table 1.

The mean time for HLQ-Br administration was 11 (±3.7) minutes. The mean time between assessments was 20.8 (±13.3) days.

Only 35 participants completed the second test occasion. The most common reasons for not returning for the retest occasion were "not answering the phone after several trials" (10 (62.5%)) and "refused to participate" (6 (37.5%)). There were no significant differences between those who did and those who did not complete the second assessment except for "frequency of care" (p=0.03), in which those completing the second assessment required higher levels of care. Details of the demographics of the 35 participants who did complete the retest occasion are also shown in Table 1.

Table 2 shows the mean scores for each scale and the reliability data (ICC, 95%CI). The results indicate that the reliability estimates of the HLQ-Br (ICC, n=35) were good (that is, ranging from 0.79 to 0.89) for seven of the nine HLQ-Br scales. On the subscale, "actively managing my health", the reliability was excellent [0.925 (95%CI 0.846–0.963)]. For the remaining scale, "navigate the healthcare system", reliability was moderate [0.725 (95%CI 0.452–0.861)].

DISCUSSION

There has been an increase in health literacy research in Brazil over the past few years[11]. The World Health Organization classifies health literacy as one of the major health promotion factors for achieving or maintaining good health, which makes the topic extremely important in terms of public health[12]. Brazilian health literacy studies have been conducted with participants in the following disease areas — cardiology, infectious and oral diseases[13-17]. Previously, two Brazilian studies[5,18] undertook health literacy research in carers of older people, however, neither of them used the Health Literacy Questionnaire[6]. Instead they preferred using the S-TOFHLA[5] and the Canadian instrument called Health Literacy[18].

Australian and Brazilian researchers recently conducted a systematic review[19] to identify studies on the psychometric properties of self-report health literacy assessment instruments, focussing on studies of older people or caregivers of older people. This systematic review reported that the Health Literacy Questionnaire[6] was the best self-report health literacy tool across the various psychometric properties.

Table 1. Sociodemographic data of the carers and of the older people being cared.

	n=51	n=35 (attended T2)	n=16 (did not attend T2)
Carers mean age (SD)	52.5 (14.0)	53.5 (14.3)	50 (13.5)
Carers sex–n (%)			
Female	41 (80.4)	26 (74.2)	15 (93.7)
Male	10 (19.6)	9 (25.8)	1 (6.3)
Schooling (years)–n (%)			
1–9	15 (29.4)	9 (25.8)	7 (43.7)
10–12	20 (39.2)	13 (37.1)	6 (37.5)
≥13	16 (31.4)	13 (37.1)	3 (18.8)
Relationship to older person you care for–n (%)			
Wife	5 (9.8)	4 (11.4)	1 (6.2)
Husband	3 (5.9)	3 (8.6)	0 (0)
Son/daughter	30 (58.8)	22 (62.9)	10 (62.6)
Grandson/granddaughter	3 (5.9)	2 (5.7)	1 (6.2)
Others	10 (9.8)	4 (11.4)	4 (25.0)
Frequency of care–n (%)*			
Every day	32 (62.7)	23 (65.7)	8 (50.0)
Several times a week	10 (19.6)	9 (25.8)	2 (12.5)
At least once a week	4 (7.8)	2 (5.7)	2 (12.5)
Less often	5 (9.8)	1 (2.8)	4 (25.0)
Dwelling–n (%)			
With the older person	28 (54.9)	19 (54.3)	8 (50.0)
Visits the person they care for	22 (43.1)	16 (45.7)	7 (43.7)
Caring by telephone	1 (1.9)	0 (0)	1 (6.2)

*p<0.05 (Chi-Square); T2: Time 2.

Table 2. Retest reliability of nine Health Literacy Questionnaire in Brazilian scales.

Scales	Included only (n=35)		
	T1 M (SD)	T2 M (SD)	ICC (95%CI)
Range 1 (lowest)–4 (highest)			
1. Feeling understood and supported by healthcare providers	3.11 (0.61)	2.98 (0.41)	0.775 (0.558–0.886)
2. Having sufficient information to manage my health	2.98 (0.50)	2.87 (0.49)	0.891 (0.774–0.946)
3. Actively managing my health	2.71 (0.62)	2.60 (0.65)	0.925 (0.846–0.963)
4. Social support for health	3.05 (0.72)	2.88 (0.58)	0.808 (0.618–0.903)
5. Appraisal of health information	3.08 (0.56)	3.05 (0.41)	0.828 (0.658–0.913)
Range 1 (lowest)–5 (highest)			
6. Ability to actively engage with healthcare providers	3.74 (1.09)	3.85 (0.84)	0.864 (0.733–0.931)
7. Navigate the healthcare system	3.58 (0.87)	3.52 (0.79)	0.725 (0.452–0.861)
8. Ability to find good health information	3.82 (0.82)	3.97 (0.74)	0.791 (0.591–0.894)
9. Understand health information enough to know what to do	4.05 (0.72)	3.77 (0.91)	0.837 (0.634–0.923)

T1: Time 1; M: Mean; SD: Standard deviation; T2: Time 2.

The validation study[7] of the Health Literacy Questionnaire in Brazil did not involve older people or caregivers of older people, therefore, testing the reliability of this instrument for this population is important. We found that reliability was good or excellent in most of the nine scales of the instrument, making it a reliable instrument to be used for assisting carers of older people, especially at the time of hospital discharge of the person being cared for.

Limitations of the present study included that the study sample was smaller than the desired sample, because of 31.3% dropouts for the second assessment. However, the final sample of thirty-five did not differ from the participants who did not return for the reassessment, except on one item (frequency of care), in which those completing the second assessment required higher levels of care. In addition, the sample was recruited from inpatient clinics at a single university hospital, and so may not be representative of carers of older people associated with other hospitals, or other recruitment avenues.

CONCLUSIONS

The Brazilian version of the Health Literacy Questionnaire is a reliable tool to assess health literacy in carers of older people.

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AUTHORS' CONTRIBUTIONS

AFJ: Conceptualization, Funding acquisition, Formal Analysis, Writing – original draft. **KH:** Writing – review & editing. **CT:** Funding acquisition, Writing – review & editing. **MF:** Data curation. **AB:** Data curation. **SS:** Writing – review & editing. **EB:** Formal Analysis, Writing – review & editing.

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ORIGINAL ARTICLE
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Ankle-brachial index and subclinical atherosclerosis in type 1 diabetes

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SUMMARY

OBJECTIVE: To assess the prevalence of altered ankle-brachial index (<0.9 or >1.3) in patients with type 1 diabetes and to compare it with the presence of subclinical atherosclerosis by carotid ultrasound.

METHODS: Prospective, cross-sectional study in which 45 adults with type 1 diabetes were evaluated (age 34±10 years, 46.7% men). The data collected included anamnesis, clinical evaluation, calculation of the ankle-brachial index (relationship between systolic blood pressure in the ankle and brachial artery), and performance of carotid ultrasound.

RESULTS: Thirty-two patients had ankle-brachial index >1.3 (66.7%) and no patient had ankle-brachial index <0.9. Carotid echocardiography was performed on 21 patients, 4 (19%) of whom had atherosclerosis. Age >35 years and ankle-brachial index >1.4 showed a good correlation with atherosclerosis (r=0.49, p=0.021; r=0.56, p=0.008, respectively). A model associating age >35 years and ankle-brachial index >1.4 showed an excellent relationship with atherosclerosis (r=0.59, p=0.004).

CONCLUSIONS: Our study showed that vascular calcification (ankle-brachial index >1.4) was frequent in this population with type 1 diabetes and associated with subclinical atherosclerosis. A model combining ankle-brachial index >1.4 and age >35 years showed an excellent correlation with atherosclerosis and can assist in clinical suspicion and optimize the request for additional tests.

KEYWORDS: Vascular calcification. Ankle-brachial index. Diabetes mellitus, type 1. Atherosclerosis.

INTRODUCTION

Type 1 Diabetes (T1D) is the most common endocrinopathy in childhood and adolescence; global statistics estimate that 382 million people live with T1D and that number tends to reach 592 million in 2035.[1] The risk of cardiovascular events and neurological complications is approximately four times higher among diabetics compared to non-diabetics.[2] Atherosclerotic cardiovascular disease, especially coronary artery disease, is the main cause of mortality and morbidity in diabetes. Furthermore, T1D patients have more vascular calcification (VC) compared to non-diabetics, and the presence of calcification is related to extensive and accelerated atherosclerosis in these individuals.[3]

The Ankle Brachial Index (ABI) is normally used for monitoring atherosclerotic disease of lower limbs and reflects the reduction of systemic arterial pressure in the lower limb, as a consequence of proximal atherosclerotic obstruction. The ABI can be also used to estimate the progression and severity of

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systemic atherosclerosis.[2] Abnormal levels (ABI <0.9 and >1.3) are associated with the risk of cardiovascular events, in addition to the higher prevalence of atherosclerotic plaque.[4]

T1D is associated with an elevated incidence of atherosclerotic disease and mortality.[5] Based on this, the early identification of subclinical atherosclerosis is essential to prevent cardiovascular disease in these patients. Then, the purpose of this study was to assess the occurrence of subclinical atherosclerosis in asymptomatic T1D adults. For this purpose, subclinical atherosclerosis was assessed by ABI and confirmed with carotid ultrasound.

METHODS

Prospective, cross-sectional study in T1D patients, followed up at the Endocrinology Outpatient Clinic of the Specialized Center for Diabetes, Obesity and Arterial Hypertension of the Central Health Region of the State Health Department in Brasília – Federal District – Brazil, from February to June 2020.

Anamnesis was performed, including evaluation of previous acute myocardial infarction (MI), stroke and peripheral arterial disease; time since diagnosis of the diabetes, presence of comorbidities - dyslipidemia, systemic arterial hypertension, smoking, family history of early coronary artery disease, sedentary lifestyle (which, according to the World Health Organization, is lower than 150 minutes of moderate intensity activity, less than 3 times a week) and continuous use of medications.

Clinical evaluation was performed with anthropometric measurements (weight (kg), height (cm), body mass index (BMI, kg/m^{-2})) and physical examination. In addition, medical records were reviewed to assess laboratory tests (blood glucose (mg/dL^{-1}), total cholesterol (mg/dL^{-1}), LDL-cholesterol (mg/dL^{-1}), HDL-cholesterol (mg/dL^{-1}), triglycerides (mg/dL^{-1}) and glycated hemoglobin (%) when available up to 6 months from the date of clinical evaluation.

To calculate the ABI, the patient remained relaxed, in the supine position. The measurement of the systolic blood pressure (SBP) was performed in the posterior tibial arteries and in the brachial arteries of each limb. The SBP measurement was performed three times on each limb, with universal cuff, using automatic blood pressure monitor (OMRON, HEM 7130, Omron Health Care Brazil, São Paulo, Brazil). The ABI was obtained by simply dividing the largest SBP obtained in each artery of the lower limb by the upper limb. The ABI was considered abnormal, <0.9 (indicative of peripheral atherosclerosis) or >1.3 (indicative of vascular calcification).[6] The patients were advised to avoid caffeine intake, tobacco use or physical exercise in the last four hours before evaluation.

Subsequently, patients underwent evaluation of atherosclerosis by Doppler ultrasound, performed by a single experienced echocardiographer, without knowing the clinical and laboratory data of the study subjects. The images were obtained using a vascular ultrasound (Vivid S6 and Vivid S60 GE Medical Systems,GE Healthcare do Brasil, São Paulo, Brazil) equipped with a 7.5–10 MHz linear matrix transducer. Atherosclerosis was defined as the increase in IMT above 1.5 mm, or the presence of carotid plaque, both detected by vascular ultrasonography.[7]

The IMT was characterized by the presence, in a two-dimensional mode, of a double line with the definition of the light-intimate and medium-adventitia interfaces. The distance between the two acoustic interfaces was considered the measure of the IMT. The IMT measurement was automatically obtained on the posterior wall of the common carotids on the right and on the left, at least 1 cm proximal to the flow divider – referred to as bifurcation. The atheromatous carotid plaque was defined as a focal structure extending across at least 0.5 mm for vessel light, and/or measuring more than 50% of the value of the adjacent IMT measurement, and/or an IMT measurement greater than 1.5 mm.[7]

Sample

The research subjects should meet the criteria of having T1D and age over 18 years. Seventy-five patients with diabetes were evaluated and 45 T1D (21 men and 24 women) were included in the study. The study excluded patients with type 2 diabetes, patients with clinical manifestations of coronary artery disease (MI and stable angina), cardiovascular disease (stroke and transient ischemic attack), peripheral obstructive arterial disease (claudication), patients with aortic aneurysm and/or heart failure and those who did not consent to participate by signing the Free and Informed Consent Form.

The research was approved by the Research Ethics Committee of Health Science Teaching and Research Foundation of the Federal District (CAE nº 17592619.9.0000.5553), guaranteeing confidentiality to the participants' information, according to Resolution 196/96 of the National Health Council, despite the research involving human beings.

Statistical analysis

The statistical analysis was performed using a statistical program (SPSS 24.0, SPSS, IBM, Armonk, NY). We categorized patients according to the presence or absence of atherosclerosis on the carotid ultrasound, and the ABI value <0.9, between 0.9 and 1.3 and >1.3. Quantitative variables were described as mean±standard deviation, and categorical variables were described in absolute numbers. Student's independent t-test and Chi-square test were used to assess the differences between

the groups. Laboratory tests were not included in the statistical analysis due to the small number of tests, which could lead to a bias in the analysis, but the data are shown in Table 1.

Spearman's non-parametric correlation was used to assess the correlation of factors – age, time since diagnosis and ABI – with atherosclerosis. The Receiver Operating Characteristic Curve (ROC) was used to assess the sensitivity and specificity, for different cutoff points of the variables associated with atherosclerosis (age and ABI). Spearman's correlation was performed to assess the association of cutoff points (age >35 years and ABI >1.4) with atherosclerosis. A model associating age >35 years and ITB >1.4 was suggested and, to validate the robustness of the model, a Spearman's correlation, the area under the ROC curve analysis (AUC) and a scatterplot were performed. All p values presented will be of the bilateral type, $p<0.05$ and $0.05\leq p\leq 0.10$ will be considered significant and marginally significant, respectively.

RESULTS

The 45 T1D individuals included in the study (53.3% women) had a mean age of 34.37±10.26 years and time of diagnosis of 16.97±8.13 years. The prevalence of hypertension was 20%, dyslipidemia 8.9%, smoking 6.7% and physical inactivity 28.9%. The BMI was an average of 24.57±4.38 $kg.cm^{-2}$. No patient had an ABI <0.9, while 71.1% (32 patients) had an ABI >1.3, as described in Table 1. Carotid ultrassound was performed on 21 patients, showing atherosclerosis in 4 patients (19% of the sample), with an equal frequency between the sexes. The mean age and time since diagnosis was higher in the atherosclerosis group (Table 1).

Table 1. Clinical and Epidemiological Characteristics of Patients with Type 1 Diabetes with and without evidence of atherosclerosis on the carotid ultrasound and with an Ankle Brachial Index (ABI) of less than 0.9, between 0.9 and 1.3 and above 1.3.

	Atherosclerosis (n=21)		Ankle Brachial Index (n=45)		
	Present (n=4)	Absent (n=17)	<0.9 (n=0)	0.9–1.3 (n=13)	>1.3 (n=32)
Age (years)	44.7±13.5*	30.4±6.4*	–	35.2±10.6	32.3±9.3
Feminine gender	2	9	–	9	15
BMI (kg/m^{-2})	23.0±5.8	24.8±4.6	–	25.0±5.5	24.3±3.86
Diagnostic time (years)	23.5±8.2*	15.7±8.7*	–	16.9±7.5	17.0±8.4
Hypertension	1	2	–	5	4
Dyslipidemia	0	2	–	1	3
Smoking	4	17	–	1	2
Sedentary lifestyle	2	5	–	4	9
ABI LL	1.48±0.04*	1.31±0.22*	–	1.11±0.11*	1.52±0.22*
ABI RL	1.37±0.10	1.30±0.21	–	1.11±0.14*	1.47±0.20*
Abdominal Circumference (cm)	73.5±10.6	77.5±4.9	–	81±9.2	78.1±10.0
Fasting glucose (mg/dL^{-1})	178±5.6 (n=2)	178.3±58 (n=10)	–	158.8±12.3 (n=7)	173.5±49.6 (n=19)
Glycosylated hemoglobin (%)	7.47±0.7 (n=3)	8.25±1.9 (n=15)	–	7.81±1.6 (n=8)	7.97±1.6 (n=24)
Total cholesterol (mg/dL^{-1})	167.6±36.0 (n=3)	179.1±36.9 (n=8)	–	168.3±37.5 (n=6)	161.2±33.5 (n=19)
LDL-cholesterol (mg/dL^{-1})	86.3±37.8 (n=3)	16.1±34.2 (n=8)		96.5±33.6 (n=6)	89.3±28.3 (n=19)
HDL-cholesterol (mg/dL^{-1})	89±37.2 (n=3)	61.5±13.6 (n=8)		56.8±14.9 (n=6)	62.1±21.1 (n=19)
Triglycerides (mg/dL^{-1})	61±7.0 (n=2)	62.5±24.2 (n=7)	–	61±8.0 (n=4)	76,3±35.2 (n=18)

ABI LL: ankle brachial index in the left limbs; ABI RL: ankle brachial index in the right limbs. *Student's t test, p<0.05.

The univariate analysis of risk factors associated with atherosclerosis was positively correlated with age (r=0.43, p=0.05) (Figure 1), with a tendency to be associated with the ABI (r=0.41, p=0.07) (Figure 2), and there was no association with the time of diagnosis (r=0.33, p=0.14).

All patients who had atherosclerosis showed an ABI >1.3 (100%). Age >35 years and ABI >1.4 were the points with the best sensitivity and specificity for the presence of carotid atherosclerosis (Table 2). In the univariate analysis, age >35 years and ABI >1.4 showed an excellent correlation with atherosclerosis (r=0.499, p=0.021; r=0.56, p=0.008, respectively) (Figures 1 and 2). In sequence, a model associating age >35 years and ABI >1.4 (r=0.599, p=0.004; AUC=0.897, p=0.016) stepped up the association with atherosclerosis. In Figure 3, we illustrate the correlation between age >35 years and ABI >1.4 and the presence of atherosclerosis.

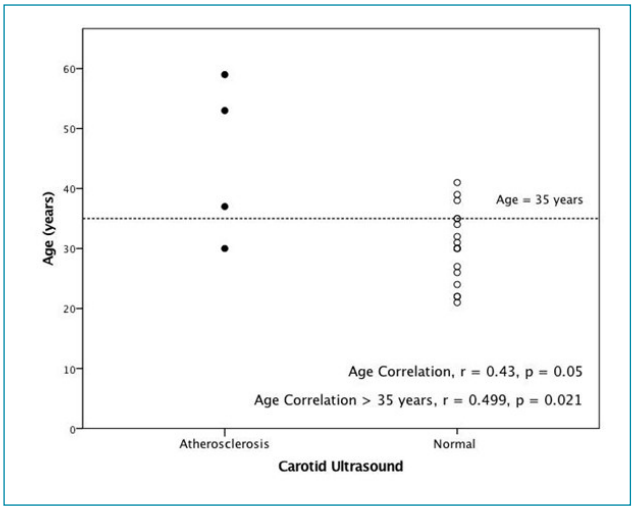

Figure 1. Scatter Plot graph of correlation between age and atherosclerosis in type 1 diabetic individuals with and without atherosclerosis by carotid ultrasound.

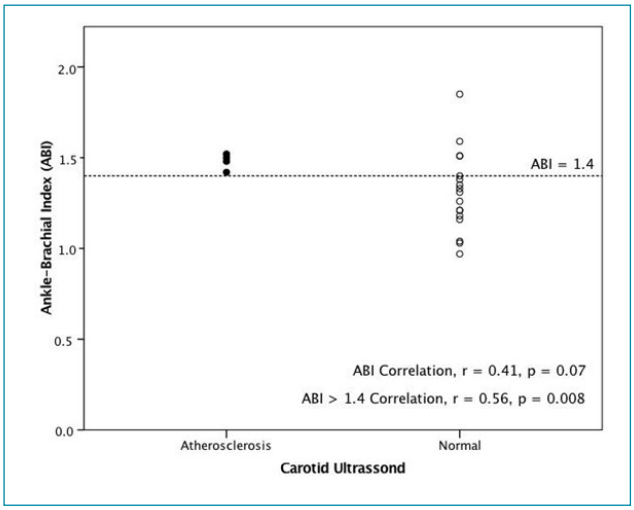

Figure 2. Scatter Plot graph of correlation between ankle brachial index (ABI) and atherosclerosis in type 1 diabetic individuals with and without atherosclerosis by carotid ultrasound.

DISCUSSION

Identifying subclinical atherosclerosis is extremely important to diagnose, treat and prevent the occurrence of cardiovascular disease (CVD) in diabetes. Our study demonstrated that a

Table 2. Evaluation of the sensitivity and specificity of different age cutoff points and ankle-brachial index in type 1 diabetic patients.

	Sensitivity	Specificity
Age (years)	(%)	(%)
30	75	59
33	75	65
35	75	83
37	50	83
40	50	99
Ankle-Brachial Index	(%)	(%)
1.32	100	53
1.34	100	59
1.36	100	65
1.39	100	71
1.41	100	77

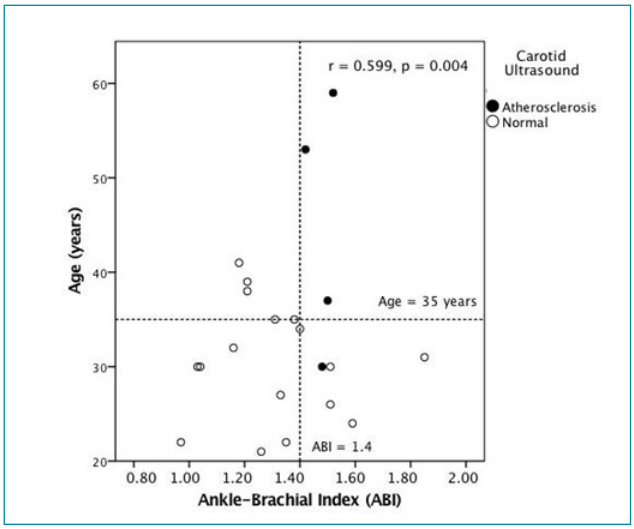

Figure 3. Scatter Plot graph of correlation between age and ankle brachial index (ABI) in type 1 diabetic individuals with and without atherosclerosis by carotid ultrasound.

simple model using age and ABI can estimate the risk of subclinical atherosclerosis in asymptomatic T1D patients with good accuracy, and the ABI can be a simple and low-cost tool that can assist in the screening of subclinical atherosclerosis.

Vascular calcification (VC) was quite prevalent in our population (66.7%) and all individuals with atherosclerosis had an ABI >1.3. There are few studies evaluating the ABI in T1D. In a cross-sectional study of 289 asymptomatic T1D adults, abnormal levels of ABI of <0.9 or >1.3 were detected in 6% and 26%, respectively. Those with abnormal ABI, 12.8%, present peripheral obstructive disease and 4.8% carotid disease.[8] Miller et al. explored relationships between an ABI >1.3 and VC in T1D and found that an ABI >1.3 is very likely to have VC.[4] Resnick et al. showed that an ABI >1.4 was an independent cardiovascular risk factor. There was a U-shaped association between ABI value and cardiovascular mortality[9]. The increased ABI also reflects coronary calcifications, suggesting diffuse atheromatous disease.[9,10] In our study, the ABI >1.4 showed a good accuracy to identify carotid atherosclerosis (Figure 2).

Our study differs from others due to the absence of an ABI <0.9, which is classically associated with atherosclerosis.[1] This factor can be attributed to the low prevalence of traditional risk factors in our population – hypertension, dyslipidemia, smoking, physical inactivity and obesity.

In diabetes, the symptoms of CVD can be atypical or silent.[1] The first manifestation can be sudden death, so early detection of atherosclerosis is extremely important, and this can be challenging in a young population. Although the IMT has been recognized as an early marker of atherosclerotic, this assessment is not routinely recommended for T1D young patients in clinical practice.[11] In our study, the association between age and elevated ABI (Figure 3) showed good accuracy and may assist in the recommendation screening for asymptomatic T1D, in addition to reinforcing the intensive control of risk factors.

Our study had some limitations. First, due to its cross-sectional design, the number of patients who were evaluated and who performed the carotid ultrasound was reduced. In addition, laboratory tests could not be analyzed, which could lead to a better design of mechanisms associated to arterial calcification and atherosclerosis. This is an initial study and more studies with larger cohorts with a higher prevalence of subclinical atherosclerosis and longitudinal trials will be needed to elucidate the clinical risk factors associated with arterial calcification and subclinical atherosclerosis. However, our study allowed us to outline a simple assessment strategy based on the patient's age and physical examination, which can be quite cost-effective for the Public Health System.

CONCLUSION

The ABI is a simple and reliable method that has proven to be efficient in estimating subclinical atherosclerosis in T1D. Vascular calcification (ABI >1.3) and the ABI >1.4 were associated with the presence of subclinical atherosclerosis in individuals with T1D. A model combination age >35 years and ABI >1.4 can be a useful and cost-effective tool to identify individuals who are most likely to have subclinical atherosclerosis and an indication to perform carotid ultrasound.

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AUTHORS' CONTRIBUTIONS

ACGBL: Conceptualization, Formal Analysis, Writing – Original Draft, Writing – Review & Editing. **MFG:** Conceptualization, Formal Analysis, Writing – Original Draft, Writing – Review & Editing. **EVR:** Conceptualization, Formal Analysis, Writing – Original Draft, Writing – Review & Editing. **LBOD:** Conceptualization, Writing – Original Draft, Writing – Review & Editing. **ANM:** Conceptualization, Writing – Original Draft, Writing – Review & Editing.

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ORIGINAL ARTICLE
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Evaluation of the American College of Radiology Thyroid Imaging, Reporting and Data System (Thyroid imaging reporting) scoring in thyroid Bethesda category on atypia and follicular lesion of uncertain significance patients

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SUMMARY

OBJECTIVE: Treatment and follow-up are controversial in patients whose thyroid fine needle aspiration biopsy (FNAB) is reported as atypia of undetermined significance and follicular lesion of uncertain significance (AUS/FLUS). We aimed the efficacy of the American College of Radiology Thyroid Imaging, Reporting and Data System (ACR TI-RADS) in preventing unnecessary thyroidectomies in patients with FNA cytology results as AUS/FLUS.

METHODS: In Bolu Abant Izzet Baysal University General Surgery Clinic, case series between 2017 and 2020 were analyzed with thyroid operated. Grouping was made according to the result of postoperative pathology: those with benign results after postoperative pathology were classified as Group 1, and those with malignant results after postoperative pathology were classified as Group 2.

RESULTS: As a result, 66 patients were found to be AUS/FLUS. A total of 28.8% of AUS/FLUS patients have been determined with cancer. In the statistical analysis of the ACR TI-RADS score between the groups, the ACR TI-RADS score in Group 1 patients (3.36) (SD 0.87) was significantly lower than that in Group 2 patients (4.11) (SD 1.04) (p=0.003). The distribution of the ACR TI-RADS scores of the patients in Group 2 was TR2: 2 (15.4%) patients, TR3: 3 (25%) patients, TR4: 5 (16.1%), TR5: 9 (90%) patients, respectively.

CONCLUSION: The ACR TI-RADS score was statistically significant in predicting malignancy in AUS/FLUS patients whose follow-ups and treatments are controversial, and the ACR TI-RADS has a limited role in preventing unnecessary thyroidectomies in patients with AUS/FLUS.

KEYWORDS: Thyroid nodule. Thyroid neoplasms. Ultrasonography. Biopsy, fine-needle. Data systems.

INTRODUCTION

The incidence of thyroid cancer is increasing worldwide, and it is the most common cancer observed in the endocrine system[1]. An excellent prognosis is achieved with early diagnosis and surgery in thyroid malignancies[2]. Fine needle aspiration (FNA) cytology is a safe method that prevents unnecessary surgical procedures for a benign nodule[3,4]. In 2007, The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) was proposed[5]. In TBSRTC, the third category has been defined as atypia of undetermined significance and follicular lesion of uncertain significance (AUS/FLUS) (BETHESDA 3). This category is a group of highly heterogeneous thyroid lesions with a high limit of cellularity. The "AUS/FLUS" category in TBSRTC caused controversy. The cases in this heterogeneous group include FNA

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cytology results that are not as benign as can be clearly reported and are not as atypical as those in the Bethesda 4 and Bethesda 5 categories[6]. In the literature, it has been stated in the thyroid FNA cytology reports that the rate of the use of AUS/FLUS is 2.1–18%[7]. In this group, the rate of malignancy in the pathological results of patients operated on varies between 6 and 48%[8]. Uncertainty in predicting the risk of malignancy in AUS/FLUS leads to confusion in patient management[9].

It is desired that ultrasonography (USG) is a noninvasive method that shows the possibility of malignancy in thyroid nodules in the confidence interval of FNA cytology. In a recent study, the most specific features for thyroid carcinoma were stated to be microcalcifications, irregular borders, and changes in anteroposterior (AP)/transverse diameter[10]. Using these features, the Thyroid Imaging, Reporting and Data System (TI-RADS) terminology was first used by Hovarth and his colleagues to determine the risk of malignancy by USG[11]. In addition, the recently implemented guidelines of the American College of Radiology (ACR) regulate the clinical management of patients with thyroid nodes with USG findings, and the guidelines help clinicians decide whether FNA cytology is required. In this approach, nodules with an ACR TI-RADS score lower than 3 are classified as benign (TR1)/nonsuspect (TR2), and biopsy is not recommended. In addition, if mild suspect nodules with an ACR TI-RADS score of 3 (TR3) are less than 25 mm, the biopsy is not recommended, but the follow-up is recommended for nodules larger than 15 mm in this category[12].

In this study, we investigated the efficacy of the ACR TI-RADS in preventing unnecessary thyroidectomies in patients with AUS/FLUS identified using FNA cytology results. Besides, we investigated the reliability of the ACR TI-RADS score in patients with AUS/FLUS.

METHODS

In Bolu Abant Izzet Baysal University (B.A.I.B.U) General Surgery Clinic, case series between 2017 and 2020 were analyzed with thyroid operated. Preoperative USG, thyroid FNA cytology results, and postoperative pathological results were examined. The preoperative thyroid FNA cytology results, the patients who were operated on, and the preoperative USG results containing data (i.e., composition, echogenicity, AP/Transverse diameter, margin, and echogenic foci) suitable for ACR TI-RADS scoring were evaluated. Patients without preoperative FNA cytology results and ACR TI-RADS scoring in USG were excluded from this study. The ACR TI-RADS scores were obtained by calculating the ACR TI-RADS score of the nodule with the highest score in USG. The FNA biopsy (FNAB) was repeated in patients whose FNAB results were presented as AUS/FLUS, and when these results were the same, the patients were scheduled for thyroidectomy, The patients whose control FNAB results were reported as AUS/FLUS and who performed thyroidectomy were included in this study. The postoperative pathological results were obtained. Thyroid USGs were performed by two radiologists who were blinded to the study. Grouping was made according to the result of postoperative pathology: those with benign results after postoperative pathology were classified as Group 1 and those with the malignant result after postoperative pathology were classified as Group 2. Patients were grouped based on histology. The intent is to predict malignancy with TI-RADS.

Statistics

Whether variables were normally distributed or not was checked by the Kolmogorov–Smirnov test. While applying a *t*-test for variables with normal distribution, the Mann–Whitney *U* test was used for the analysis of variables that do not show normal distribution. A crosstabs test was used to correlate between the ACR TI-RADS groups and its subgroups. The χ^2 test was used for gender analysis. All the analyses were performed with the Statistical Package for Social Sciences 25.0 for Windows (SPSS Inc., Chicago, IL, USA), and the results with a level of $p<0.05$ were considered to be statistically significant.

RESULTS

Preoperative USG was performed in accordance with the ACR TI-RADS scoring. A total of 66 patients with preoperative FNA cytology and AUS/FLUS were included in this study. When the patients were grouped according to the result of postoperative pathology, there were 47 patients in Group 1 and 19 patients in Group 2. The classification of the patients in the groups according to the postoperative pathological results is shown in Table 1. Of the 259 patients with preoperative FNA cytology, the results of 66 patients were found to be AUS/FLUS. This constitutes 25.48% of all biopsy results. This rate is higher than the rates in the literature (2.2–18%).

In the statistical analysis of the age values of 66 patients with AUS/FLUS according to the FNA cytology, the age of the patients in Group 1 was 47.51 (SD 9.54) and the age of the patients in Group 2 was 50.26 (SD 9.74) (p=0.96). When the gender distributions are examined, there was no statistically significant difference (p=0.26).

In the statistical analysis of ACR TI-RADS score between the groups, the ACR TI-RADS score (3.36) (SD 0.87) in Group 1 patients was significantly lower than the ACR TI-RADS score (4.11) (SD 1.04) in Group 2 patients (p=0.003) (Table 2). It was observed that there were statistical differences between Group 1 and Group 2 in composition (p=0.007), echogenicity (0.009), and

margin (p=0.009) subgroups that form the basis of ACR TI-RADS scoring. However, there were no statistical differences between Group 1 and Group 2 in shape (p=0.165) and echogenic foci (p=0.935) subgroups that form the basis of ACR TI-RADS scoring.

The distribution of patients according to TI-RADS groups is as follows: TR2 had 13/66 (19.7%) patients, TR3 had 12/66 (18.2%) patients, TR4 had 31/66 (47%) patients, and TR5 had 10/66 (15.2%) patients. It was observed that the most patients were in the TR4 group and the least patients were in the TR5 group. Comparing the groups and ACR TI-RADS scores of 66 patients with Bethesda 3 scores, ACR TI-RADS distributions, and numbers of patients, respectively, TR2 had 11 (84.6%) patients in Group 1 and 2 (15.4%) patients in Group 2; TR3 had 9 (75%) patients in Group 1 and 3 (25%) patients in Group 2; TR4 had 26 (83.9%) patients in Group 1 and 5 (16.1%) patients in Group 2; TR5 had 1 (10%) patient in Group 1 and 9 (90%) patients in Group 2 (Table 3). Cancer rates are very close between TR2 (15.4) and TR4 (16.1) due to the high number of benign patients in the TR4 group.

When the ACR TI-RADS scores of 19 patients with malignant AUS/FLUS were evaluated according to the results of the postoperative pathology, no biopsy should have been performed in two patients in the TR2 category. According to the ACR TI-RADS algorithm, biopsies should not have been performed for three patients in the TR3 because the nodule sizes were below 2.5 cm. In the TR4 category, two patients whose nodule size was less than 1.5 cm should not have been biopsied and USG follow-up should have been performed. Thus, seven patients could not be diagnosed with thyroid cancer. In the TR5 category, FNAB was required according to the ACR TI-RADS system because the nodule sizes of all patients were above 1 cm.

Table 1. Distribution of patients by pathological results.

Groups	Postoperative pathological results	n
Group 1	Nodular adenomatous hyperplasia	17
	Follicular hyperplasia	8
	Nodular colloidal goiter	6
	Adenomatous nodule	4
	Lymphocytic thyroid	4
	Follicular adenoma	3
	Subacute lymphocytic thyroid	1
	Hurthle cell adenoma	2
	Hashimoto thyroiditis	1
	Cavernous hemangioma	1
Group 2	Papillary thyroid carcinoma	10
	Minimal invasive follicular carcinoma	1
	Papillary microcarcinoma	7
	Noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP)	1

DISCUSSION

The follow-up and treatment of the results in the AUS/FLUS category are still controversial. While the average usage rate of AUS/FLUS ranges between 2.1 and 18% of all thyroid FNABs, the postoperative malignancy rate was reported to vary between 6 and 18% in AUS/FLUS[7,8].

It is aimed to exclude cases that do not clearly exhibit benign features with the AUS/FLUS category. AUS/FLUS is a heterogeneous category with various abnormal features, but it is assumed to be at low risk of malignancy. It also includes samples with cellularity on the limits/low quality. Nodules with AUS/FLUS are often resected if suspicious clinical and USG features are present and there are abnormal results in recurrent FNA cytology. Resection is not performed in patients who are reported as benign in recurrent FNA cytology[13]. The USG of thyroid nodules is the most effective diagnostic tool to predict malignancy and select lesions that require further evaluation[14]. The USG–FNA cytology is the most cost-effective procedure that provides useful diagnostic information about advanced

Table 2. Test results of American College of Radiology Thyroid Imaging, Reporting and Data System and subgroups.

	Group 1 (n=47)	Group 2 (n=19)	p-value
TI-RADS	3.36 SD 0.87	4.11 SD 1.04	0.003
Composition	1 (0–2)	1 (1–2)	0.007
Echogenicity	0 (0–3)	3 (0–3)	0.009
Shape	0 (0–2)	0 (0–2)	0.165
Margin	0.060 (0.03–0.15)	0.057 (0.03–0.10)	0.009
Echogenic foci	0 (0–3)	0 (0–3)	0.935

TI-RADS: Thyroid Imaging Reporting and Data System.

Table 3. Comparison of groups with American College of Radiology Thyroid Imaging, Reporting and Data system.

TI-RADS	% of total patients	Patients					
		Group 1		Group 2		Total	
		n	(%)	*n*	(%)	*n*	(%)
Tr2	13/66 (19.7)	11	84.6	2	15.4	13	100
Tr3	12/66 (18.2)	9	75.0	3	25.0	12	100
Tr4	31/66 (47)	26	83.9	5	16.1	31	100
Tr5	10/66 (15.2)	1	10	9	90	10	100

TI-RADS: Thyroid Imaging, Reporting and Data System.

clinical management[15]. The clinical and practical purpose of this diagnostic tool is to reduce the number of unnecessary surgical procedures in patients with benign nodules and to identify people at high risk of malignancy. While 47 of the 66 patients with AUS/FLUS in our study were benign as a result of postoperative pathology, 19 (28.78%) of the patients were reported as malignant. The rate of 28.78% that we reported was found to be higher than the rate of 6–18%[7] AUS/FLUS malignancy, which is stated in the world literature.

There are many guidelines regarding the USG evaluation of thyroid nodules[16]. Several USG-based methods were developed to classify the risk of malignancy of thyroid nodules in adults. Two of these methods are Kwak TI-RADS and ACR TI-RADS (which is officially stated by the ACR in 2017 for the management of thyroid nodules)[12]. ACR TI-RADS is based on evaluating the USG properties of nodules in five categories (i.e., composition, echogenicity, shape, shape, and echogenic foci) where each category is given 0–3 points. While each of the features in the first four categories has a single score derived from exclusive choices, there may be more than one feature in the echogenic foci category. In ACR TI-RADS, the scores are given for all USG features in a nodule, i.e., the more suspicious features get higher scores. In other words, the possibility of malignancy associated with each feature is weighted and considered differently. Along with the maximum diameter of the nodule, the TR level recommends that FNA cytology, follow-up by USG, or any other procedure should not be performed to the patient.

Total scores determine the level of ACR TI-RADS of the nodule, ranging from TR1 (benign) to TR5 (suspicious of high malignancy)[12]. Compliance with ACR TI-RADS will result in less benign nodule biopsy, as the threshold diameters for mild and moderately suspect nodules (TR3 and TR4) are larger than other systems. However, it will result in less malignant nodule cytology. Therefore, ACR TI-RADS recommends observation and following of some nodules that do not meet the size criteria for FNA cytology[17].

In our study, we showed that ACR TI-RADS was statistically significant in predicting malignancy in patients with AUS/FLUS. We concluded that unnecessary surgery can be prevented by the ACR TI-RADS assessment in patients with AUS/FLUS. Although it was statistically significant, when examined proportionally, it was observed that only TI-RADS appeared as a robust tool in predicting malignancy with only the TR5 group (90%), but this was not valid for other TR groups. Consequently, it was concluded that seven patients with AUS/FLUS, whose postoperative results were reported as malignant, could not be diagnosed. This number constitutes a high rate of 36.8% of 19 patients with AUS/FLUS who were malignant according to the results of pathology.

All of the patients were operated on by evaluating preoperative FNAB. This study has two limitations. One is that this is a retrospective study, and the other is that the number of cases is limited in our study.

CONCLUSION

The ACR TI-RADS score was statistically significant in predicting malignancy in AUS/FLUS patients whose follow-ups and treatments are controversial, and ACR TI-RADS has a limited role in preventing unnecessary thyroidectomies in patients with AUS/FLUS.

AUTHORS' CONTRIBUTIONS

OC: Conceptualization, Data curation, Formal Analysis, Writing – original draft. **MS:** Writing – original draft, Writing – review & editing. **BO:** Data curation, Formal Analysis. **HY:** Data curation.

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Coronavirus disease 2019 pandemic and anxiety: a longitudinal study in 287 Brazilians

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SUMMARY

OBJECTIVE: The aim was to study the prevalence of symptoms of anxiety and post-traumatic stress in a sample of Brazilians and their relationship with sex, age, and work situation, and compare if these symptoms change with 8 weeks of quarantine.

METHODS: Online survey to collect epidemiological data and apply the Beck Anxiety Inventory (BAI) and Impact of Event Scale-Revised (IES-R). Eight weeks later, the researchers requested the same requirements to complete the BAI and IES-R and compare the results.

RESULTS: The sample of 287 answered the first and second questionnaires, being 72.8% women, with a median age of 22 years. In the first interview, the median BAI was 12 (7–19) and the median IES-R was 27.0 (15–40); in the second, the median BAI was 11 (6–22) and the IES-R was 30 (15–41) with $p<00001$ and 0.09, respectively. Anxiety levels were worse in females ($p<0.0001$ for both BAI and IES-R) and in those who worked/studied in the area of health ($p=0.001$ for BAI and 0.01 for IES-R). There was a negative correlation between age and anxiety ($p<0.0001$ for BAI and IES-R).

CONCLUSIONS: A high prevalence of anxiety that lowered after 8 weeks were found. Anxiety was worse in females, in younger people, and in those who worked/studied in the area of health.

KEYWORDS: COVID-19. Pandemics. Quarantine. Anxiety.

INTRODUCTION

In March 2020, the World Health Organization (WHO) recognized the coronavirus disease 2019 (COVID-19) outbreak as pandemic[1]. No drug can be considered efficient against this infection nor has an effective vaccine been developed until now. So, to avoid the infection spreading, social distancing and quarantine have been implemented, bringing segregation, rupture of previous habits, and economical losses. In this context, additional stress can be triggered by a fear of contamination and anxiety about the loss of one's own life or that of loved ones.

The infections have been identified first in China at the end of 2019, and investigators have found a reduction in positive emotions and life satisfaction and an increased rate of depression, anxiety, and even suicidality in this population during the period of isolation[2-4].

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The same pattern has been observed in other regions. A German study showed generalized anxiety in 44.9%, depression in 14.3%, and psychological distress in 65.2% of the study sample which were found to be more common in women and younger people[5]. Another study conducted in the USA among people aged from 18 to 30 years showed high levels of depression (43.3%), high anxiety scores (45.4%), and high levels of post-traumatic stress disorder (PTSD) symptoms (31.8%) which were less frequent in Asian Americans than in whites[6]. They also observed that anxiety was less likely to be reported in Hispanics/Latinos[6]. A large Iranian study showed that anxiety was higher among women, among people who followed corona-related news closely, among those in the age group of 21–40 years and those who have had a relative with the disease[7]. A systematic review by Xiong et al.[8] corroborated the findings of high levels of anxiety, depression, PTSD, psychological distress, and stress in several countries (i.e., China, Spain, Italy, Iran, USA, Turkey, Nepal, and Denmark), bringing attention to the fact that unemployment, student status, and presence of chronic/psychiatric illnesses should be added to the previously mentioned associated risk factors.

An interesting observation, comparing the physical and mental health of citizens living in countries with an opposite point of views about wearing face masks (China that encouraged face masks and Poland that discouraged them), showed that the infrequent use of face masks was associated with more physical symptoms, more frequent medical consultation, COVID-19 testing, hospitalization, and also deterioration of emotional status. This study highlights the importance of health education not only in preventing the disease from spreading but also in reducing its psychological impact[9].

Additionally, it was noted that there was an increase in the post-traumatic stress symptoms when quarantine exceeded 10 days[10].

The health care workers who were quarantined may have more significant symptoms of post-traumatic stress than people in other work areas. The COVID-19 pandemic has placed a significant strain on health care institutions that were required to allocate all the efforts and resources in appropriated protective equipment and minimize non-essential services, and in the front-line health care staff exposed to the disease and the psychological adversities brought by patient's care. Chew et al.[11], studying 1,146 individuals, observed that health care workers not medically trained were particularly vulnerable to psychological adversities. Besides, health care workers who experienced physical symptoms had worse psychological outcomes than those who did not. Some of the known reasons are fear of spreading the infection to colleagues and family members and the dilemma of applying for sick leave in an already strained area. In this context, the health care worker may develop fear, stigmatization, and feeling of exclusion while displaying the physical indicators of virus infection[12].

Not only health care workers have been affected by the COVID-19 pandemic but also others in whom the social isolation constrained their jobs and brought income reduction and recession[13]. Two-third of the 1,423 respondents involved in a cross-sectional study from Vietnam reported an important reduction in their quality and quantity of working which was more accentuated among females[13].

Returning to work during the COVID-19 epidemic is another situation that may be associated with psychological effects. About 10.8% of responders of 673 questionnaires in China showed PTSD without significant differences between technicians/workers and executive/managers. Anxiety (3.8%), depression (3.7%), stress (1.5%), and insomnia (2.3%) were similarly detected although at a lower rate. Factors associated with the severity of psychiatric problems were the presence of physical symptoms, poor physical health, considering the return to work as a health hazard, and marital status[14].

Resilience to stress may suffer from cultural and social influences. Therefore, it may diverge according to the study population. Herein, we studied a sample of Brazilian patients aiming to know the prevalence of anxiety and PTSD and if the persistence of quarantine would change this prevalence.

METHODS

This study was approved by the local Committee of Ethics in Research under protocol 30725520.7.0000.0103. This is a prospective study based on an Internet questionnaire. Electronic informed consent was obtained before answering the questions. The first questionnaire was applied from March 20 to 30 through Google forms announced on Facebook, Instagram, and WhatsApp groups and was intended for individuals aged 18 years or above. Data collection included epidemiological information, questions on social distancing and quarantine, the Beck Anxiety Inventory (BAI)[15], and Impact of Event Scale-Revised (IES-R)[16].

The IES-R is a self-application instrument for screening the symptomatology of PTSD which demonstrates good discriminating validity and diagnostic utility and can be used in any stage (acute, chronic, and late) of the development of symptoms. It uses a Likert scale on which an individual answers the questions based on 7 days before the application of the questionnaire. The scale has 22 items distributed across 3 subscales (i.e., avoidance, intrusion, and hyperstimulation) that contain the criteria for evaluating PTSD available in DSM-IV. The score for each question ranges from 0 to 4 points and the calculation

of the score of each subscale is obtained through the average of the items; the total score is the sum of the scores of the subscales. According to this instrument, PTSD can be classified as follows: 0–23 as absent, 24–32 as mild, 33–36 as moderate, and >37 as severe psychological impact[16].

IES-R has been used and previously validated to measure post-traumatic disorders symptoms during the COVID-19 pandemic in several countries[17-19].

The Beck anxiety scale or Beck inventory is a self-report questionnaire with 21 multiple choice questions, which is used to measure the severity of an individual's anxiety. These questions are about how the individual has felt in the previous week and it is expressed as common symptoms of anxiety (such as sweating and feelings of anguish). Each question has four possible answers, and the one that most closely resembles the individual's mental state should be signaled. The instrument has a maximum score of 63 points, and the participants are classified according to the degrees of anxiety: minimum: 0–10, mild: 11–19, moderate 20–30, and severe: 31–63[15].

Patients who participated in this survey were contacted again after 8 weeks and invited to answer again the IER-S and Beck inventory to verify if the symptoms have changed with isolation time.

Obtained data were analyzed in tables of frequency and contingency. Data distribution was studied by using the Shapiro–Wilk test. The Mann–Whitney *U* test was used to compare two numerical data (i.e., Beck inventory and IES-R according to epidemiological variables). The Wilcoxon test was used to compare two paired numeric data (comparison of two results of Beck inventory and two results of IES-R). The chi-squared test was used to compare the categorical values of Beck and the categorical values of the IES-R between the two evaluations. The correlation of age with Beck inventory and results of IES-R was done by using the Spearman's test. The adopted significance was 5%. Tests were calculated using GraphPad Prism® version 6.01 software.

RESULTS

1. Results of the first evaluation:

The survey was answered by 287 individuals. Their epidemiological data are shown in Table 1.

In this sample, the mean result of the Beck inventory was 12 (range 0–46; IQR 6–22). According to this instrument, 121/267 (42.1%) had minimal anxiety, 78/287 (27.1%) had mild anxiety, 56/287 (19.5%) had moderate anxiety, and 31/287 (10.8%) had severe anxiety.

The median IES-R was 30.0 (range 0–76; IQR 15–41). In 117/287 (40.7%), PTSD was absent; in 56/287 (19.5%), PTSD was mild; in 23/287 (8.0%), PTSD was moderate, and in 92/287 (32.0%), PTSD was severe.

Table 1. Epidemiological profile of the study sample.

Sex – male/female	78/209 – 27.1/72.8%
Median age – years (IQR)	22.0 (20.0–25.0)
Working/studying in health area (%)	96/287 (33.4)
Stayed on quarantine (%)	266/287 (92.6)

IQR: interquartile range.

According to gender, the comparison of BAI scores and IES-R showed that males had a median value of 7.5 (IQR 3.0–14.0) and females had a median value of 14 (IQR 8.0–25.0) with p<0.0001; the median IES-R in males was 22.0 (IQR 7.0–31.2) and in females it was 32.0 (IQR 18.0–44.0), with p<0.0001.

The comparison of the results of Beck anxiety inventory in those who were working/studying in the health area was 14.0 (IQR 8.0–24.0) and in those who were not working in this area was 10.0 (IQR 4.0–15.0) with p=0.001. The IES-R in those who were related to the health area was 31.0 (IQR 17.0–42.0) and in those who were not related to the health area was 23.0 (13.2–34.0) with p=0.01.

The comparison of BAI in those who were keeping quarantine had a median value of 13.0 (IQR 7.0–23.0) and in those who were not keeping it had a median value of 6.0 (IQR 2.5–11.5), with p=0.0003. The median IES-R in those who were on quarantine was 30.0 (IQR 17.0–41.0) and in those who were not keeping it was 12.0 (IQR 6.5–24.5), with *p*=0.0001.

A correlation study between age and Beck inventory results showed p<0.0001 (rho=–0.24; 95%CI –0.35 to –0.12). The correlation of age with IES-R also showed p<0.0001 (rho=–0.22; 95%CI –0.33 to –0.10).

2. Results of the second evaluation:

During the period between the two evaluations, 239/287 (83.2%) kept the quarantine, 8/287 (2.7%) who were not on quarantine previously entered in isolation, 27/287 (9.4%) left the quarantine, and 13/287 (4.55) who were not on isolation in the first evaluation continued not keeping isolation.

The comparison of Beck anxiety inventory between the first and second visits is shown in Figure 1 in which the second evaluation showed lower levels.

The comparison of IES-R in the two evaluations is shown in Figure 2.

The variation of scores in the Beck inventory between the second and first evaluation (delta Beck) had a median value of

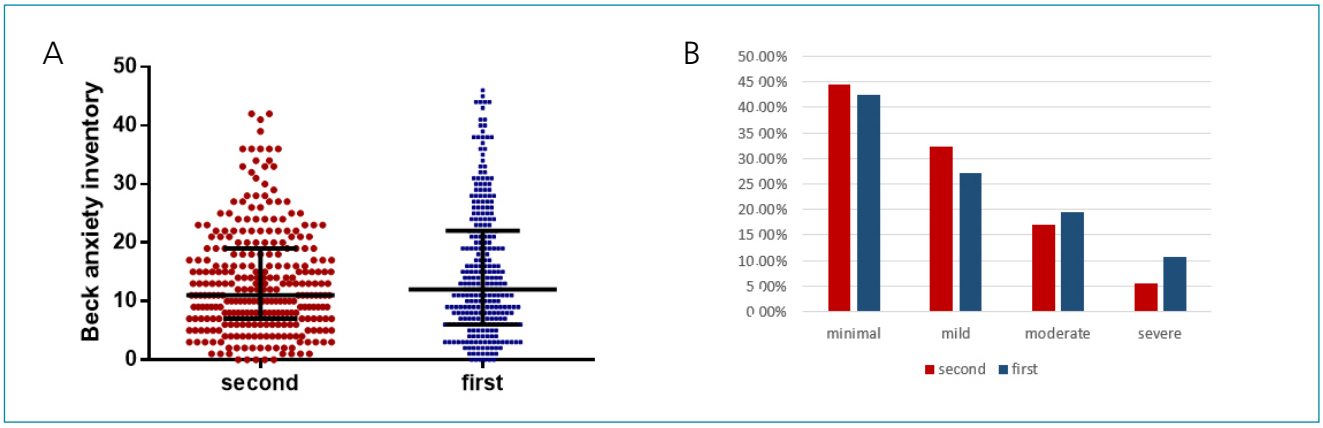

Figure 1. Comparison of Beck anxiety inventory between second and first evaluation. (A) Numeric values: p<0.0001; second evaluation: median value of 11.0 (IQR 7–19); first evaluation: median value of 12.0 (IQR 6.0–22.0). (B) Categorical values: p=0.08; second evaluation: minimal=44.5%, mild=32.4%, moderate=17.0%, and severe=5.5%; first evaluation: minimal=42.5%, mild=27.1%; moderate=19.5%, and severe=10.8%. IQR, interquartile range.

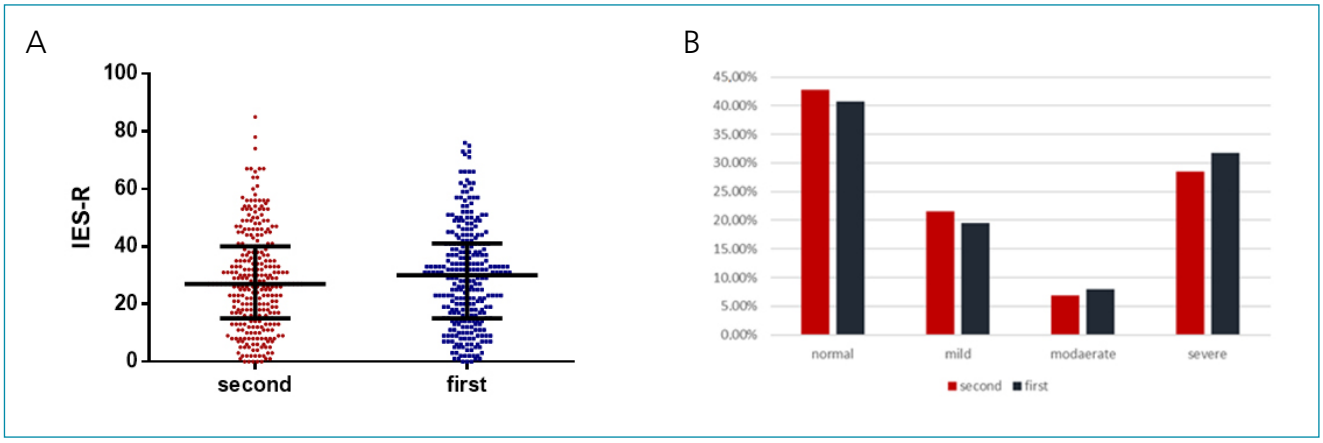

Figure 2. Comparison of IES-R (Impact of Event Scale-Revised) between the two evaluations. (A) Numeric values p=0.09; second evaluation: median values of 27.0 (IQR 15.0–40.0); first evaluation: median values of 30.0 (IQR 15.0–41.0). (B) Categorical values: p=0.76; second evaluation: normal=42.8%, mild=21.6%, moderate=6.9%, and severe=28.5%; first evaluation: normal=40.7%, mild=19.5%, moderate=8.0%, and severe=31.7%. IQR, interquartile range.

–1.0 (IQR from –5.0 to +2.0); the median variation of IES-R (delta IES-R) was –1.0 (IQR –8.0 to +6.0).

The delta Beck and delta IES-R did not change according to gender (p=0.10 and p=0.11, respectively) and according to keeping quarantine at second visit (p=0.63 for Beck inventory and *p*=0.28 for IES-R).

The IES-R did not change if the individual was working/studying in the health area (p=0.19) but the Beck inventory had a significant decrease as follows: median value of 0 (IQR –2.7 to +4.0) for those who were not working in the health area and –2.0 (IQR –7.0 to +2.0) for those who were working in the health area, with p<0.0001.

A small and positive correlation was found between age and delta Beck (rho=0.14; 95%CI 0.02–0.25; *p*=0.01) but not with delta IES-R (rho=0.01; 95%CI –0.10 to +0.12; p=0.83).

DISCUSSION

Our results have shown that almost half of the study sample had some degree of anxiety and suffered some amount of psychological impact as measured by IES-R with the COVID-19 pandemic. The results were worse in females, in those who were working/studying in the health area, and in those who were keeping quarantine. We also noted that Beck inventory and IES-R score had a negative correlation with age, being worse in

younger people. Interestingly, the second evaluation which was performed 8 weeks later showed some degree of improvement. Gender and keeping quarantine did not influence the observed changes over time but the BAI improved in those with higher age and those who were working/studying in the health area.

Finding worse results for female individuals was also observed in other surveys done in Germany, China, and Iran [2,5,7]. In these three studies, similar to our results, younger people had more anxiety, despite the results unknown for the severity of COVID-19 infection in older people with co-morbidities[1].

Females show more anxiety in general[11]. Although it is difficult to separate social and environmental factors from hormonal factors that can contribute to this preference, there are some suggestions that gonadal hormones may influence mental health. Animal studies in mice at puberty suggest that testicular hormones may have anxiolytic effects in males which are not consistent with the role of ovarian hormones in increasing anxiety[20].

Individuals who were linked to the area of health showed worse outcomes. A better knowledge of the infectious process spreading and of the possible consequences of the infection may lead to a more realistic point of view, contributing to higher levels of anxiety. Fear of contamination in the work/study area may also have contributed to these results. However, it was this group of individuals who showed some improvement in the anxiety levels in the second evaluation. Growing information about the pathophysiology and treatment of the disease overcoming the initial contradictory information overload may have contributed to this improvement. So, a positive emotion regulation may have occurred. However, it is important to note that only the BAI improved but not the IES-R. IES-R is an instrument with better discriminating validity and diagnostic utility for PTSD that can be considered a subtype of anxiety[21].

A Chinese study noticed that there was a decrease in the IES-R score after 4 weeks when compared with the first assessment that was carried out during the beginning of the COVID-19 pandemic, which is different from the results obtained in this study[22].

This study has the following limitations. It is an Internet survey so that it reaches only people with computer resources and may not include individuals with lower income and people in rural areas,[23] creating a selection bias. It also has a limited period between the two interviews. However, it shows the big impact of the COVID-19 pandemic on the mental status of the study sample. This aspect should not be neglected as it may lead to further social and economic losses. Measurements to reduce this impact are accepted although it is not easily taken. Physical distancing does not mean emotional distancing and the use of all communication resources should be stimulated to reduce the feeling of loneliness and helplessness imposed by quarantine. Exercises and meditation are nonpharmacological interventions known to have some positive effects on the anxiety symptoms[24] and may be of help in selected individuals. Telemedicine through telephone or computer networks has offered an alternative option for doctor–patient communication[25], including online psychotherapy that has been shown a cost-effective way to reduce psychological symptoms[26]. Internet cognitive behavioral therapy has already been successfully used in other conditions such as insomnia[27], chronic pain situations such as rheumatoid arthritis[26], and in the treatment of comorbid depressive symptoms among individuals who have PTSD [27].

CONCLUSIONS

A high prevalence of anxiety in the study sample was found. Females, younger people, and people who were working/studying in the area of health are associated with worse results. The level of anxiety showed a small reduction over time.

AUTHORS' CONTRIBUTIONS

ACMS, AZM, PZM, RAW, and RNYK: Conceptualization, Data Curation, Methodology, Writing – Original Draft. **TLS:** Data Curation, Investigation, Writing – Review and Editing.

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Some inflammatory markers and chest computerized tomography in patients with severe acute respiratory syndrome coronavirus infection

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SUMMARY

OBJECTIVE: We retrospectively assessed whether there was a relationship between lung complications and some easily accessible markers to predict the presence of pulmonary consolidation in patients with coronavirus disease 2019 (COVID-19).

METHODS: According to the polymerase chain reaction and chest computerized tomography results, the study was categorized into three groups. Group 1 (n=87) included the patients with polymerase chain reaction (+), group 2 (n=55) included the patients with polymerase chain reaction (-) and chest computerized tomography (+), and group 3 (n=77) included the patients with polymerase chain reaction (-) and chest computerized tomography (-), respectively.

RESULTS: High-sensitivity C-reactive protein and increased age were associated with higher computerized tomography (CT) scores.

CONCLUSION: Increased age and C-reactive protein (CRP) may suggest pulmonary infiltration on chest CT in patients with COVID-19.

KEYWORDS: Coronavirus disease 2019. X-ray computed tomography. Diagnosis. C-reactive protein.

INTRODUCTION

The novel coronavirus, severe acute respiratory syndrome coronavirus (SARS-CoV-2), had an epidemic potential in 2019 and finally spread all over the world, with its status changing to a pandemic[1,2]. Many patients with pneumonia face intubation with a death risk. After serial autopsy reports, the clinical and pathological effects of the disease have become clear[3,4]. One of the main problems was a hyperactive-immune response that propagates lung dysfunction and atheroembolic events[4-5]. For this reason, many studies have been conducted to detect real pathophysiological problems.

Infections and inflammatory events cause some systemic reactants to be released, like CRP, procalcitonin, transferrin, into the body within hours or days. Since patients with COVID-19 pneumonia have a secondary infection, especially after ventilatory use, the possibility of lung disease should be considered[6]. These acute-phase reactants can be further increased in case of secondary infection.

Hu et al. have firstly described a new marker called systemic serum inflammatory index (SII) for prognosis prediction in hepatocellular carcinoma[7]. Subsequently, especially in cancers, other studies about the superiority of this index superiority compared to other indices, such as neutrophil-lymphocyte ratio, (NLR) were published[8,9]. However, a heated debate broke out about which index was the most appropriate. Although the systemic inflammatory burden of COVID-19 in patients

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with pneumonia is well-known, it has not been investigated in the literature as to whether NLR and SII correlate with lung involvement levels, and other clinical and laboratory parameters in patients with COVID-19 infection.

The common problem associated with these markers is whether they are specific or always correlate with disease severity[10-12]. This study, therefore, aimed to assess whether there is an association of lung involvement severity resulted from COVID-19 with NLR, SII, and other inflammatory markers, such as high-sensitivity C-reactive protein (Hs-CRP), procalcitonin, or ferritin, which are easy to use in practice.

METHODS

Study design and protocol

After having been approved by the local Ethics committee, the study was retrospectively conducted in patients who were admitted to the emergency department and treated after a diagnosis of SARS-CoV-2 from April 2020 to August 2020. The diagnosis and treatment of COVID-19 followed the COVID-19 Management Guidelines of the National Science Commission of Turkey.

A total of 219 consecutive patients were admitted with pre-diagnosed SARS-Cov-2. All samples for real-time polymerase chain reaction (RT-PCR) assays were obtained from a nasopharyngeal swab. All patients were detected by RT-PCR samples and chest computerized tomography (CT). In the study, group 1 included 87 consecutive SARS-Cov-2 patients who were diagnosed with RT-PCR that was done by using commercial kits (SARS-Cov-2 Double Gene RT-PCR kit, Bioksen R&D Technologies Ltd., Istanbul, Turkey) with endorsement by the Turkish Health Minister. According to the manufacturer, the analytical sensitivity of the kit is 99.4% and its specificity is 99%. Group 2 included 55 patients who had negative RT-PCR findings with positive chest CT. Group 3 included 77 patients who had influenza-like-illness with a negative RT-PCR and chest CT findings.

Age, gender, the presence of hypertension, hyperlipidemia, smoking, and diabetes mellitus, as well as clinical status and progression, such as fever, dyspnea, intubation, or death, were collected from hospital records. Blood count parameters and biochemical findings were noted.

Chest computerized tomography evaluation

A 16-row multidetector scanner (GE Medical Systems, Bright Speed 16) was used to scan the study population. After raw data obtained from the Sectra PACS System, images were processed to make multi-reconstructive images.

Chest CT image analysis was calculated for every patient as done previously by Ding X et al.[13] Two experienced radiologists who were blinded to the patient's RT-PCR result analyzed CT findings and scores. In case of disagreement, a third radiologist made the final decision. On chest CT scans, ground-glass opacification, consolidation, and linear opacities were evaluated. Pulmonary lesions were noted according to the anatomical lobe structures of the lungs, as follows: left upper, left lower, right upper, middle, and lower.

The involvement of each lobe in terms of quartiles was assigned a 1 to 4 score, namely mean CT score can change between 0 and 20. Scores were given according to the percentage involvement of five separate lung lobes: zero point for no involvement, 1 point for <1–24% involvement, 2 points for 25–49% involvement, 3 points for 50–74% involvement, and 4 points for >75% involvement (Figure 1A). Fifteen randomly selected CT scans were re-examined by the same observer and a second observer to detect inter and intra-observer variability. The inter- and intra-observer correlation coefficients of CT scores were ≥0.95.

Statistics

The study parameters were analyzed by predictive analytics software (SPSS Inch, Chicago, Illinois, USA). Categorical parameters were shown absolute and relative frequencies (n%). For all variables, the normality test distributions were performed using the Kolmogorov-Smirnov method. While the mean±standard deviation was presented for normal distributions, the median (min-max) was presented for non-normal ones. A chi-squared test was used for comparing the categorical data of the three groups. If the expected number of categorical variables in any group was less than five, the Fisher p-value test was accepted. Normally distributed data were analyzed with the ANOVA test, non-normally distributed data were compared by the Kruskal Wallis test. Dunn's pairwise comparison test was used in case the Kruskal Wallis test presented any difference. The Tukey test was used for the pairwise comparison of ANOVA test results. Linear regression analyses were used to find whether there is an association between CT scores and other variables. A 2-tailed $p<0.05$ was considered significant.

RESULTS

The clinical characteristics of the study population are shown in Table 1 in terms of cardiovascular risk factors, such as age, male gender, hypertension, hyperlipidemia, smoking, and diabetes

mellitus, as well as symptoms. The laboratory data concerning patient groups are presented in Table 2.

The percentage of age, gender, hyperlipidemia, smoking and diabetes mellitus, hemoglobin, red cell width, mean platelet volume, D dimer, eGFR, calcium, sodium, potassium, and ferritin levels were similar in these three groups (Table 1). Groups 2 and 3 had a higher hypertension rate, CT lesion presence, and higher mean CT scores compared to group 1. Fever, dyspnea, cough, headache, sore throat, hospitalization time, intubation, and death rates were higher in groups 1 and 2 compared to group 3. Group 1 and 2 variables were similar in terms of symptoms, hospitalization times, mean CT scores, intubation, and death rates.

Group 2 had the highest WBC, neutrophil, N/L ratio, SII, hs-CRP, and fibrinogen levels in the study population. Compared to group 3, groups 1 and 2 had a higher N/L ratio, Hs-CRP, AST, and procalcitonin levels (for all p values<0.05) (Figure 1B). SII and fibrinogen levels were similar in groups 1 and 3.

hsCRP was correlated with N/L ratio (r=0.387, p<0.001) and SII (r=0.298, p<0.001). N/L ratio and SII were not correlated with CT scores and ferritin levels. Both fibrinogen and procalcitonin were correlated with N/L ratio (r=0.278, p=0.003 and r=0.436, p<0.001), and SII was correlated with procalcitonin (r=0.255, p=0.007), but not with fibrinogen levels. Mean CT scores were correlated with CRP (r=0.331, p<0.001), ferritin (r=0.336, p<0.001) and fibrinogen (r=0.280, p=0.003) levels, but not with procalcitonin.

Univariate linear regression analysis showed mean CT scores had correlations with increased age, hs-CRP, fibrinogen, ferritin, but not with N/L ratio, SII, and procalcitonin. However, age and hs-CRP levels appeared to be the predictors in the multivariate analysis. (Table 2).

The ROC analysis showed that hs-CRP levels ≥4.3 mg/dL had good diagnostic value to discriminate lung involvement within the study population with 80% sensitivity and 62% specificity (area under the curve (AUC)=0.781, 95%CI 0.720–0.842, p<0.001). However, ferritin and fibrinogen levels were not good for the prediction of lung involvement compared to that of hs-CRP in the ROC analysis, p=0.255 and p=0.238, respectively.

DISCUSSION

We retrospectively studied patients with COVID-19 and those with a suspicion of COVID-19 due to chest CT lesions, as well as those with influenza-like symptoms. Our study findings showed that despite not having had positive RT-PCR tests, patients with chest CT lesions had a higher inflammation response compared to COVID-19 patients. Moreover, these patients had similar symptoms and clinical events to COVID-19 patients, such as

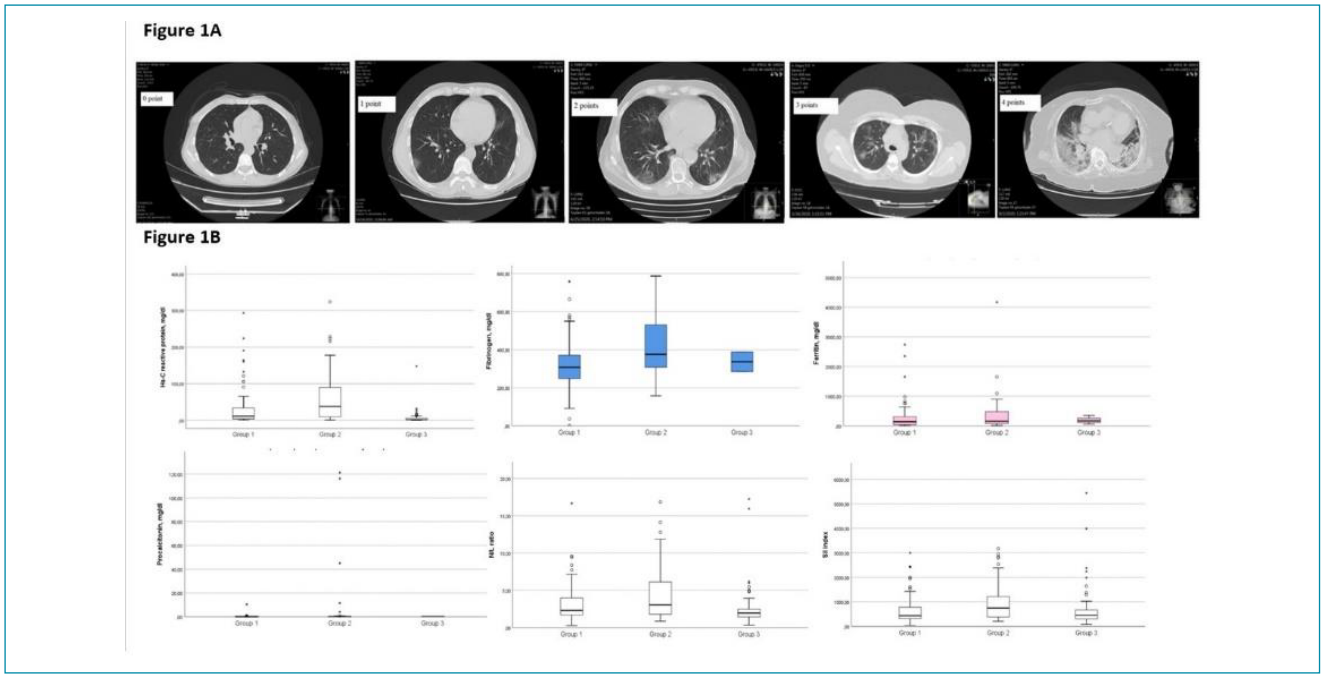

Figure 1. (A): As a result of a visual assessment of the percentage of involvement of each of the five separate lobes of the lung, scores were obtained according to the following percentages of involvement: zero point for no involvement, 1 point for <1–24% involvement, 2 points for 25–49% involvement, 3 points for 50–74% involvement, and 4 points for >75% involvement. (B) Inflammation markers were shown as boxplots in the three study groups.

Table 1. Comparison of demographic, clinical characteristics, blood cell counts, and biochemical levels of laboratory data with respect to the patient groups in the study population.

	Group 1 n=87	Group 2 n=55	Group 3 n=77	p-value
Age, years	49(19–84)	56(20–87)	52(25–86)	0.081
Gender, male, n (%)	38(43.7)	32(58.2)	44(57.1)	0.065
Hypertension, n (%)	15(17.2)	22(40)	24(31.2)	0.037[a,b]
Hyperlipidemia, n (%)	8(9.1)	4(7.3)	10(13)	0.284
Smoking, n (%)	40(46)	31(56.4)	24(31.2)	0.052
Diabetes Mellitus, n (%)	13(14.9)	32(21.8)	8(10.4)	0.384
Fever, n (%)	41(47.1)	28(50.9)	2(2.6)	<0.001[b,c]
Dyspnea, n (%)	22(25.3)	23(41.8)	1(1.3)	<0.001[a,b,c]
Cough, n (%)	55(63.2)	31(56.4)	3(0.4)	<0.001[b,c]
Headache, n (%)	7(8)	4(7.3)	1(1.3)	0.047[b,c]
Sore throat, n (%)	14(16.1)	6(10.9)	1(1.3)	0.001[b,c]
Diarrhea, n (%)	4(4.6)	0	0	0.077
Hospitalization time, day	8.5(0–62)	6(2–48)	3(2–8)	0.034[b,c]
Mean CT scores	4(0–15)	4(1–16)	0	0.029[b,c]
CT lesion presence	28(32.1)	55(100)	0	<0.001[a,b,c]
Intubation, n (%)	8(9.2)	5(9.1)	0	<0.001[b,c]
Death, n (%)	5(5.7)	4(7.3)	0	<0.001[b,c]
Hemoglobin, gr/dL	13.3±2.1	13.2±1.9	13.5±1.7	0.475
Red cell width	14.4±1.7	14.6±2.5	13.9±1.9	0.057
WBC, x10^9/L	6.2±3.1	9.1±4.9	7.5±2.6	0.001[a,c]
Neutrophils, x10^9/L	3.3(0.1–14.2)	6(1.32–17.5)	3.9(1.5–11.8)	0.022[b,c]
Lymphocytes, x10^9/L	1.5±1.1	1.61±1.3	2.22±1.4	0.007[b,c]
Platelets, x10^9/L	209±92	249±101	251±78	0.006[a,b]
MPV	8.9±1.9	8.6±0.8	8.6±1.1	0.278
N/L ratio	2.31(0.2–16.7)	3.1(0.9–17)	1.94(0.3–17.2)	0.001[a,b,c]
SII	437(30–3,000)	750(209–3,107)	465(89–5,437)	<0.001[a,c]
Hs-CRP, mg/dL	11.1(0.5–293)	38(0.3–323)	2.3(011–147)	<0.001[a,b,c]
D dimer, mg/dL	0.55(0.2–188)	0.68(0.2–21)	0.62(0.19–1.27)	0.228
eGFR, mL/min/1.73m^2	97±23	90±25	94±21	0.241
AST, IU/L	25(12–306)	24(14–432)	17.6(8–48)	0.001[a,c]
ALT, IU/L	21(0–172)	24(9–432)	18.5(6–48)	0.047[c]
Calcium, mg/dL	9.2±0.6	9.3±0.7	9.4±0.35	0.850
Sodium, mEq/L	138±3.7	137.1±3.9	139.5±2.4	0.123
Potassium, mEq/L	4.23±0.5	4.15±0.7	4.41±0.5	0.121
Fibrinogen, mg/dL	323±95	412±156	336±73	0.010[c]
Procalcitonin, mg/dL	0.61(0.2–14)	0.41(0–121)	0.16(0–0.20)	0.012[b,c]
Ferritin, mg/dL	139(11–2,736)	157(23–4,167)	170(57–350)	0.370

If p-values <0.05; [a]: for comparing group 1 vs 2; [b]: for comparing group 1 vs 3; [c]: for comparing group 2 vs 3; CT: computerized tomography; AST: aspartate aminotransferase; ALT: alanine aminotransferase; eGFR: estimated glomerular filtration rate; Hs-CRP: high-sensitivity C-reactive protein; MPV: mean platelet volume; N/L: neutrophil/lymphocyte; SII: systemic serum inflammatory index; WBC: white blood cells

Table 2. Dependent variable: CT scores of lung involvement.

	Non-standardized Beta ± SE	Adjusted R^2 Model	p-value
Age	0.066±0.15	0.091	<0.001
Gender, male	-0.038±0.68	-0.006	0.654
Hypertension	1.611±0.77	0.024	0.037
Hyperlipidemia	1.627±1.59	0.001	0.294
Smoking	-0.113±0.69	-0.007	0.870
Diabetes Mellitus	0.988±0.90	0.001	0.275
N/L ratio	0.000±0.11	-0.007	0.997
SII	0.001±0.01	-0.047	0.818
Hs-CRP	0.019±0.05	0.086	<0.001
D dimer	0.011±0.02	-0.006	0.624
Fibrinogen	0.009±0.03	0.088	0.001
Procalcitonin	0.008±0.02	-0.008	0.764
Ferritin	0.002±0.01	0.035	0.017
Multivariate			
Age	0.059±0.15	0.156	0.002
HsCRP	0.017±0.06	0.156	0.003

Hs-CRP: High-sensitivity C-reactive protein; N/L: Neutrophil/lymphocyte; SII: Systemic serum inflammatory index.

fever, cough, headache, sore throat, mean CT scores, hospitalization time, or death and intubation rates.

Since disease progression in elderly patients is mostly accelerated by an inappropriate immune response compared to young patients[6], increased age was a risk factor of extensive involvement of lung lobes in the study.

CRP concentration is an extremely useful and nonspecific biochemical marker for inflammation. CRP measurement contributes significantly to organic disease screening, monitoring response to inflammation, detecting accompanying infections, and treating infection[14]. Compared to bacterial infections, CRP levels in viral infections are lower, but they may be high in some organ-involving viruses[15]. CRP is more valuable than white blood cell count in determining infection[16-17]. In line with earlier studies, a positive correlation between lung involvement severity in SARS-CoV-2 patients and CRP levels was not a surprise. Some authors recommend that CRP levels be used to assess the severity and outcome of patients with SARS-CoV-2[18-19]. CRP values can predict the diagnosis of early severe SARS-CoV-2 infection, before CT findings[18-19]. Tan et al.[11] found a moderate correlation of CRP with chest findings, which is in line with our results.

SII has never been evaluated in the concept of COVID-19 infection to date; however, we found SII and N/L ratio are not suitable for positive CT scores. N/L ratio had a better correlation with Hs-CRP and procalcitonin than SII. Since viral infections are a common reason for lymphopenia and neutrophilia, increased N/L ratio levels are expected[12]. Even though we did see the highest rising values of N/L ratio in group 2, in which all patients had positive CT scores, no correlation with N/L ratio was seen among the chest scores.

Surprisingly, we detected the highest fibrinogen levels in group 2. Because of higher embolic events reported, the evaluation of fibrinogen levels is particularly important in SARS-CoV-2 patients. The correlation of fibrinogen levels with N/L ratio and mean chest score may be important for anticoagulant therapy strategies in these patients, most of whom might be COVID-19 patients.

Even though they have negative PCR results, the fact that the greater mean CT scores were positively correlated with hs-CRP, ferritin, and fibrinogen levels should lead clinicians to evaluate patients with high serum levels of hs-CRP, ferritin, and fibrinogen by chest CT scanning.

There is no significant increase in procalcitonin levels in viral infections and systemic immunological diseases. Unlike cytokines and CRP, there is no significant increase in procalcitonin levels in necrosis, inflammation, and viral infections. Therefore, it is accepted that procalcitonin is specific for bacterial

infections[10]. For this reason, we believe the reason for a lack of correlation between procalcitonin and lung involvement severity is a superinfection in a few patients.

As for ferritin levels correlated with lung involvement, we believe increased ferritin levels are caused by higher intracellular replication of the virus due to pneumonia. Infected host cells need iron to synthesize viral particles. As such, we believe that increased ferritin levels are a result of lung cell necrosis.

Study limitations

We did not present all data for the early, peak, and recovery stages of the disease courses. We only evaluated the clinical characteristics and laboratory data of all patients at the time of chest CT to provide clear data concerning the correlation of disease severity with lung lesions. A study showed that the greatest time of lung involvement severity during the disease was about ten days after the onset of the symptoms[12]. For this reason, chest CT imaging and laboratory data of our study population were obtained approximately ten days after the onset of symptoms.

CONCLUSION

The study findings made us accept that the patients who have negative RT-PCR findings with positive chest CT should be considered and treated as SARS-Cov-2 patients because they have similar CT lesions to that of COVID-19 patients. Besides, monitoring serum Hs-CRP concentrations is important for cases of suspected lung involvement in COVID-19 patients.

AUTHORS' CONTRIBUTIONS

AA: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project Administration, Resources, Software, Supervision, Validation, Visualization, Writing-Original Draft, Writing-Review & Editing. **TIKÖ:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Validation, Visualization, Writing-Original Draft, Writing-Review & Editing. **GGŞ:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Validation, Visualization, Writing-Original Draft, Writing-Review & Editing. **MME:** Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Project Administration, Resources, Validation, Visualization, Writing-Original Draft, Writing-Review & Editing. **DÖG**: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing-Original Draft, Writing-Review & Editing. **ŞA:** Conceptualization, Project Administration, Supervision, Visualization, Writing-Review & Editing.

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Motor development of infants (6–12 months) with low birth weight

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SUMMARY

OBJECTIVE: The aim of this study was to describe the motor development (MD) and growth of infants born with low birth weight (LBW) versus adequate birth weight (ABW) by using the Alberta Infant Motor Scale (AIMS).

METHODS: The cross-sectional study including LBW infants (aged 6–12 months) followed at an outpatient clinic from a University Hospital in Brazil and a group of infants of the same age with ABW. The variables were recorded as maternal, birth, and infant conditions. The infants were assessed for MD using the AIMS.

RESULTS: In total, 98 infants (38 LBW versus 60 ABW) were evaluated and no statistically significant differences were found in demographic characteristics and in the AIMS results. The AIMS results of the total sample were suspicious or abnormal MD in 44 (45%) of total infants. Higher frequency of suspected or abnormal motor behavior was found in the age group between 9 and 12 (54.6%) months.

CONCLUSIONS: A frequency of 45% of suspected or abnormal behavior was observed in the evaluated infants, with a higher frequency of occurrence in those aged 9–12 months (54.6%).

KEYWORDS: Infant, low birth weight. Motor skills.

INTRODUCTION

Low birth weight (LBW) is defined by the World Health Organization as birth weight of <2.500 g, regardless the gestational age (GA), including preterm newborns, those with intrauterine growth restriction or small for GA infants. LBW is considered a global public health problem and is associated with a series of functional consequences[1,2].

In 2019, it was estimated 20.5 million live births with LBW, mostly (91%) in low/middle income countries[2]. In Latin America, the rate observed was 8.7%[2]. In Brazil approximately 8.5% of live births were born LBW[3], and in São Paulo it was 9.5%[3].

Children with LBW was at risk for growth, and motor developmental (MD) delays with a broad spectrum of alterations such as cognitive, behavioral, and learning disabilities[4]. Functional changes usually become more apparent over the years, resulting in difficulties in reading and writing in the school phase[5].

In the first 5 years of life, the motor acquisition of child represents the integrity and functionality of other systems[6]. The early identification of possible MD delay, and timely intervention can lead to a better prognosis for children at risk for developmental disorders[7].

The Alberta Infant Motor Scale (AIMS) is considered a dynamic assessment scale, as it describes the acquisitions achieved by the child and enables the analysis of the components necessary for the acquisition of certain skills. It emphasizes movement patterns and skills in different gravitational situations, as well as weight distribution, posture, and antigravity movement. It is a low cost and easy-to-apply instrument[6,8].

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Although infants with LBW are at higher risk for growth and MD delay and developing short- and long-term diseases[4], a few studies[9-11] evaluated the MD in the first year of life applying AIMS. Thus, the main objective of this study was to evaluate the MD of infants aged 6–12 months with LBW by AIMS.

METHODS

This cross-sectional clinical trial included infants born with LBW (LBW Group) and healthy infants (aged between 6 and 12 months) of the same age and also born at term with adequate birth weight (ABW Group). The study was approved by the Ethics Committee (No. 1.904.715). Infants diagnosed with central or peripheral nervous system malformations, encephalopathy, congenital heart disease, genetic syndromes, or the Apgar score <7 in the fifth minute were excluded.

The variables were noted as follows: information about birth (i.e., type, clinical condition of mother, complications, birth GA, birth weight, height, and head circumference), length of stay in the nursery and neonatal complications, the Apgar score, resuscitation procedures, and maternal conditions (e.g., age, parity, chronic diseases, complications during pregnancy, tobacco/alcohol/drug use, socioeconomic status, and education).

Gestational age and birth weight were used to classify the newborn as appropriate for GA (AGA), small for GA (SGA), or large for GA (LGA)[12]. For MD and nutritional status assessment, all premature infants (GA ≤37 weeks) had their GA corrected to 40 weeks[2]. For anthropometry, at the time of MD evaluation, weight (g), length (cm), and head circumference (cm) measurements were obtained[13]. The indicators shown as *Z*-score for age were weight/age, height/age, body mass index (BMI), and head circumference/age[13].

Environment/routines of the infant were as follows: use of a walker, habit of placing the child in prone position, and attending in a day care during the period of the evaluation. MD evaluation was performed by using the AIMS[8] applied by two trained physiotherapists. The total score was converted into a percentile curve, and the MD ratings of infant were included[8] as follows:

1. Normal or typical motor performance when >p25 on the scale percentile curve;
2. Suspicious motor performance between p5 and p25; and
3. Abnormal motor performance when <p5.

At the moment of the MD evaluation the infant was positioned in a firm surface, and specific stimuli were provided to apply the tests according to the age range to be evaluated, manipulating the child only when necessary[8].

All evaluations were recorded by filming, with the purpose of performing the disagreement/agreement analysis between the two physiotherapists. In case of disagreement, a third trained physiotherapist analyzed the videos. The children were worn only with diapers, and the evaluations were performed between feedings, as long as they were active and awake (Brazelton Scale at level 4 or 5)[14].

Statistical analysis

The data were recorded in an Excel spreadsheet (Office®) and analyzed using the Statistical Package for Social Sciences for Windows (SPSS, Chicago, IL, USA) 25.0 (IBM®). Qualitative variables were presented as absolute numbers and percentages, compared by using the Pearson's chi-square test. The normality of continuous variables was assessed using the Shapiro-Wilk test. Those that presented parametric distribution were presented as mean±standard deviation, compared by using the Student's *t*-test. Variables with nonparametric distribution were presented as median and 25–75% interquartile range and compared using the Mann-Whitney *U* test. The variables were considered statistically significant when p≤0.05.

RESULTS

In this study, 69 eligible infants were attended at the LBW outpatient clinic, and 17 infants were excluded due to congenital malformations and 14 due to loss of follow-up, resulting in the inclusion of 38 infants with LBW, and 60 with ABW.

The maternal sociodemographic and gestational characteristics of infants can be observed in Tables 1 and 2. In LBW group, the mothers had a higher percentage of complications during pregnancy (87% versus 45%; p<0.001), the most frequent were infectious and specific pregnancy hypertensive disease (Table 1).

Among the evaluated infants, the mean birth weight of LBW and ABW groups were 2.218.0±166.4 and 3.232.8±416.4 g, respectively (p<0.001). The LBW group had a higher frequency of SGA infants (42% versus 10%; p<0.001), as well as neonatal complications (57% versus 12%; p<0.001) (Table 2).

The 66% of LBW infants were born premature (GA: 35.5±1.7 weeks). The average length of stay after birth was <10 (8.9±6.8) days. Four newborns from the LBW group (11%) remained in the intensive care unit, but the hospitalization time was <24 h.

At the time of the AIMS evaluation, the mean real and corrected age of infants were 273.5±77.2 days in LBW group versus 215.6±60.7 days in ABW group. It was observed that 14 (37%) of the LBW group received breast milk versus 49 (82%) in ABW group (p<0.001). No statistical differences were observed between the groups concerning their nutritional conditions. There was no correlation between MD assessment and nutritional status in the groups.

Table 1. Maternal sociodemographic and gestational characteristics of infants with low birth weight and adequate birth weight, 2020.

Variables	LBW (n = 38)		ABW (n = 60)		p-value
	n	%	n	%	
Maternal age (years)*	29.9±6.8		26.7±6.4		0.015†
Maternal schooling					
Elementary school	11	28.9	23	38.5	0.219‡
High school	20	52.6	35	58.3	
University education	7	18.4	2	3.2	
ABEP					
A	0	0.0	0	0.0	0.258‡
B	3	7.9	1	1.7	
B1	12	31.6	10	16.7	
C1	16	42.1	35	58.3	
C2	6	15.8	11	18.3	
D–E	1	2.6	3	5.0	
Prenatal initiation (months)*	1.0±0.16		1.13±0.34		0.036†
Number of pregnancies*	1.6±1.3		2.2±0.9		0.024†
Smoking					
Yes	1	2.6	2	3.3	0.649‡
No	37	97.4	58	96.7	
Alcoholism/drugs					
Yes	1	2.6	2	3.3	0.649‡
No	37	97.4	58	96.7	
Gestational complications					
Yes	33	86.8	27	45.0	<0.001‡
No	5	13.2	33	55.0	
Type of complications					
Cardiovascular	13	34.2	3	5.0	<0.001‡
Respiratory	1	2.6	0	0	
Genitourinary	5	13.1	2	3.3	
Hematological	4	10.5	4	6.7	
Infectious	15	42.8	14	23.3	
Metabolic	4	10.5	0	0	
Neurological	1	2.6	0	0	
Others	11	28.9	4	6.7	
Type of delivery					
Vaginal	4	10.0	34	56.7	<0.001‡
Cesarean section	34	89.5	26	43.3	

LBW: low birth weight; ABW: adequate birth weight; n: number; %: absolute percentage; ABEP: Associação Brasileira de Empresas de Pesquisa; *data in averaged±standard deviation of the mean; †student's *t*-test significance level; ‡Chi-square test significance level.

Table 2. Conditions at birth and anthropometry at the date of motor assessment of infants with low birth weight and adequate birth weight, 2020.

Variables	LBW (n=38)		ABW (n=60)		p-value
	n	%	n	%	
Gender					
Male	19	50.0	34	56.7	0.540†
Female	19	50.0	26	43.3	
Birth weight (g)*	2218±166.4		3232.8±416.4		<0.001‡
Adequate for gestational age					
SGA	16	42.1	6	10.0	<0.001†
AGA	22	57.9	42	70.0	
LGA	0	0.0	12	20.0	
Neonatal complications					
Yes	21	57.5	7	11.7	<0.001†
No	17	42.5	53	88.3	
Type of complications					
Infectious	4	11.4	0	0	<0.001†
Respiratory	12	31.6	0	0	
Metabolic	5	13.1	0	0	
Icterus	7	18.4	8	13.3	
Others	5	13.1	0	0	
Z-score height					
Short stature	7	18.4	2	3.4	0.026†
Adequate height	31	81.6	57	96.6	
Z-score BMI					
Thinness	1	2.6	1	1.7	0.897†
Eutrophic	31	81.6	48	80	
Overweight/obesity	6	15.8	11	18.3	
Z-score HP					
Adequate	38	100	60	100	

LBW: low birth weight; ABW: adequate birth weight; n: number; %:absolute percentage; SGA: small for gestational age; AGA: adequate for gestational age; LGA: large for gestational age; BMI: body mass index; HP: head perimeter; * data in averaged±standard deviation of the mean; †Chi-square test significance level; ‡Student's *t*-test significance level.

Through the interview with parents/legal guardians of the infants, it was obtained that 82% of the LBW group and 90% of the ABW group were under the parental care and did not attend at a day care.

The AIMS results observed were suspicious or abnormal in 18 (47%) in the LBW group versus 26 (43%) in the ABW group (p=0.522). There were no statistically significant difference between the groups, regarding total score and test components (Table 3). However, the median score of the seated position was lower in the LBW group versus the ABW group [i.e., 7.0 (3.0–11.2) versus 9.5 (5.0–12.0)] (p=0.087).

It was observed that at the age of 6–9 months, 44.4% of the infants in the LBW group had suspicious or abnormal/atypical motor behavior, and in the ABW group the frequency was 37.2%. Infants aged 9–12 months had a higher frequency of suspicious or abnormal behavior (i.e., 54.6% in the LBW group).

Table 3. Total and component score of Alberta Infant Motor Scale in children with low birth weight and adequate birth weight, 2020.

AIMS	LBW (n=38)	ABW (n=60)	p-value
Total score	27.5 (19.0;43.0)	31.5 (22.2;42.7)	0.233*
Classification			
Normal	20 (52.6)	34 (56.7)	0.522†
Suspicious	12 (31.6)	21 (35.0)	
Abnormal	6 (15.8)	5 (8.3)	
Prone			
Score	10.5 (7.0,17.0)	12.0 (7.0,17.7)	0.483*
Supine			
Score	8.0 (6.0,9.0)	7.5 (6.0,9.0)	0.888*
Seated			
Score	7.0 (3.0,11.2)	9.5 (5.0,12.0)	0.087*
Standing			
Score	3.0 (2.7,6.3)	3.0 (3.0,5.0)	0.553*

AIS: Alberta Infant Motor Scale; LBW: low birth weight; ABW: adequate birth weight; n: number, and data expressed as median and interquartile range; *Mann-Whitney *U* test significance level; †Chi-square test significance level.

DISCUSSION

This study observed that MD of infants with LBW (i.e., between 6 and 12 months of corrected age) assessed by AIMS was similar to the group of healthy infants born at term with ABW in São Paulo, Brazil. In both groups, there was a high percentage of suspicious or abnormal MD performance.

There are several standardized tests and scales that help identifying children at risk for MD delay, which can be used for screening and diagnosis and for therapeutic planning if any abnormality is detected[15]. Choosing the best test for assessing MD in infants remains a challenge[15,16]. The detection of changes in MD in the first year of life has a high predictive value for the medium and the long-term global developmental changes[16].

During the evaluation of the MD in children from 1 month to 2 years old, it was identified that the motor domain was the first to present a delay, starting around 10 months of age, followed by the language domain[17]. Those minor deviations in MD between 9 and 15 weeks of age are associated with receptive and expressive language delay at 1.5 and 2.5 years, concluding that motor function delays may precede delays in other domains[17,18].

In this study, the results of MD assessment by AIMS were similar in both groups. It is noteworthy that the characteristics that represent risk factors for MD delay such as the Apgar score, socioeconomic status, and maternal education were similar in all evaluated infants. In both groups, more than 40% of infants were classified by AIMS as having suspicious or abnormal MD.

Some studies[19-21] suggest that the poorer performance of Brazilian children could be related to the fact that instruments suffer interference from cross-cultural adaptation. Other possible elements involved could be the distinct socioeconomic, ethnic, and cultural factors and the greater daily exposure of Brazilian children to biological and environmental risk factors[19,21].

The maternal practices, such as the preference for the supine position due to the concern with the sudden infant death syndrome, could be another factor associated with the observed differences[18]. The lack of habit of Brazilian parents in leaving their children in prone position, even when they are awake, may be a risk factor[22]. Another study[23] verified the influence of maternal practices on the MD of healthy infants between 6 and 12 months of age, suggesting that practices that encourage the adoption of four-support posture and the use of the floor have a positive influence in the MD.

In this study, when infants were stratified in trimesters of age, there was a higher percentage of suspected or abnormal MD between 9 and 12 months (54.6%) than between 6 and 9 months (44.4%). These results are similar to other studies[24] using the AIMS and concluded that in the first 3 months and from 13 months of life, the AIMS curve is not as sensitive for detecting MD delays between 4 and 12 months of age.

Independent sitting is a posture that a child acquires between 6th and 7th month of life that is not a locomotion posture such as crawling and walking, but it is a stabilizing

posture necessary for the development of balance, coordination, and motor control, requiring static and dynamic muscle control, which may occur later in children with LBW[22]. The acquisition of postures in MD in the early years of life is influenced by the environment of the child and by the sociocultural context[19-23].

This study has some limitations. It is a cross-sectional study including a convenience sample. The validation proposed of a version of AIMS for the Brazilian population not yet normative for the analysis of motor performance in this population[20]. Previous studies[19-21] observed that the MD of Brazilian children were lower than those observed in Canada, except at 18 months. The reference values for AIMS are still the values determined by the Canadian study[16-21].

CONCLUSIONS

The motor development of LBW infants assessed by AIMS was similar to that of ABW infants, and approximately 60% of the sample was of premature newborns. A frequency of 45% of suspected or abnormal behavior was observed in the evaluated infants, with a higher frequency of occurrence in those aged 9–12 (54.6%) months.

AUTHORS' CONTRIBUTIONS

VCWPG: Investigation, Methodology, Project administration, Writing – original draft. FISS: Formal analysis, Statistical analysis, Writing – review & editing. CJ: Methodology, Validation. MWLS: Formal analysis, Methodology, Validation, Writing – review & editing.

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Commercial blood cell separation systems versus tube centrifugation methods for the preparation of platelet-rich plasma: a preliminary cross-sectional study

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SUMMARY

OBJECTIVE: Clinical studies claim that platelet-rich plasma (PRP) accelerates tissue healing due to its high concentration of growth factors and that the combination with leukocytes improves the antimicrobial effect of the concentrate. Most of these studies obtained PRP using different separation systems, and few analyzed the content of the PRP used for treatment. This study characterized the composition of PRP and white blood cells (WBC) from a single donor produced by three commercially available PRP separation systems and two anticoagulated general analytical tubes.

METHODS: Five patients donated 50 mL of blood, which was processed to produce PRP and WBC using three PRP concentration systems (i.e., Easy PRP Kit, GloPRP, and Wego) and two tubes for general analysis anticoagulated with ethylenediaminetetraacetic acid (EDTA) and citrate. Platelets and WBC in combination with their concentrates were analyzed by automated systems in a clinical laboratory.

RESULTS: There were no significant differences in the average concentrations of PRP platelets and WBC between GloPRP and the tubes for general analysis with EDTA and citrate; however, the Easy PRP Kit gave results much superior to the rest of the methods, especially comparing it with the Wego Kit, whose concentrates were especially low, even nonexistent for WBC.

CONCLUSIONS: The Easy PRP Kit concentrates WBC-rich PRP, resulting in increased WBC concentrations, compared with low WBC-low PRP of GloPRP and general tube methods for EDTA and citrate analysis and the even lower concentration of PRP from the Wego Kit, with the absence of leukocytes.

KEYWORDS: Platelet-Rich Plasma. Leukocytes. Blood.

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INTRODUCTION

Platelet-rich plasma (PRP) is a blood product formed by concentrating platelets in a small volume of plasma and is used for a wide variety of applications[1].

The mechanism of action of PRP is unknown; however, the release of high concentrations of growth factors from platelet alpha granules as platelets degranulate is believed to play a critical role[2].

The PRP was first recognized as an effective agent for bone and tissue repair within the field of dentistry and oral maxillofacial surgery[3]. Its applicability then spread to the fields of plastic surgery by demonstrating an evidence of improved skin graft wound healing[4,5].

A number of commercial systems are available that result in platelet products with a wide range of platelet and leukocyte concentrations, although there is limited information available regarding the optimal platelet and leukocyte contents necessary to achieve a desired biological effect, and it may be that specific products are better for certain applications[6].

There is no single definition for PRP in the literature, and the "PRP" products include wide ranges of platelet and leukocyte concentrations that reflect the various separation methods and the lack of consensus on specific composition of the final product, and furthermore, platelet numbers are the primary concern, but optimal leukocyte concentrations in PRP is a topic of discussion in this study[6,7].

Platelet concentrations in PRP commonly range from a 3- to 5-fold increase over whole blood (WB) or a minimum concentration of 300,000–1,000,000 platelets/mL[8].

Therefore, the purpose of this study was to assess the concentration of platelets and white blood cells (WBC) of PRP produced by three new commercial systems and two tubes for general analytical tests, in order to verify if the kits reach the minimum concentration and if the tubes for general analytical tests are also capable of achieving it, or if, on the contrary, it is necessary to resort to the commercial systems created to alkalize the ideal concentrations, and the tubes for general analysis are not useful in order to concentrate platelets and WBC.

METHODS

Participant recruitment

Five healthy volunteers (i.e., two men and three women, aged 31–64 years) who were patients at the 2010 Podiatric Clinic, León, Spain were invited to participate in this study. Individuals were free from all chronic illnesses and not taking any regular medication, including aspirin and nonsteroidal anti-inflammatory drugs. Prior to recruitment, the objectives and procedures involved in this study were explained to participants, and each signed an informed consent form. All individuals fulfilled the inclusion criteria and consented to be included as participants in this study.

Challenge PRP production methods and PRP preparation

Blood (60 mL) was collected by venipuncture from the antecubital vein of each donor using a 21 G×1.9 cm butterfly needle (BD Vacutainer, Franklin Lakes, NJ). WB was drawn into two plain 4.5-mL BD Vacutainer ethylenediaminetetraacetic acid (EDTA) tubes, two plain 4.5-mL BD Vacutainer sodium citrate (SC) 3.2% tubes, and SC pre-filled syringes of the three commercial blood separation systems, namely, the classic PRP Kit (cl-PRP Kit) based on gel PRP tubes (Wego, Shenzhen, China), the Easy PRP Kit® (Mesotech, Naples, Italy), and GloPRP® (Glofin, Ösalo, Finland) systems. An additional 2 mL of WB was collected into an EDTA tube to determine the basal count of platelets and leukocytes (WBC) in WB, including neutrophils, lymphocytes, and monocytes among WBC fractions (Echevarne Laboratory, HM San Francisco Hospital, León, Spain).

The PRP preparation technique was adapted from previously published methods[9-11] and following the indications of the manufacturer's kits. All PRP protocols were based on a single-centrifugation step for 5–10 min (Nahita centrifuge, Ibor Médica, Spain), except for Glo-PRP system for which a double centrifugation process (1,200×*g* for 5 min followed by 1,200×*g* for 10 min) was used. After centrifugation, all tubes and systems were allowed to rest for 5 min to facilitate the settling of platelets onto the buffy coat. The PRP was collected using a needle attached to a 5-mL syringe measuring 21 G×40 mm under the naked eye visualization. With the tube stoppers removed, the needle tip was positioned so as to just touch the buffy coat. The syringe plunger was gently raised to vacuum up the platelets on the buffy coat, and the needle tip was slowly moved along the buffy layer[12].

A total volume of 0.3–0.5 mL buffy coat was extracted from each tube into the collection syringe. A total PRP of 2.0–2.5 mL per participant was collected and transferred into an EDTA tube for analysis. The number of platelets and WBC present in the venous blood and the PRP were determined. The ratio of platelet levels in PRP to venous blood (PRP:WB ratio) was calculated to determine the ability of the current method to concentrate platelets and WBC[12]. The platelet and WBC analyses, both in the WB (baseline) and after centrifugation by the five methods of preparing PRP, were measured in milliliters.

Statistical analysis

The descriptive analysis of the characteristics of participants was performed. Continuous variables were reported using mean and standard deviation as well as lower and upper limits for a 95%

confidence interval (95%CI) or median and interquartile range (IQR). The categorical data were presented as frequencies and percentages. A repeated-measures one-way ANOVA followed by the Dunnett's multiple comparison test and ANOVA followed by the Tukey's multiple comparison test were performed to determine the differences in platelets and WBC contents between the WB and PRP obtained by each concentrator method and between systems used in this study, under the assumption that all the data suitably measured followed an approximately Gaussian distribution according to the Shapiro-Wilk normality test.

For all analyses, a value of $p<0.05$ was considered statistically significant. The data obtained were analyzed using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

Five healthy patients were recruited, according to the sample size of the previous studies[6] with an average age of 47.40±15.58 years (95%CI 28.03–66.76 years) and a body mass index of 28.79±4.81 (95%CI 22.82–34.77).

The platelet and WBC counts in WB and in the PRP obtained with each system are summarized in Table 1. All methods, except the cl-PRP system, significantly increased WB platelet density (i.e., Easy PRP Kit and citrated tubes, $p<0.0001$; EDTA tubes, $p=0.001$; and the GloPRP system, $p=0.011$). However, the mean increase in the platelet factor (PRP:WB ratio) provided by the Easy PRP Kit system was much greater (by 3.56 times versus 1.36–2.03 times basal platelets for Easy PRP Kit versus the other methods; $p<0.05$) (Figure 1), reaching a significantly higher PRP platelet concentration than the other blood concentration methods. The platelet density achieved in the PRP was not significantly different between EDTA tubes, citrated tubes, GloPRP, or cl-PRP systems. The mean increase in the WBC factor was relatively less. The Easy PRP Kit system also showed the highest PRP:WB ratio (2.41 times versus 0.00–1.13 times baseline WBC for Easy PRP Kit versus all other systems), reaching WBC densities significantly higher than WB and those obtained with any other system. The cl-PRP system did not concentrate WBC, and the densities reached by the rest of the systems were not significantly different from those measured in WB (Figure 1).

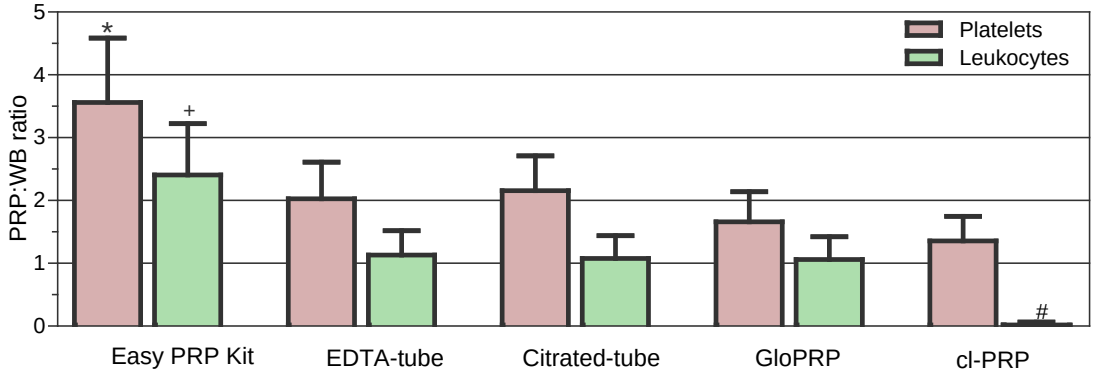
As shown in Figure 2, the concentration PRP:WB ratio for the different WBC fractions was markedly different among systems, being lower for neutrophils compared with lymphocytes and monocytes.

Table 1. Platelet and white blood cells count (cells×10^6/mL) in whole blood and platelet-rich plasma obtained by different concentrator methods.

	Platelets			WBC		
	Mean±SD (95%CI)	Median (IQR)	p	Mean±SD (95%CI)	Median (IQR)	p
WB	291.00±64.09* (211.43–370.57)	304.00 (122.50)	0.81	8.02±2.12† (5.38–10.65)	7.53 (3.03)	0.05
Easy PRP Kit	1008.00±224.12‡ (729.33–1286.00)	1023.00 (398.52)	0.99	18.53±4.45§ (13.01–24.05)	18.60 (8.82)	0.40
EDTA tube	573.19±127.49 (414.88–731.49)	582.14 (226.63)	0.99	8.72±2.09 (6.12–11.32)	8.76 (4.15)	0.40
Citrated tube	613.79±147.80 (430.28–797.31)	670.95 (279.39)	0.51	8.28±1.99 (5.81–10.74)	8.31 (3.94)	0.40
GloPRP system	468.97±104.31 (339.45–598.49)	476.30 (185.43)	0.99	8.16±1.96 (5.73–10.60)	8.20 (3.89)	0.40
cl-PRP	384.61±85.55 (278.39–490.83)	390.62 (152.07)	0.99	0.0±0.0// (0.0–0.0)	0.0 (0.0)	–

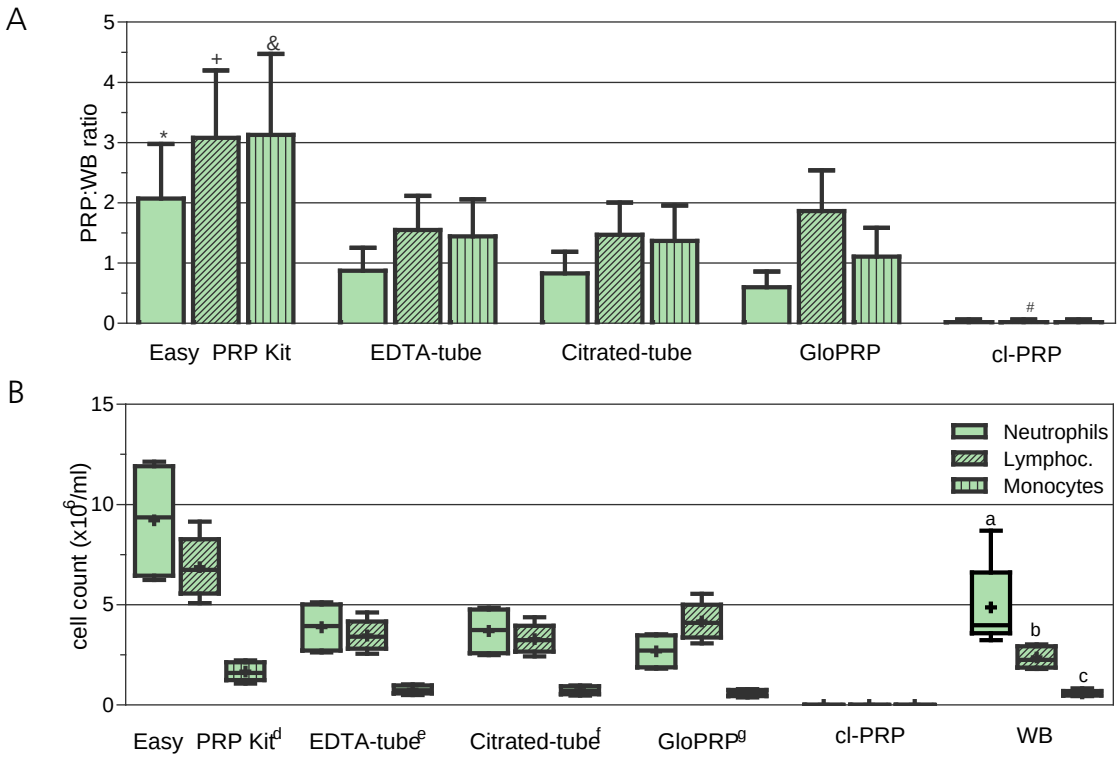
*$p<0.0001$ versus Easy PRP Kit and citrated tube, $p=0.001$ versus EDTA tubes and $p=0.011$ versus GloPRP system; †$p<0.0001$ versus Easy PRP Kit and cl-PRP; ‡$p<0.0001$ versus GloPRP and cl-PRP, $p=0.0011$ versus EDTA tube and $p=0.0031$ versus citrated tube; §$p<0.0001$ versus all methods; //$p\leq0.0005$ versus EDTA tube, citrated tube, and GloPRP.

SD: standard deviation; CI: confidence interval; IQR: interquartile range; p: Shapiro-Wilk test with a statistical significance for a p-value<0.05, with a 95%CI; WB: whole blood; PRP: platelet-rich plasma; EDTA: ethylenediaminetetraacetic acid.



*p≤0.0192 versus all methods. +p≤0.0026 versus all methods. #p≤0.013 versus EDTA tube, citrated tube, and GloPRP.
WBC: white blood cells; WB: whole blood; PRP: platelet-rich plasma; SD: standard deviation; EDTA: ethylenediaminetetraacetic acid.

Figure 1. Fold change of platelets and white blood cells versus basal whole blood (platelet-rich plasma:whole blood ratio) in the concentrates obtained by each blood separation method (mean±standard deviation). Platelet-rich plasma:whole blood ratio of 1; no cell concentration.



*p≤0.0065 versus all methods; +p≤0.0148 versus EDTA tube, citrated tube, and cl-PRP. &p≤0.014 versus EDTA tube, citrated tube, and cl-PRP. #p≤0.0195 versus EDTA tube, citrated tube, and GloPRP. ᵃp<0.0001 versus Easy PRP Kit and cl-PRP, p=0.033 versus GloPRP; ᵇp<0.0001 versus Easy PRP Kit and cl-PRP, p=0.0024 versus GloPRP; ᶜp<0.001 versus Easy PRP Kit and cl-PRP; ᵈp≤0.0014 versus all methods for WBC fractions; ᵉp≤0.0036 versus cl-PRP for WBC fractions; ᶠp≤0.0058 versus cl-PRP for WBC fractions; ᵍp≤0.013 versus cl-PRP for lymphocytes and monocytes.
WBC: white blood cells; WB: whole blood; PRP: platelet-rich plasma; EDTA: ethylenediaminetetraacetic acid.

Figure 2. The efficiency of the different blood separation methods to concentrate neutrophils, lymphocytes, and monocytes. (A) Platelet-rich plasma:whole blood ratio. (B) Box plot (minimum to maximum) of cell count (×10⁶/mL) recovered from each concentrate (median: line in the middle of the box; mean: +).

DISCUSSION

In this study, we demonstrated a general difference in the platelet factor increase and platelet recovery rates in the PRP concentrated by the commercial Easy PRP Kit and the remaining PRP preparing methods, which was a surprising finding given that the starting blood volume (i.e., 11 mL for Easy PRP Kit, 9 mL for GloPRP and the cl-PRP Kit, and 8 mL for EDTA and citrate general analytical tubes) and the centrifugation patterns (i.e., rotation force and the number of centrifugation steps) used were similar to other methods used or even more unfavorable (i.e., GloPRP used two-step centrifugation procedure to prepare PRP), producing equal volumes of PRP (2 mL). However, both arguments alone do not explain the performance of Easy PRP Kit system, since with the increase of only 3 mL of blood volume, it concentrated between 40 and 60% more platelets than the others, and the centrifugal force accommodated was identical for different methods. Thus, based on the recent theoretical predictions by Piao et al. to establish the optimal conditions for PRP preparation, we believed that both the geometry of the device and the use of relatively short spinning times (4–5 min) play an important role in the recovery rates of the platelets[13]. However, it is important to note that the increase in the platelet factor for the Easy PRP Kit system in this study (3.56 times the basal platelet count) was far from the system efficiency displayed by the manufacturer (7–9 times). Consequently, the skill of the clinician and the familiarity with the use of the device are the essential factors to predict the platelet capture efficiency of the different PRP separation systems in the clinical setting and, thus, to be able to satisfactorily advise patients on the PRP content provided by each one of them.

Interestingly, both tube-based methods, which achieved between 56 (EDTA) and 60% (SC) of the total platelets concentrated by the Easy PRP Kit system, exhibited a higher efficiency than the commercial GloPRP systems (i.e., 46% of the total platelets recovered by Easy PRP Kit), which included a two-step centrifugation process, or the cl-PRP Kit (38%) to concentrate platelets. These results confirm that the anticoagulant incorporated into the system modifies the efficiency of the separation and concentration of the blood fractions as previously described by Amaral et al.[14] Furthermore, since all systems were anticoagulated with SC, the use of EDTA as anticoagulant would result in a particularly low platelet recovery rate for the GloPRP and the classic PRP Kit.

In this study, we also found a significant difference in the WBC obtained by each blood separation method. The Easy PRP Kit system again demonstrated the highest increase in the WBC factor, achieving WBC densities significantly higher than those measured in WB. In contrast, leukocytes in the concentrates obtained by the blood tube collection-based methods or by GloPRP produced virtually no increase from WB levels.

This study revealed that regardless of the specific efficiencies of each system, the cell concentration factor was blood cell size dependent; the smaller the blood cell size, the greater the concentration factor. Apart from platelets, highly concentrated based on the size, the concentration factor among leukocytes was higher for lymphocytes>monocytes>neutrophils. Neutrophils, along with monocytes/macrophages, are the effectors of the expected innate immune response of the WBC fraction and have a greater representation in WB and higher microbicidal activity in comparison[15]. Thus, this cell size-based efficiency, which implies an overrepresentation of lymphocyte fraction in the WBC extract, would be suitable for our therapeutic expectations with WBC if methods with poor efficiency in WBC concentration, such as EDTA or SC tubes or GloPRP system, were used. These methods significantly concentrated lymphocytes, but decreased the basal neutrophil concentration in WB. For this reason, if we are truly interested in the clinical use of platelets in combination with "leukocytes," it would be highly recommended to know in advance the concentration power of the system for the different WBC fractions.

CONCLUSION

The Easy PRP Kit concentrates WBC-rich PRP, resulting in increased WBC concentrations, compared with low WBC-low PRP of GloPRP and general tube methods for EDTA and citrate analysis and the even lower concentration of PRP from the Wego Kit, with the absence of leukocytes.

AUTHORS' CONTRIBUTIONS

BTR: Data curation, Formal analysis, Investigation, Writing – review & editing. **RBBV:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. **DS:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **NG:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **MELI:** Conceptualization, Writing – original draft, Writing – review & editing. **DLL:** Data curation, Formal analysis, Investigation, Writing – review & editing. **LA:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing.

ETHICS STATEMENT

The Research Ethics Committee of the Rey Juan Carlos University of Madrid approved this study.

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A comparison of computerized tomography findings of COVID-19 infection by gender and age groups

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SUMMARY

OBJECTIVE: To compare the computed tomography (CT) imaging findings of coronavirus disease 2019 (COVID-19) by gender and age groups.

METHODS: The patients with COVID-19 (n=1,024) were divided into nine age groups (0–9 years, 10–19 years, 20–29 years, 30–39 years, 40–49 years, 50–59 years, 60–69 years, 70–79 years, and 80 years and above). The CT findings were retrospectively analyzed according to the age groups and gender.

RESULTS: Under 20 years of age, except for the ground-glass opacity and consolidation, no other finding was observed. Airway changes and crazy-paving pattern were more common over 80 years. While the tree-in-bud pattern was more common in the 20–29 age group than in other age groups, the halo sign was mostly seen at the age of 30–39 years. Unlike other groups, the thin reticular pattern was more common in patients aged 60–79 years. When the findings were compared by gender, the rates of centrilobular nodules (p=0.006), airway changes (p=0.004), and tree-in-bud pattern (p=0.050) were significantly higher in males than in females.

CONCLUSION: The chest CT findings of COVID-19 show significant changes according to age and gender. The findings that are more common in elderly and male patients should be carefully evaluated in terms of the prognosis of the disease.

KEYWORDS: COVID-19. Computerized tomography. Age. Gender.

INTRODUCTION

The novel coronavirus disease 2019 (COVID-19) is an infectious disease, which was first identified in December 2019 and declared a pandemic in March 2020, spreading rapidly to the whole world and causing the death of 1,280,000 people as of November 11, 2020[1]. COVID-19 is a viral disease that spreads rapidly from person to person and can cause serious health problems, such as pneumonia, necrotizing encephalopathy, systemic and pulmonary thromboembolism, acute respiratory distress syndrome, respiratory failure, and sepsis[1-3]. Approximately, 15–20% of patients have the serious form of the disease, and the mortality rate is around 2–3%[4].

Recent studies have highlighted the importance of a chest CT examination in COVID-19 patients with false negative RT-PCR results and reported CT sensitivity as 98%[5,6]. However, chest CT images may show different imaging features in COVID-19 patients depending on the stage and severity of the disease[7].

The typical CT features of COVID-19 pneumonia are bilateral and multifocal ground-glass opacities (GGO). Classically, lesions are predominant in the peripheral, posterior, and basal parts of the lungs[8]. Despite the large number of

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studies on the CT findings of COVID-19, there are only limited studies evaluating the distribution of findings by gender and age. Most of the available studies present the comparison of pediatric and adult patients without age grouping.

The aim of this study was to evaluate the distribution of the CT findings of the COVID-19 disease by age and gender.

METHODS

This retrospective study was carried out with 1,024 patients who were admitted in the Sakarya University Education and Research Hospital between January 1 and April 1, 2020 with the suspicion of COVID-19. The study protocol was approved by the local ethics committee of the Faculty of Medicine of the University (IRB No. 71522473/050.01.04/221). The RT-PCR assay of the nasopharyngeal or oropharyngeal swab samples and chest CT imaging data of 1,024 patients were recorded within the scope of the study. The inclusion criteria were having a positive PCR-RT test and complete CT findings. The exclusion criteria were having a chronic lung disease or a history of any lung pathology. The sample was divided into nine groups based on age decades.

Computed tomography protocol

For the investigation of COVID-19 pneumonia, all the patients underwent nonenhanced chest CT examinations in the supine position during end-inspiration. The CT scans of the patients were obtained with a 64-section multidetector CT scanner (Aquilion 64, Toshiba, Japan) using the following protocols: tube voltage 120 kV, automatic tube current 120–380 mA, thickness 5 mm, slice interval 5 mm, rotation speed 0.5 s, and helical pitch 1.0875:1 or 1.375:1 for the adults, and tube voltage 100 kV, automatic tube current 30–100 mA, thickness 3–5 mm, slice interval 1 mm, rotation speed 0.6 s, and helical pitch 0.969:1 for the pediatric patients. Lung window images at 0.625–1 mm thickness were reconstructed using the iterative technique. The informed consent for the CT examination was obtained from all patients or from their parents. The chest CT features were reviewed by two radiologists (AK and YG with 10 and 15 years of experience in thoracic imaging, respectively) based on consensus. Any controversy between the two radiologists evaluating thorax CT findings was resolved by consulting a third experienced radiologist (MHO with more than 20 years of experience in thoracic imaging). The imaging findings were evaluated comparatively by gender and age groups.

Statistical analysis

Statistical analysis was performed with SPSS v23.0 (IBM, New York, NY, USA), and $p<0.05$ indicated a statistically significant difference. Quantitative variables were expressed as mean or median values and standard deviation. Categorical variables were compared with the Pearson's chi-square test.

RESULTS

A total of 1,024 patients, 546 men (53.32%) and 478 women (46.68%), were included in the study. The average age was 46.29±17.99 years. The distribution of the CT findings is shown in Figure 1. GGO was present in 715 patients (69.82%), consolidation in 350 (34.18%), intra-interlobular septal thickening in 152 (14.84%), crazy-paving pattern in 96 (9.38%), thin reticular pattern in 86 (8.40%), airway changes in 72 (7.03%), air bronchogram in 62 (6.05%), fibrosis in 48 (4.69%), lymphadenopathy in 35 (3.42%), intraparenchymal vascular enlargement in 28 (2.73%), pleural effusion in 25 (2.44%), air bubbles in 21 (2.05%), tree-in-bud pattern in 20 (1.95%), halo sign in 20 (1.95%), reverse halo sign in 17 (1.66%), centrilobular nodule in 16 (1.56%), and pericardial effusion in 11 (1.07%).

The results of the comparison of the CT findings by gender are given in Table 1. In the males, the rates of centrilobular nodules (2.56%), airway changes (9.16%), and tree-in-bud pattern (2.75%) were significantly higher compared to the females (0.42, 4.60, and 1.05%, respectively) ($p=0.006$, 0.004, and 0.050, respectively). The statistically significant findings according to the gender comparison are shown in Figure 2.

When the CT findings were compared between the age groups, there were significant differences in terms of the rates of GGO, consolidation, intra-interlobular septal thickening, thin reticular pattern, fibrosis, extrapulmonary involvement (pleural effusion), extrapulmonary involvement (lymphadenopathy), air bubble, air bronchogram, airway changes, pericardial effusion, and crazy-paving pattern. The results of the comparison of the CT findings by age are given in Table 2.

Under 20 years of age, except for the GGO and consolidation, no other finding was observed.

The airway changes and crazy-paving pattern were more common over 80 years.

While the tree-in-bud pattern was most frequently seen in the 20–29 years age group, the halo sign was more common in the patients aged 30–39 years compared to the other age groups. Unlike other ages, the thin reticular pattern was more common in the 60–79 years age group. The higher frequencies of all these findings in different age groups were statistically significant ($p<0.001$).

Some of the CT findings are shown in Figure 3.

Furthermore, the chi-square trend analysis revealed that the significant relationship of the CT findings with age had an increasing trend as the age progressed.

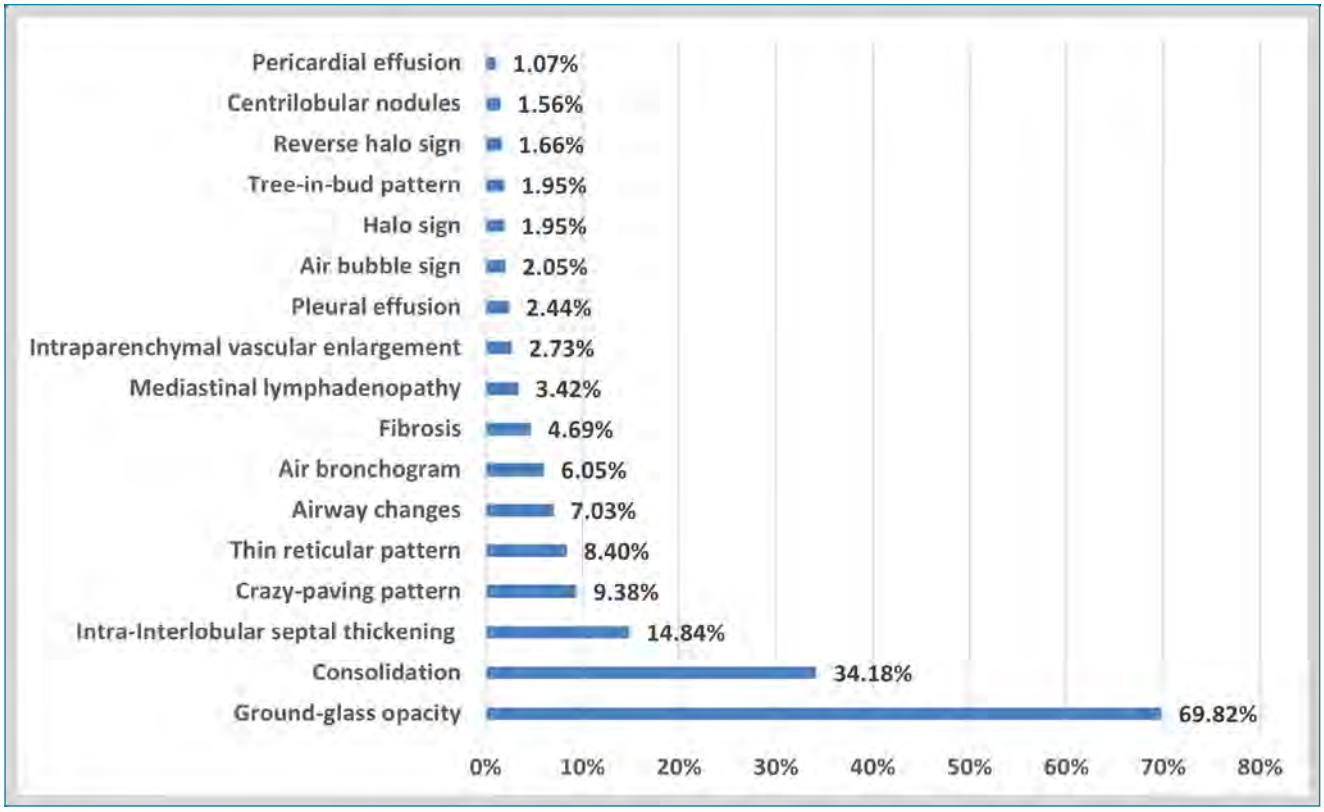

Figure 1. Distribution of computed tomographic findings of COVID-19.

Table 1. Distribution of parenchymal abnormalities according to gender.

	Male		Female		p-value
	n	%	n	%	
Ground-glass opacity	384	(70.3)	331	(69.25)	0.706
Consolidation	185	(33.88)	165	(34.52)	0.830
Intra-interlobular septal thickening	89	(16.30)	63	(13.18)	0.161
Thin reticular pattern	52	(9.52)	34	(7.11)	0.165
Centrilobular nodules	14	(2.56)	2	(0.42)	0.006*
Intraparenchymal vascular enlargement	20	(3.66)	8	(1.67)	0.051
Halo sign	8	(1.47)	12	(2.51)	0.228
Reverse halo sign	11	(2.01)	6	(1.26)	0.343
Fibrosis	30	(5.49)	18	(3.77)	0.192
Pleural effusion	13	(2.38)	12	(2.51)	0.893
Mediastinal lymphadenopathy	23	(4.21)	12	(2.51)	0.135
Air bubble sign	13	(2.38)	8	(1.67)	0.426
Air bronchogram	39	(7.14)	23	(4.81)	0.119
Airway changes	50	(9.16)	22	(4.60)	0.004*
Pericardial effusion	3	(0.55)	8	(1.67)	0.082
Tree-in-bud pattern	15	(2.75)	5	(1.05)	0.050*
Crazy-paving pattern	54	(9.89)	42	(8.79)	0.546

*p<0.05 is statistically significant.

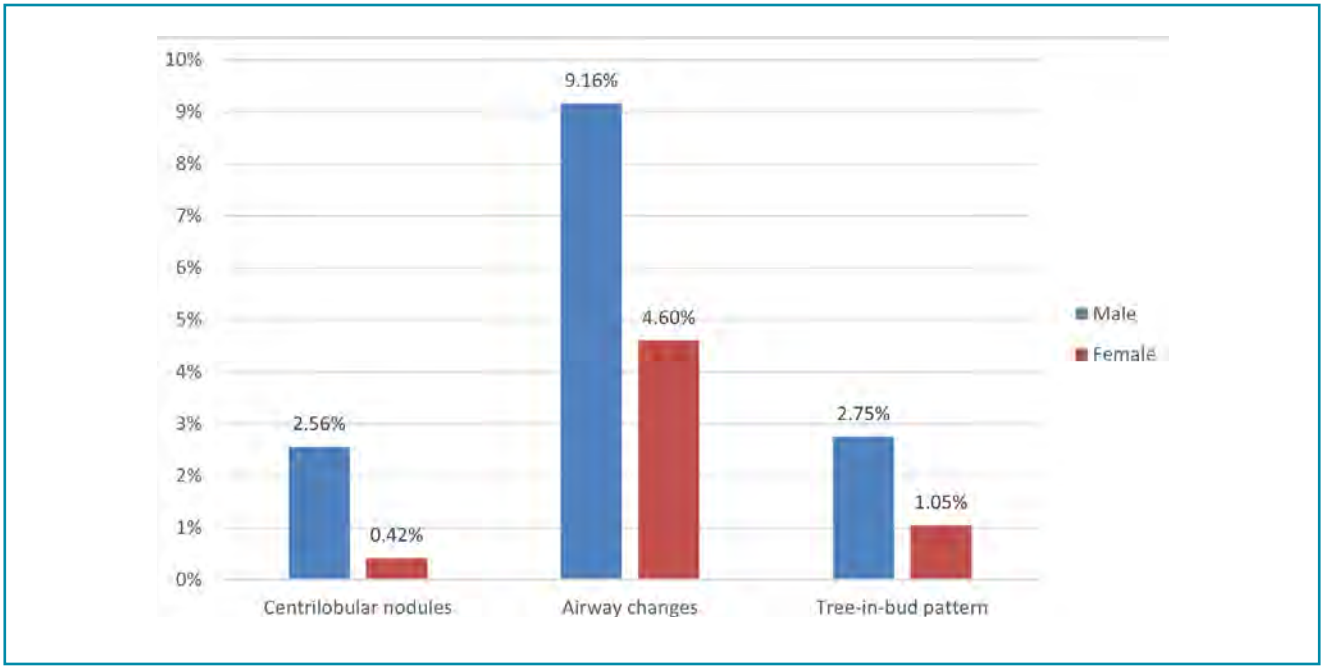

Figure 2. Statistically significant findings by gender.

Table 2. Distribution of parenchymal abnormalities according to age.

	0–9 age		10–19 age		20–29 age		30–39 age		40–49 age		50–59 age		60–69 age		70–79 age		≥80 age		p
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	
Ground-glass opacity	4	(66.67)	10	(27.78)	54	(36.24)	131	(60.65)	161	(72.85)	124	(83.78)	103	(93.64)	85	(93.41)	43	(91.49)	<0.001*
Consolidation	3	(50.00)	4	(11.11)	24	(16.11)	54	(25.00)	61	(27.60)	62	(41.89)	57	(51.82)	55	(60.44)	30	(63.83)	<0.001*
Intra–interlobular septal thickening					2	(1.34)	16	(7.41)	17	(7.69)	26	(17.57)	33	(30.00)	40	(43.96)	18	(38.30)	<0.001*
Thin reticular pattern					2	(1.34)	6	(2.78)	15	(6.79)	11	(7.43)	25	(22.73)	21	(23.08)	6	(12.77)	<0.001*
Centrilobular nodules					4	(2.68)			3	(1.36)	2	(1.35)	3	(2.73)	3	(3.30)	1	(2.13)	0.408
Intraparenchymal vascular enlargement					1	(0.67)	3	(1.39)	12	(5.43)	3	(2.03)	5	(4.55)	2	(2.20)	2	(4.26)	0.105
Halo sign			1	(2.78)	5	(3.36)	8	(3.70)	2	(0.90)	2	(1.35)	1	(0.91)			1	(2.13)	0.323
Reverse halo sign					1	(0.67)	2	(0.93)	4	(1.81)	7	(4.73)	1	(0.91)	2	(2.20)			0.142
Fibrosis							7	(3.24)	6	(2.71)	12	(8.11)	4	(3.64)	9	(9.89)	10	(21.28)	<0.001*
Pleural effusion							1	(0.46)	2	(0.90)	2	(1.35)	3	(2.73)	7	(7.69)	10	(21.28)	<0.001*
Mediastinal lymphadenopathy					3	(2.01)	1	(0.46)	2	(0.90)	4	(2.70)	11	(10.00)	7	(7.69)	7	(14.89)	<0.001*
Air bubble sign					2	(1.34)			2	(0.90)	2	(1.35)	5	(4.55)	8	(8.79)	2	(4.26)	<0.001*
Air bronchogram					5	(3.36)	5	(2.31)	9	(4.07)	6	(4.05)	8	(7.27)	17	(18.68)	12	(25.53)	<0.001*
Airway changes					4	(2.68)	7	(3.24)	8	(3.62)	6	(4.05)	14	(12.73)	21	(23.08)	12	(25.53)	<0.001*
Pericardial effusion											1	(0.68)	1	(0.91)	6	(6.59)	3	(6.38)	<0.001*
Tree-in-bud					7	(4.70)	5	(2.31)	3	(1.36)	1	(0.68)	2	(1.82)			2	(4.26)	0.168
Crazy-paving pattern					1	(0.67)	6	(2.78)	14	(6.33)	18	(12.16)	23	(20.91)	20	(21.98)	14	(29.79)	<0.001*

*p<0.05 is statistically significant.

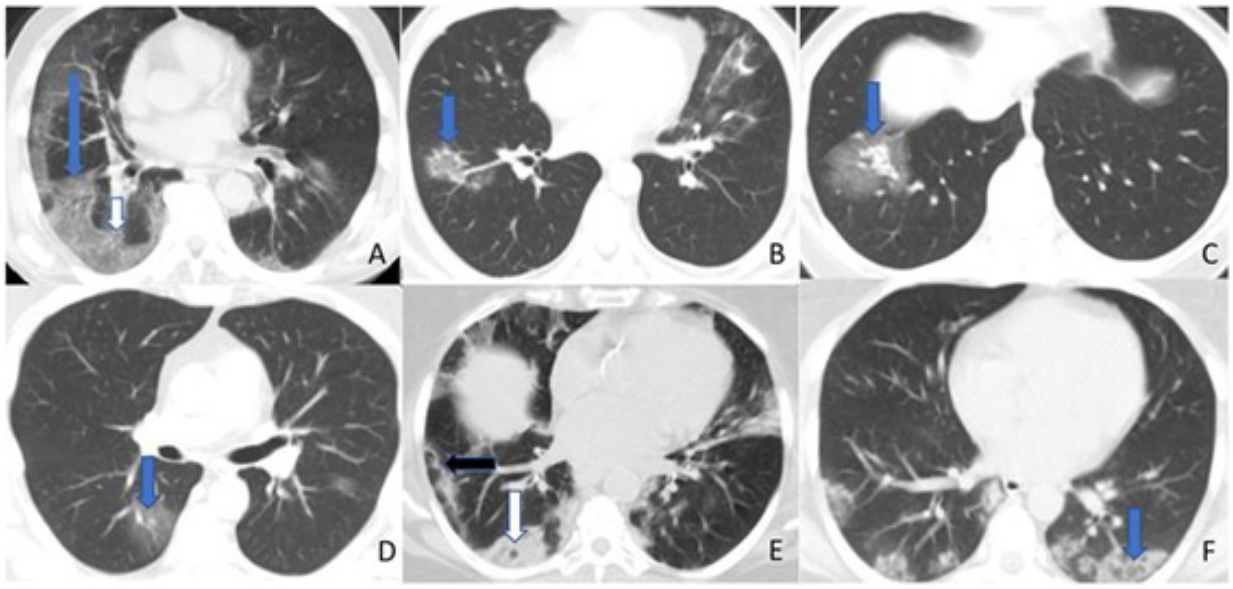

Figure 3. **(A)** Multiple ground-glass opacities and consolidation with a thickened intra-interlobular septum (blue arrow). The air bronchogram sign (white arrow). **(B)** Reversed halo sign in the right lower lobe upper segment (blue arrow). **(C)** Halo sign, central denser consolidation surrounded by ground-glass opacity of crescent shape in right lower lobe (blue arrow). **(D)** Multiple patchy ground-glass opacities with consolidation scattered in the peripheral zone of lower lobe, presenting with poorly defined boundaries and vascular thickening (blue arrow). **(E)** Ground-glass opacities with the air bubble sign in the upper lobe of the right lung (white arrow) and ground-glass opacities with subpleural line in the right inferior lobe (black arrow). **(F)** Crazy-paving pattern, multiple ground-glass opacities distributed in the peripheral area, ground-glass opacities with consolidation, and thickened intralobular septum (blue arrow).

DISCUSSION

This retrospective study showed that the CT manifestations of patients of different ages and gender were not exactly the same. Therefore, determining their CT features can help deepen our understanding of differences in disease characteristics between different age groups and can assist in clinical diagnosis and treatment decision-making.

In patients infected with COVID-19, the mortality increases with the increase in age, which is attributed to the higher incidence of comorbid diseases in advanced ages[9]. However, the disease can also have a severe course in young people without any comorbidity or may present with mild symptoms in elderly individuals with certain comorbidities. Zhu et al. found no significant difference in the development and progression stages of the disease in young and elderly patients[10]. However, many previous studies show that being old, male, and having comorbid diseases are poor prognostic factors for COVID-19[11]. Li et al. stated that the mortality rate among elderly patients was significantly higher in males but they also noted no significant difference in mortality according to age[12].

Chest CT is the key imaging modality in the early diagnosis of COVID-19, with the most common CT findings being patchy subsegmental or multiple GGOs with bilateral segmentation[13]. In all ages, the most common finding of COVID-19 pneumonia is GGO. The multiple and bilateral incidence of GGO was found to be statistically significant in elderly patients. In addition, a strong relationship was observed between age and the number of involved lobes[14]. Wang et al. reported that non-subpleural distribution, single lesions, and limited number of involved lobes were common in patients under 40 years of age[15]. Similarly, the most common finding in this study was GGO, with its incidence significantly increasing over the age of 60 years.

Song et al. stated that consolidation was seen more frequently in young patients and GGO in elderly patients, and the area of the lungs involved was more in elderly patients[16].

However, we found that the consolidation incidence increased over the age of 70 years.

In this study, the halo sign was more common in 30–39 years. Xia et al. stated that the halo sign accounted for up to 50% of pediatric cases as a typical sign in pediatric patients[17]. In this study, the rate of the halo sign in pediatric patients was only 2.78%. The incidence of subpleural line and pleural thickness is higher in elderly patients than young people[10]. Wang et al. reported that moderate pleural thickening and bronchiectasis were more common in elderly patients[15]. Gu et al. stated that the interlobular thickening and honeycomb pattern findings were more common in elderly people[18]. In this study, the thin reticular pattern finding was more common in the patients aged 60–79 years, and airway changes and the crazy-paving pattern were more common over 80 years. The higher rates of both pleural and airway findings in elderly patients were consistent with the literature.

Gu et al. reported that nodules were more common in younger people[18]. We found no significant difference in terms of nodule incidence. In this study, the more common finding in the young age group was the tree-in-bud pattern, which was mostly seen in the 20–29 years age group.

According to the reports of the World Health Organization, COVID-19 infection is more common in men, but the effect of gender difference on prognosis remains unclear[19]. Ueyama et al. stated that COVID-19 infection was more severe in men, but there was no significant difference in mortality between the genders[20]. Gu et al. found no significant difference between genders in their study with 50 patients[18]. In this study, the more common findings in men compared with women were centrilobular nodules, airway changes, and tree-in-bud pattern. The higher frequencies of all these three findings in men were statistically significant.

There are some limitations in this study. We used single imaging for each patient and we did not evaluate the progress of COVID-19 with dynamic imaging. This study mostly emphasized on the CT imaging features of COVID-19 and the differences between age groups and gender. We did not explore the relationship between the COVID-19 imaging findings and disease prognosis or mortality.

CONCLUSION

Multiple lobes being affected, subpleural lesions, crazy-paving pattern, bronchodilation, and pleural thickening are more common COVID-19 findings in elderly patients than in young people. Each age group has its own characteristics, and having knowledge on these features can help better understand the disease, make accurate diagnosis, and manage follow-up. There were significant differences in the imaging findings of the patients with confirmed COVID-19 pneumonia by age and gender. These differences became more evident in advanced ages. We consider that this study will contribute to the literature in terms of showing that the COVID-19 disease progresses asymptomatically or mildly in younger ages and progresses seriously in advanced ages, with the mortality rate being markedly higher among the elderly patients.

AUTHORS' CONTRIBUTIONS

AK: Data curation, Investigation, Resources, Validation, Writing-original draft. **ZK:** Formal analysis, Methodology, Software. **OT:** Project administration, Writing-review & editing. **YG:** Visualization. **MHO:** Conceptiualization. **OFA:** Supervision. **FG:** Finding acquisition.

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ORIGINAL ARTICLE
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The association between serum uric acid to high density lipoprotein-cholesterol ratio and non-alcoholic fatty liver disease: the abund study

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SUMMARY

OBJECTIVE: Non-alcoholic fatty liver disease, which is characterized by lipid being deposited into hepatocytes, affects nearly one in three adults globally. Inflammatory markers were suggested to be related with hepatic steatosis. Uric acid to HDL cholesterol ratio is proposed as a novel inflammatory and metabolic marker. We aimed to compare Uric acid to HDL cholesterol ratio levels of patients with Non-alcoholic fatty liver disease to those of healthy controls and find out potential correlations between Uric acid to HDL cholesterol ratio and other inflammatory and metabolic markers of Non-alcoholic fatty liver disease.

METHODS: Patients with a diagnosis of Non-alcoholic fatty liver disease who were on clinical follow-up in our institution were enrolled in the study as the Non-alcoholic fatty liver disease group, while healthy volunteers were enrolled as the control group. The Uric acid to HDL cholesterol ratio of the groups was compared and potential correlations were studied between Uric acid to HDL cholesterol ratio and fasting blood glucose, transaminases, serum lipids (triglyceride, LDL-cholesterol), weight, and body mass index.

RESULTS: The Uric acid to HDL cholesterol ratio of the Non-alcoholic fatty liver disease (13±5%) group was significantly higher compared to the Uric acid to HDL cholesterol ratio of the control (10±4%) group ($p<0.001$). Uric acid to HDL cholesterol ratio was significantly and positively correlated with fasting blood glucose, transaminases, triglyceride, body weight, waist circumference, hip circumference, and body mass index. A ROC analysis revealed that a Uric acid to HDL cholesterol ratio level greater than 9.6% has 73% sensitivity and 51% specificity in determining Non-alcoholic fatty liver disease.

CONCLUSION: Due to the inexpensive and easy-to-assess nature of Uric acid to HDL cholesterol ratio, we suggest that elevated Uric acid to HDL cholesterol ratio levels be considered a useful tool in diagnosing hepatic steatosis.

KEYWORDS: Inflammation. Liver steatosis. Uric acid. HDL cholesterol.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is characterized by lipid being deposited into hepatocytes. It affects nearly one in three adults globally, especially in developed territories. The clinical spectrum of the disease includes hepatic steatosis, steatohepatitis, fibrosis, and even cirrhosis[1]. In addition to hyperlipidemia, the burden of chronic inflammatory also contributes to the pathogenesis of NAFLD[2]. Indeed, inflammatory markers, including C-reactive protein (CRP), mean platelet volume (MPV), red cell distribution width (RDW), and mean platelet volume to platelet count ratio were suggested to be associated with hepatic steatosis[3-7].

Uric acid is an end product of the metabolism of purine (adenine and guanine). High serum uric acid levels can trigger inflammation since antigen-presenting cells have been reported to sense uric acid as a cause of endogenous pro-inflammatory signal[8]. In fact, decreased uric acid levels are associated with reduced inflammatory burden[9]. Higher uric acid levels are associated with the development of various conditions that are associated with chronic low-grade inflammation, such as, type 2 diabetes mellitus, obesity, and metabolic syndrome[10-12]. It is also associated with the control level of diabetes mellitus and correlates with glycated hemoglobin (HbA1c) levels in diabetic subjects[13,14]. Accordingly, elevated serum uric acid levels were reported to be associated with non-alcoholic fatty liver disease in the literature[15-17]. Hepatic steatosis is suggested to be promoted by elevated serum uric acid levels[18].

Uric acid to HDL cholesterol ratio (UHR) is proposed as a novel inflammatory and metabolic marker in recent research studies. It has higher sensitivity and specificity compared to other criteria of metabolic syndrome in diagnosing the disease[19]. Moreover, HbA1c and fasting plasma glucose (FPG) levels of type 2 diabetic patients were significantly and positively correlated with serum uric acid levels[20]. It is also considered to be related with cardiac conditions[21]. In addition, high UHR levels were associated with increased risk of NAFLD in a study by Zhang et al[22].

In the present study, we aimed to compare the UHR levels of patients with NAFLD to those of healthy controls. We also aimed to observe potential correlations between UHR and other inflammatory and metabolic markers in NAFLD.

METHODS

Study population

Patients with a diagnosis of NAFLD who were on clinical follow-up in the gastroenterology and internal medicine outpatient clinics of our institution between January 2019 and January 2020 were enrolled in this retrospective study. Control subjects consisted of healthy volunteers that visited our institution for a routine check-up. Patients under 18 years of age, pregnant women, or patients with any other type of liver disease were not included in the study. Patients with active infection, inflammatory diseases (i.e. rheumatoid arthritis), and malignant conditions were also excluded. The local ethics committee approved the study protocol (approval number: 2020/202).

Laboratory analyses

Age, gender, height, body weight, waist circumference, and hip circumference of the subjects were obtained from the patients' files and database of the institution. The waist to hip ratio was calculated dividing the waist circumference by the hip circumference in centimeters. The body mass index (BMI) was calculated dividing the body weight in kilograms by the height in meters squared. Cigarette smoking, alcohol drinking, and physical exercise history of the subjects were also recorded. Fasting blood glucose (FBG), fasting insulin, aspartate and alanine transaminases (AST and ALT), gamma-glutamyl transferase (GGT), uric acid, total cholesterol, LDL cholesterol, HDL cholesterol, and serum triglyceride of the subjects were also obtained and recorded. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following equation: (FBG x fasting insulin)/405. Insulin resistance was considered to be present when HOMA-IR was greater than 2.5. UHR was obtained dividing serum uric acid levels by HDL-cholesterol levels. General characteristics and laboratory variables of the study groups were compared.

Statistical analyses

Statistical analyses were conducted with statistic software (SPSS 15.0 for Windows, IBM Co., Chicago, Il, USA). Distribution of the variables among study groups was analyzed with Kolmogorov-Smirnov test. Variables with normal distribution were compared using independent samples t-test and these variables were expressed as mean±standard deviation (SD). On the other hand, variables without normal distribution were compared using the Mann Whitney-U test and these variables were expressed as median (min–max). Chi-square test was used to compare categorical variables among study groups. Correlation between study variables was analyzed with Pearson's correlation test. UHR sensitivity and specificity in selecting NAFLD patients were analyzed with a receiver operating characteristic (ROC) curve. When p-value was lower than 0.05, it was considered statistically significant.

RESULTS

Once subjects who did not meet the inclusion criteria were excluded, a total of 117 subjects, 60 patients with NAFLD and 57 healthy volunteers, was enrolled in the study. The median ages of the NAFLD and control groups were 49 (27–81) years and 46 (18–73) years, respectively (p=0.19). Thirty-three out of 60 subjects (55%) in the NAFLD group were men and 27 (45%) were women, while 27 out of 57 subjects (47%) in the control group were men and 30 (53%) were women (p=0.41).

The height (p=0.94), waist to hip ratio (p=0.25), and HDL cholesterol (p=0.06) of the study and control groups were not significantly different.

The body weight (p<0.001), BMI (p<0.001), waist circumference (p<0.001), hip circumference (p<0.001), fasting insulin (p<0.001), FBG (p<0.001), AST (p=0.001), ALT (p=0.001), GGT (p=0.003), triglyceride (p<0.001), total cholesterol (p=0.002), LDL cholesterol (p=0.04), uric acid (p<0.001), and HOMA IR (p<0.001) levels of the NAFLD group were significantly higher than those of the control group. Table 1 shows the general characteristics and laboratory data of the study cohort.

The rates of smokers (p=0.72), alcohol drinkers (p=0.12) and subjects that exercise regularly (p=0.52) were not statistically different between NAFLD and control groups.

The UHR of the NAFLD (13±5%) group was significantly higher compared to the UHR of the control (10±4%) group (p<0.001).

In a correlation analysis, UHR was significantly and positively correlated with FBG (r=0.23, p=0.01), ALT (r=0.20, p=0.03), triglyceride (r=0.4, p<0.001), body weight (r=0.39, p<0.001), waist circumference (r=0.4, p<0.001), hip circumference (r=0.22, p=0.02), and BMI (r=0.29, p=0.002).

In a ROC analysis, a UHR level greater than 9.6% has 73% sensitivity and 51% specificity in determining NAFLD (Figure 1).

Table 1. General characteristics and laboratory data of the study population.

		NAFLD group	Control group	p
Sex	Men (%)	33 (55)	27 (47)	0.41
	Women (%)	27 (45)	30 (53)	
	Mean±SD			
UHR (%)		13±5	10±4	<0.001
Uric acid (mg/dL)		5.6±1.3	4.6±1	<0.001
LDL cholesterol (mg/dL)		123±37	107±41	0.04
Total cholesterol (mg/dL)		208±44	182±43	0.002
	Median (Min–Max.)			
Height (cm)		168 (130–184)	167 (140–195)	0.94
Weight (kg)		84 (63–120)	68 (46–105)	<0.001
BMI (kg/m^2)		30.1 (25–45)	25 (17.3–35)	<0.001
Hip circumference (cm)		110 (90–157)	100 (75–126)	<0.001
Waist circumference (cm)		103 (85–140)	88 (59–102)	<0.001
Waist to hip ratio (%)		0.9 (0.8–1.1)	0.9 (0.6–1)	0.25
Fasting insulin (uIU/mL)		14.3 (7–64)	8.6 (3.1–17-5)	<0.001
FBG (mg/dL)		99 (80–127)	91 (69–99)	<0.001
AST (U/L)		23 (11–266)	18 (9–157)	0.001
ALT (U/L)		28 (8–160)	18 (6–111)	0.001
GGT (U/L)		26 (9–180)	17 (7–177)	0.003
Triglyceride (mg/dL)		160 (53–414)	95 (31–455)	<0.001
HDL cholesterol (mg/dL)		45 (26–71)	47 (28–103)	0.06
HOMA-IR		3.34 (1.1–14)	1.8 (1–4.7)	<0.001

NAFLD: non-alcoholic fatty liver disease; UHR: uric acid to HDL cholesterol ratio; BMI: body mass index; FBG: fasting blood glucose; AST: transaminases; ALT: transaminases; GGT: gamma-glutamyl transferase; HOMA-IR: homeostasis model assessment of insulin resistance.

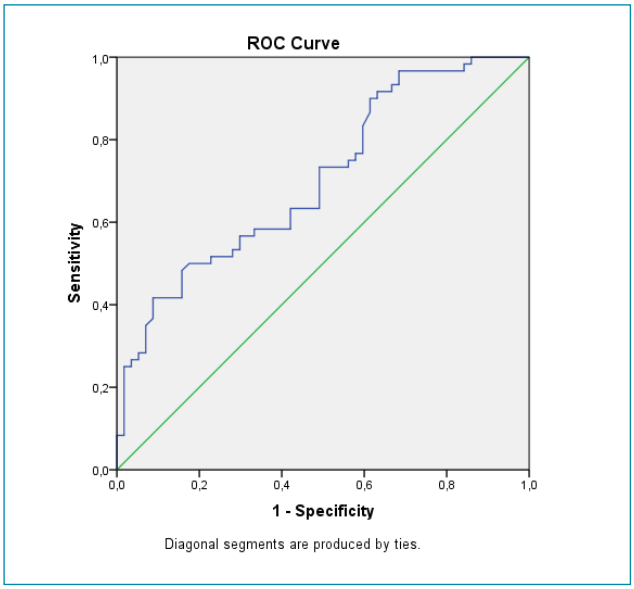

Figure 1. The ROC curve of uric acid to HDL cholesterol ratio in determining non-alcoholic fatty liver disease.

DISCUSSION

The present study showed that UHR is significantly increased in subjects with NAFLD compared to the healthy population. Moreover, UHR has significant positive correlation with other determinants of NAFLD, such as, BMI, waist circumference, hip circumference, blood glucose, ALT, and triglyceride levels. Finally, the present study demonstrated that increased UHR has high sensitivity and considerable specificity in selecting NAFLD subjects.

Uric acid is an end product of the metabolism of purine and is associated with a variety of chronic conditions. Elevated serum uric acid levels were suggested to be linked with type 2 diabetes mellitus and hypertension[23]. Indeed, the authors showed that 1 mg/dL increase in serum uric acid levels increases the risk of incident hypertension 1.2 fold[24]. In another study, it has been claimed that high uric acid levels predicted the development of type 2 diabetes mellitus[25]. A meta-analysis in type 2 diabetic subjects suggested that increased uric acid levels were an independent marker of vascular complications and mortality in this population[26]. The risk of microvascular complications of type 2 diabetes mellitus is increased in subjects with high uric acid levels and low total bilirubin blood levels[27]. The combination of uric acid and HDL cholesterol has been proposed as a novel and more sensitive marker of metabolic and inflammatory conditions. UHR has been shown to be higher in metabolic syndrome and suggested to have greater sensitivity and specificity than any other criteria used to select subjects with metabolic syndrome[19]. Since hepatic steatosis was associated with metabolic syndrome[28], a similar increase in the UHR in subjects with hepatic steatosis could be expected. In 2020, the authors reported elevated UHR levels in subjects with non-alcoholic fatty liver disease compared to controls[22]. However, since the study population consisted of subjects with a BMI lower than 24kg/m^2, this association was only applied for lean adults. In the present study, the BMI of the subjects with hepatic steatosis was significantly higher than the BMI of control subjects. Additionally, UHR was significantly correlated with BMI in the study population.

Increased UHR was reported in other conditions as well. Higher UHR has been reported in patients with coronary artery fistula compared to control subjects with normal coronary arteries[21]. Furthermore, in a recent study, elevated UHR was reported in poorly controlled diabetic subjects compared to well-controlled diabetic subjects and non-diabetic controls[20]. UHR was significantly and positively correlated with waist circumference, body weight, body mass index, fasting glucose, and HbA1c levels in a study mentioned in the literature[20]. Similarly, we reported that UHR was positively correlated with FBG, body weight, BMI, and waist circumference. In addition, we found a positive correlation between UHR and ALT, triglyceride, and hip circumference.

UHR is calculated dividing serum uric acid levels by HDL cholesterol and is an inexpensive, easy-to-assess tool. Therefore, it could be measured repeatedly during the follow-up of subjects with hepatic steatosis. Elevated serum uric acid levels are associated with hepatic steatosis, as reported in a Chinese study[29]. Furthermore, the authors reported decreased HDL cholesterol levels in subjects with non-alcoholic fatty liver disease[30]. Thus, uric acid to HDL cholesterol ratio could be a better predictor of hepatic steatosis. In the present study, despite uric acid and UHR levels were significantly increased in patients with hepatic steatosis compared to healthy controls, the HDL cholesterol of the study subjects was not statistically different.

Our study confirmed that UHR could be a marker of hepatic steatosis, and due to its inexpensive and easy-to-assess nature, it might also be useful to follow the treatment of the disease. However, our study did not answer whether elevated UHR in hepatic steatosis begins to decrease after lifestyle modification or medical treatment of the subjects with liver steatosis. A prospective study, rather than a retrospective report, could answer this question.

The present study has two limitations. First, the retrospective design, which could make the results of the study difficult to interpret. Second, a relatively small study population. However, to the best of our knowledge, this is the first study in the literature that reported both a significant association between UHR and hepatic steatosis, and a

significant correlation between UHR and other metabolic risk factors of liver steatosis.

CONCLUSION

We suggest that elevated UHR be considered an indicator of hepatic steatosis in otherwise healthy subjects. Since obtaining UHR by simply dividing serum uric acid levels by HDL cholesterol levels is easy and inexpensive, UHR may be useful to diagnose and follow subjects with hepatic steatosis.

AUTHORS' CONTRIBUTIONS

MAK: Conceptualization, Writing – Original Draft, Writing – Review & Editing. **OK:** Conceptualization, Writing – Original Draft, Writing – Review & Editing. **BMAT:** Conceptualization, Data Curation, Writing – Original Draft, Writing – Review & Editing. **GK:** Conceptualization, Data Curation, Writing – Original Draft, Writing – Review & Editing. **GA:** Conceptualization, Writing – Original Draft, Writing – Review & Editing. **MED:** Data Curation. **TTD:** Data Curation, Writing – Original Draft. **SB:** Writing – Original Draft

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ORIGINAL ARTICLE
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IARS2 regulates proliferation, migration, and angiogenesis of human umbilical vein endothelial cells

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SUMMARY

OBJECTIVE: In this study, we aimed at investigating the role of isoleucyl-tRNA synthetase in the growth, migration, and angiogenesis of human umbilical vein endothelial cells and the underlying molecular mechanism.

METHODS: To assess the role of isoleucyl-tRNA synthetase, we silenced isoleucyl-tRNA synthetase in human umbilical vein endothelial cells using lentiviral 2 specific short hairpin RNAs (short hairpin RNAs 1 and 2) and examined silencing efficiency using real time quantitative polymerase chain reaction and western blot analyses. Short hairpin RNAs 1- isoleucyl-tRNA synthetase had greater knockdown efficiency, it was used in the entire downstream analysis. Short hairpin RNAs 1- isoleucyl-tRNA synthetase silencing effects on cell proliferation, cell colony generation, cell migration, as well as angiogenesis were assessed using cell counting kit-8, colony development, cell migration, and angiogenesis tube formation assays, respectively.

RESULTS: Compared to the control group, anti- isoleucyl-tRNA synthetase short hairpin RNAs significantly silenced isoleucyl-tRNA synthetase expression in human umbilical vein endothelial cells, and suppressed their proliferation, migration, and angiogenic capacity. To characterize the underlying mechanism, western blot analyses showed that isoleucyl-tRNA synthetase knockdown suppressed phosphorylation of extracellular-regulated kinase ½ and protein-serine- threonine kinase, as well as expression of vascular endothelial growth factor, GSK-3β, and β-catenin.

CONCLUSIONS: We have shown, for the first time, the critical role of isoleucyl-tRNA synthetase in human umbilical vein endothelial cells. Our data show that isoleucyl-tRNA synthetase knockdown suppresses human umbilical vein endothelial cell proliferation, migration, and angiogenesis. We have also shown that isoleucyl-tRNA synthetase knockdown suppresses phosphorylation of extracellular-regulated kinase ½ and protein-serine- threonine kinase, as well as expression of vascular endothelial growth factor, GSK-3β, and β-catenin. Together, these data highlight isoleucyl-tRNA synthetase as a potential antitumor anti-angiogenic target.

KEYWORDS: Human umbilical vein endothelial cells. Isoleucine-tRNA ligase. Angiogenic proteins. Cell proliferation. Cell migration

INTRODUCTION

Angiogenesis, the physiological process through which new blood vessels are formed, provides oxygen and nutrients to actively proliferating tumor cells[1]. To support the high proliferative degree of cancer cells, tumors should rapidly form new vascular networks[2]. Multiple clinical studies show that tumors are angiogenesis dependent. This process is needed to support the growth of tumors beyond 1–2 mm^3. Thus, anti-angiogenesis has arisen as a prospective anti-cancer approach[3]. Angiogenesis is strongly modulated by an equilibrium between pro- and anti-angiogenic factors. Multiple factors like VEGF, neuropilin-1, tyrosine kinase with Ig, and bFGF are involved in angiogenesis. Elevated tumor VEGF levels are a key feature in tumor angiogenesis[4]. While multiple promising anti-angiogenic therapy

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trials have been carried out, outcomes have been unsatisfactory[5]. Thus, profound comprehension of the molecular basis of angiogenesis is needed to design potential anti-angiogenic therapies.

Aminoacyl-tRNA synthetases (ARSs) constitute a family of 20 essential enzymes (one for each amino acid) that bind amino acids to their respective tRNAs during protein biosynthesis[6]. In higher eukaryotes ARSs have been shown to be critical for cell growth, differentiation, cytokine activity, tumorigenesis, and angiogenesis[7].

IARS2 (isoleucyl-tRNA synthetase), which belongs to class I mitochondrial ARS family, is a 1012 amino acid protein found in the mitochondrial matrix. IARS2 is documented to enhance cell proliferation, inhibit apoptosis, and enhance tumor ability to form RKO cells[8]. IARS2 knockdown suppresses cell proliferation, cell colony formation, and triggers cell cycle interruption in the gastric cancer cell line, AGS[9]. Taken together, these reports implicate IARS2 in tumor progression. However, the role of IARS2 in angiogenesis remains uncertain. In this study, we used shRNA to silence IARS2 in HUVECs and investigated its effects and underlying mechanisms in modulating proliferation, migration, and angiogenesis.

METHODS

Cell culture

We obtained HUVEC and HEK293T cells from the cell bank of the Chinese Academy of Science (Shanghai, China). They were grown in DMEM enriched with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin. We grew the cells in a humidified environment, at 37°C, 5% CO_2, in an incubator.

Transfection

Lentiviral IARS2 shRNAs and the negative control were designed and produced by Hanbio (Shanghai, China). The target sequences of anti-IARS2 shRNAs were: shRNA-1, 5 '-cagtggtttataaacatcacggata-3' and shRNA-2, 5'-cagcaaggaagagagcaccttataa-3'. They were constructed using pHBLV-U6-ZsGreen-Puro vector. Cells were cultured in 6 cm dishes and transfected with 10 µL of the lentivirus in the presence of polybrene at a final concentration of 10 µg/mL for 48 hours. Stably transfected cells were selected using 3 µg/mL puromycin for 7 days. RT-qPCR and western blot analyses were used to confirm knockdown.

Western blot analysis

Total proteins were isolated from HUVEC and the BCA protein assay Kit (Beyotime, Cat No. P0012) was employed to quantify them. Thirty micrograms of each protein sample were resolved by 10% SDS-PAGE. We transfer-embedded the proteins onto PVDF membranes. After blocking with 5% milk for two hours, we incubated the membranes overnight with the indicated primary antibodies at the membranes at 4°C. After that, we conjugated the membranes with the respective secondary antibodies at RT for two hours. Signal was then developed using FluoChem E system (Bio-Techne) and quantified using ImageJ. The following antibodies were used at 1:1000: rabbit anti-IARS2 (Proteintech, Cat No. 17170-1-AP), rabbit anti-β-catenin (Santa Cruz Biotechnology, Cat No. sc-7963), rabbit anti-AKT (Proteintech, Cat No. 10176-2-AP), rabbit anti-Phospho-Akt (Cell Signaling Technology, Cat No. 4060S), rabbit anti-GSK3β (Proteintech, Cat No. 22104-1-AP), mouse anti-β-actin (Cell Signaling Technology, Cat No. 4967S), rabbit anti-VEGF (Bioss Biotech, Cat No. bs-1665R), rabbit anti-extracellular signal-regulated kinase (ERK1/2) (Cell Signaling Technology, Cat No. 9102S), and rabbit anti-phospho-ERK1/2 (Cell Signaling Technology, Cat No. 4376S).

Cell proliferation and colony formation

To test the proliferative impacts of IARS2 knockdown, we planted 100 µL of 3×10^3 cells/well onto in 96-well culture plates and grew them for five days. Cell proliferation was then measured by adding 10 µL CCK-8 reagent (Dojindo Laboratories, Cat No. CK04) into every well and incubating the plate for two hours at 37°C, followed by 450 nm absorbance reading on a microplate reader. Colony formation analysis was done by culturing 400 cells/well in 6 cm dishes for two weeks, and successive staining was accomplished using crystal violet. We counted the colonies with >50 cells.

Cell migration assessment

We employed the Wound healing test to inspect cell migration. We planted 4×10^5 cells/well in 6-well plates and incubated overnight to 90% confluence. We then a made a wound via scratching the monolayer using a 200 µL pipette tip. Cell migration over the wounded area was then measured after 24 hours.

Transwell chambers without Matrigel were used to measure cell migration capacity. The amount of 5×10^4/well in 200 µL of serum-free medium was cultured on the upper chamber for 48 hours. Subsequently, membrane fixation was accomplished using 100% methanol for 10 minutes. Successive staining was carried out using 0.1% crystal violet for 30 minutes and cell migration was examined under an inverted microscope.

Tube formation assessment

The amount of 50 µL/well of Matrigel was used to coat 96-well plates. After that, we incubated the plates at 37°C for 30 minutes. We subsequently added HUVECs (4×10^4 cells/well) in 100 µL serum-free DMEM on the solidified Matrigel. After 24 hour incubation, the capillary-like structures were then imaged under a digital microscope system.

Real time quantitative polymerase chain reaction analysis

RNA purification from the cells was accomplished using TRIzol reagent following the protocol provided by the manufacturer. Reverse transcription was done using PrimeScript™ RT Master Mix (Takara, Cat No RR036B). RT-qPCR analysis was carried out on an ABI 7500 real-time PCR machine (Applied Biosystems) using SYBR Green Premix Ex Taq™ (Takara, Cat No RR820B). The amplification conditions were 95°C for 30 seconds, 40 cycles at 95°C for 5 seconds, and 60°C for 34 seconds. The primer sequences for IARS2 and β-actin were: IARS2, forward primer, 5'-actgcccgaagtttgtggg-3', reverse primer, 5'-cggtatctg-ccactattcgagtt-3'; and β-actin, forward primer, 5'-tgtaaggttgtc-cagttcaaaagact-3', reverse primer, 5'-ccagctcaccatggatgatg-3'. β-actin was employed as a normalization gene and data were assessed using the delta-delta Ct approach.

Statistical analysis

GrahpPad Prism 7.0 software was employed to conduct the statistical analyses. All data were expressed as mean±SD of three independent experiments. We employed the two-tailed student's t-test to compare the differences between groups. $p<05$ signified statistical significance.

RESULTS

Expression of Isoleucyl-TRNA Synthetase 2 was repressed by short hairpin RNAs-IARS2 in human umbilical vein endothelial cells

To assess the role of IARS2, we knocked down IARS2 in HUVEC cells using two specific shRNAs (shRNA1 and 2) and examined IARS2 titers by western blot analysis, as well as RT-qPCR assays (Figure 1). Because shRNA1-IARS2 had greater knockdown efficiency, it was used in the entire downstream analysis.

Isoleucyl-TRNA Synthetase 2 knockdown suppresses human umbilical vein endothelial cells proliferation, migration, and angiogenesis

CCK-8 analysis revealed that IARS2-silenced HUVECs grew slower than control cells (Figure 2A). Colony formation assays showed that IARS2-silenced cells formed significantly fewer colonies compared to control cells (Figure 2B–C). Similar observations were made from wound healing assays (Figure 2D–E). Additionally, Transwell® migration assay (without Matrigel) revealed suppressed cell migration upon shRNA1-IARS2 knockdown compared to the control group after 48 hours (Figure 2F–G).

Tube-formation assay constitutes a well-documented *in vitro* test for angiogenesis and is centered on the capacity of HUVECs to develop three-dimensional capillary-like tubular structures. To evaluate the role of IARS2 in angiogenesis, control or IARS1-silenced HUVECs were planted on a thin layer of Matrigel and fixed after 24 hours. Tube formation analysis showed that IARS2 knockdown significantly suppressed tube formation, the branches of capillary tube in HUVECs knockdown IARS2 were significantly reduced by 67.0% compared to the control group (Figure 2H), suggesting that IARS2 knockdown suppressed angiogenesis.

Isoleucyl-TRNA Synthetase 2 knockdown alters cell signaling pathways

To elucidate the mechanism by which IARS2 knockdown suppressed HUVECs proliferation, migration, and angiogenesis, we examined cell signaling pathways associated with these processes using a western blot analysis. This analysis found that AKT, ERK and GSK3-β/β-catenin signaling pathways may be involved in IARS2-mediated functions. IARS2 knockdown suppressed phosphorylation of ERK1/2 and AKT. IARS2 knockdown also decreased the expression of VEGF, GSK-3 β, and β-catenin (Figure 3).

DISCUSSION

Multiple studies have shown that tumors are angiogenesis dependent and angiogenesis is a well-known mediator of tumor growth and metastasis. Thus, anti-angiogenic therapies are promising anti-cancer strategies[10].

ARSs expression profiles have been reported as potential cancer prognosis biomarkers because they are associated with overall patient survival for all types of cancer on the human protein atlas repository[11]. Multiple studies have implicated the role of ARSs in tumorigenesis, angiogenesis, and inflammation[12], highlighting their potential as anti-cancer targets.

Past studies suggest that ARSs, including tyrosyl-tRNA synthetase (YARS), tryptophanyl-tRNA synthetase (WARS), threonyl-tRNA synthetase (TARS), seryl-tRNA synthetase (SARS), and glutamyl-prolyl-tRNA synthetase (EPRS) influence angiogenesis. SARS and EPRS are thought to influence angiogenesis by modulating the expression of angiogenic factors, including VEGF[13]. However, few studies have examined the role of IARS2 in angiogenesis.

IARS2 is involved in various diseases, i.e., liver disease, hypotonia, and intellectual disability[14]. IARS2 silencing suppresses cell proliferation in non-small cell lung cancer, induces cell cycle interruption, and enhances apoptosis[15]. In this study, we found that IARS2 knockdown suppresses HUVEC

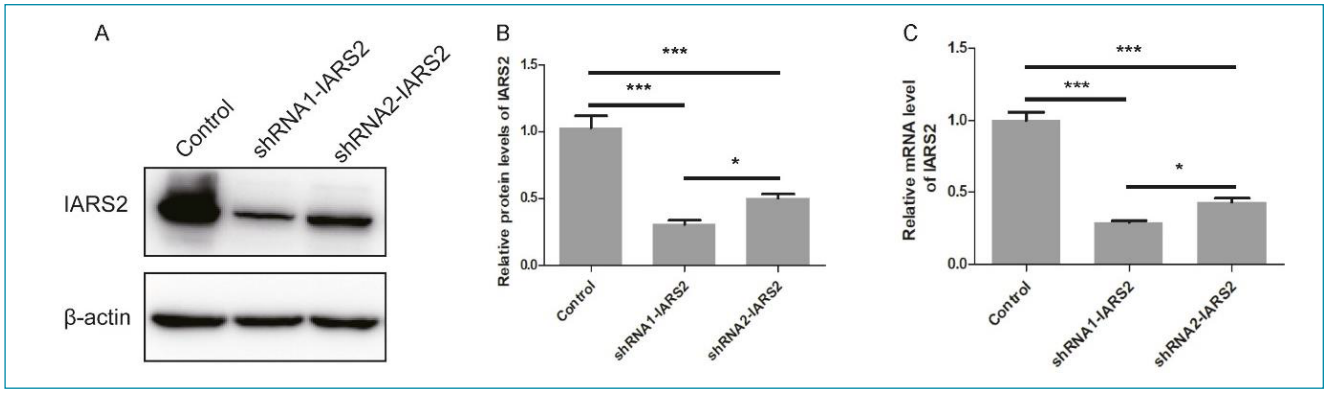

Figure 1. Expression of Isoleucyl-TRNA Synthetase 2 was repressed by short hairpin RNAs 2 in human umbilical vein endothelial cells. **(A)** Human umbilical vein endothelial cells were infected with 2 anti- Isoleucyl-TRNA Synthetase 2 lentiviral short hairpin RNAs or control, and Isoleucyl-TRNA Synthetase 2 protein level measured by western blotting. **(B)** Measurement of Isoleucyl-TRNA Synthetase 2 silencing efficiency by protein level analysis in the human umbilical vein endothelial cells. **(C)** Real time quantitative polymerase chain confirmed Isoleucyl-TRNA Synthetase 2 knockdown. Data are indicated as the mean±SD of three independent assays (*p<0.05, **p<0.01, and ***p<0.001).

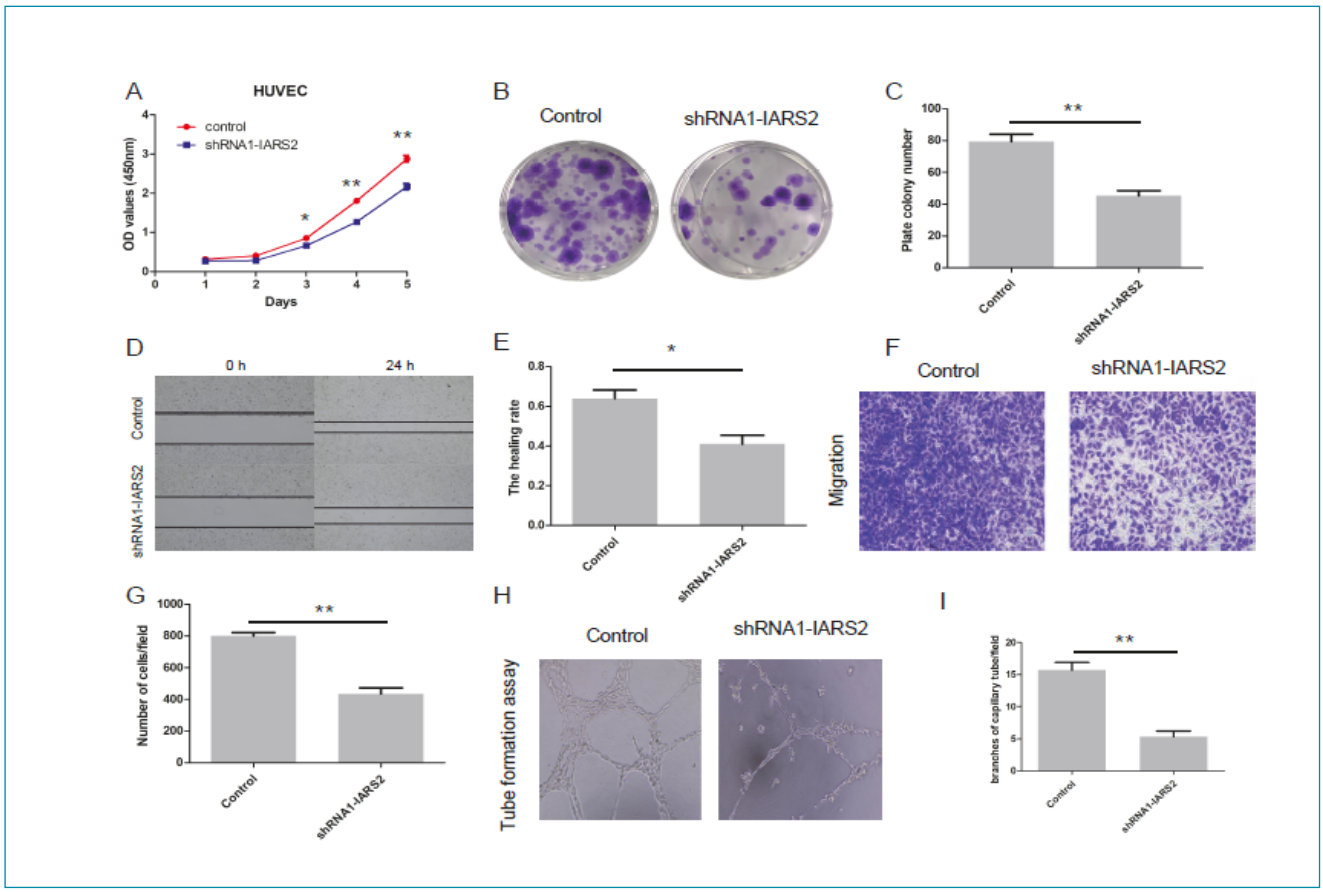

Figure 2. Isoleucyl-TRNA Synthetase 2 knockdown silencing repressed proliferation, migration and angiogenesis of human umbilical vein endothelial cells. **(A)** Cell proliferation was measured using CCK8 analysis. **(B)** Cell colony formation assay images. **(C)** Quantitative analyses of the relative colony number. **(D)** Human umbilical vein endothelial cells migration assay at 0 and 24 h post-wounding. **(E)** Quantification of the above-mentioned assay. **(F)** Human umbilical vein endothelial cells migration upon incubation in permeable supports for 48 h was assessed using crystal violet staining. **(G)** Quantification of the aforementioned assay. **(H)** Illustrative images of tube formation in Human umbilical vein endothelial cells treated with control or Isoleucyl-TRNA Synthetase 2 short hairpin RNAs. **(I)** The branches of capillary tube formed by Human umbilical vein endothelial cells. Data are indicated as mean±SD of three independent experiments (*p<0.05, **p<0.01, and ***p<0.001).

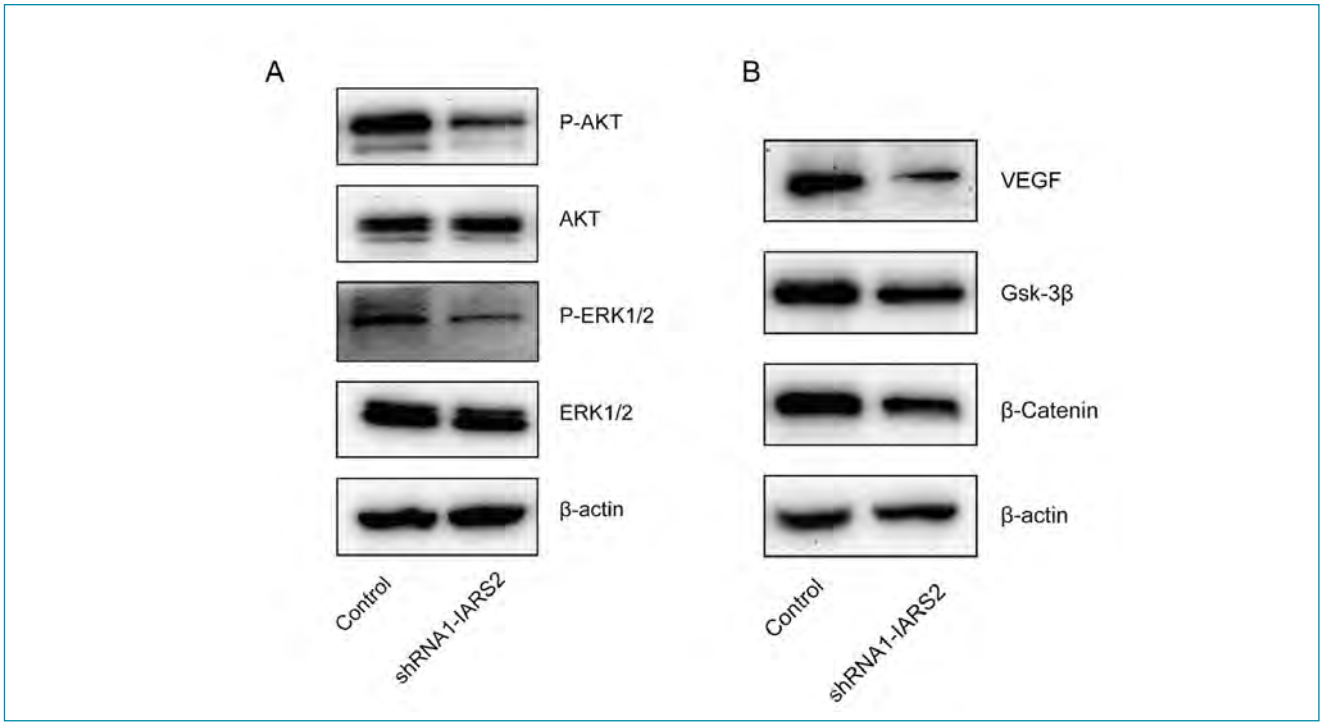

Figure 3. Alterations in signaling pathways upon Isoleucyl-TRNA Synthetase 2 knockdown. **(A)** Western blot analyses of the phosphorylation of extracellular signal-regulated kinase 1/2 and the phosphorylation of protein-serine- threonine kinase in Isoleucyl-TRNA Synthetase 2 knockdown human umbilical vein endothelial cells. **(B)** Western blot analyses of vascular endothelial growth factor, GSK-3β and β-catenin, upon Isoleucyl-TRNA Synthetase 2 knockdown ***versus*** control knockdown. Data are shown as mean±SD of three independent experiments (*p<0.05, **p<0.01, and ***p<0.001).

proliferation, migration, and angiogenesis. IARS2 knockdown also suppressed phosphorylation of ERK1/2, as well as AKT, and reduced expression of VEGF, GSK-3β, and β-catenin. These results enhance our understanding of angiogenic mechanisms and highlight IARS2 as a potential anti-angiogenic target.

Multiple studies have implicated Wnt/β-catenin, PI3K/AKT, Ras/Raf/MEK/ERK, as well as VEGF, in tumor cell metabolism, growth, proliferation, survival, as well as angiogenesis[16]. AKT and ERK pathway inhibitors suppress angiogenesis[17]. Our data show IARS2 knockdown suppresses phosphorylation of ERK1/2 and AKT. VEGF is a crucial angiogenic factor with central roles in physiologic and pathologic tumor angiogenesis. It modulates all the main steps of angiogenesis-linked processes consisting of endothelial cell proliferation, migration, and tube formation[18]. Here, we find that VEGF protein levels are remarkably reduced by IARS2 knockdown. Wnt/β-catenin signaling also mediates angiogenesis, vascular remodeling, and differentiation and Wnt signaling inhibition may suppress angiogenesis. GSK3β directly phosphorylates β-catenin at multiple sites causing its degradation and thus negatively modulating Wnt/β-catenin signaling[19]. IARS2 knockdown inhibited β-catenin and GSK3β protein levels. However, GSK3β may negatively correlate with β-catenin. Thus, the cross-talk between signaling pathways is complex and evidences the need for additional studies.

CONCLUSIONS

In summary, we have shown, for the first time, the critical role of IARS2 in HUVECs. Our data show that IARS2 knockdown suppresses HUVEC proliferation, migration, and angiogenesis. We have also shown that IARS2 knockdown suppresses phosphorylation of ERK1/2 and AKT, as well as expression of VEGF, GSK-3β, and β-catenin. Together, these data highlight IARS2 as a potential antitumor anti-angiogenic target.

AUTHORS' CONTRIBUTIONS

YMY: Conceptualization**. LX:** Conceptualization, Formal Analysis. **HRL:** Writing – original draft. **TQZ:** Writing – review & editing. GQ: Supervision. **LFL:** Supervision. **MHW:** Funding acquisition.

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Investigation of patients with coronary slow flow in terms of periodontal health status

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SUMMARY

OBJECTIVE: This study aims to evaluate the relationship between periodontal health status and coronary slow flow phenomenon.

METHODS: One hundred and two patients who underwent coronary angiography with the diagnosis of stable angina pectoris were included in the study. Patients were divided into two groups: patients with coronary slow flow (Test group) (n=51), and patients with normal coronary angiography (Control group) (n=51). Diagnosis of slow coronary flow was made according to Beltrame criteria by coronary angiography. Demographic characteristics of the participants were recorded. The periodontal health was assessed by clinical periodontal parameters such as probing depth, clinical attachment level, gingival index, plaque index, and bleeding on probing.

RESULTS: There were no significant differences between groups as regards the frequencies of hypertension, smoking ($p>0.05$). As for the periodontal parameters of the study groups, probing depth, gingival index, plaque index, bleeding on probing, and clinical attachment level values were statistically higher in the test group compared to the control group ($p<0.05$).

CONCLUSIONS: Periodontitis might be accepted as one of the underlying causes of coronary slow flow. Patients with coronary slow flow should be evaluated for an underlying periodontal disease, and treatment of periodontal disease can protect against future cardiovascular events.

KEYWORDS: Periodontal diseases. Angiography. Slow-flow phenomenon. Coronary artery disease.

INTRODUCTION

The coronary slow flow (CSF) phenomenon first introduced in 1972 by Tambe et al. in six patients with chest pain, is the late removal of opaque material from normal and/or near-normal epicardial coronary vessels during coronary angiography[1]. The prevalence in patients undergoing coronary angiography for stable angina pectoris is about 1–5%[2]. CSF is associated with several clinical cases such as arrhythmia, angina pectoris, acute myocardial infarction (MI), and sudden death[3]. Information about the etiology of CSF is insufficient. microvascular endothelial dysfunction, Inflammation, and increased coronary microvascular resistance are recommended as the underlying physiopathological causes[4].

Periodontitis is a multifactorial chronic inflammatory disease caused by dysbiotic plaque biofilms and described as progressive destruction of supportive tissues of teeth[5]. It does not affect only the oral cavity and influences general health. It may induce endotoxemia, bacteremia, and

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systemic low-grade inflammation. Furthermore, recent studies reported that periodontitis is commonly seen in patients with cardiovascular disease[6,7].

Although the role of periodontitis as an independent risk factor for atherosclerotic CVDs is established, there is no study evaluating a possible relationship between periodontitis and CSF. In the present study, we aimed to assess the association between periodontal health and coronary slow flow phenomenon.

METHODS

Study population

This is a prospective study that was conducted in the School of Medicine of Bolu Abant Izzet Baysal University between June 2019 and February 2020. The study has been conducted following the principles of the Declaration of Helsinki and approved by the local Institutional Review Board. Written informed consent was obtained from all subjects.

Type I errors (0.05), targeted power (0.80), and effect size (0.50) due to probing depth (PD) value ($p<0.05$) by G* power 3.1.9.4 software program (Heinrich Heine University, Dusseldorf, Germany) were considered for the calculation of the sample size[8]. The minimum required sample size was calculated as 51.

A total of 102 patients who experienced coronary angiography with the diagnosis of stable angina pectoris were enrolled for the study. While patients with coronary slow flow were determined as the test group (group 1, n=51), patients with normal coronary angiography were assigned as a control group (group 2, n=51). Demographic characteristics of the participants were recorded. The periodontal health of subjects was also evaluated by clinical periodontal parameters.

Diagnosis of slow coronary flow was made according to Beltrame criteria; Angiographic evidence of CSFP, described by: a) No evidence of obstructive epicardial coronary artery disease (CAD) (no lesions ≥40%), b) Delayed distal vessel contrast opacification as evidenced by either: TIMI 2 flow (requiring ≥3 beats to opacify the vessel) or corrected TIMI frame count >27 frames (images acquired at 30 frames/s), c) Delayed distal opacification in at least 1 epicardial vessel[9].

Individuals with the following conditions were excluded: patients who had previously undergone percutaneous intervention or bypass surgery, had periodontal treatment within 6 months, are using antiarrhythmic drugs, had coronary ectasia, diabetes mellitus, systemic diseases predisposing to periodontal diseases, including immune deficiency and autoimmunity; used antibiotics and/or anti-inflammatory drugs in the last 6 months.

Periodontal examination

All clinical periodontal parameters were evaluated by the same periodontist (G.U), and a calibration exercise was done to obtain acceptable interexaminer reproducibility. Periodontal examinations were performed with a Williams probe (Hu-Friedy, Chicago, IL, USA). The clinical parameters of PD, clinical attachment level (CAL) gingival index (GI)[10], and plaque index (PI)[11] were measured for every tooth present in the oral cavity. The measurements were performed at six sites (mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual, distolingual) and the results were recorded in approximation to the nearest whole millimeter. The distance from the bottom of the pocket to the cementoenamel junction was defined as CAL and this distance between these two points was measured and recorded (Figure 1). The mean PD and the mean CAL values were calculated by dividing the total score of all teeth by the total number of teeth examined during the study. The periodontal probe was carefully and gently introduced into the gingival sulcus to calculate the percentage of BOP, even one site with BOP was recorded as (+) for each tooth.

Loe & silness gingival index[10]

Score 0: Normal gingiva; Score 1: Slight inflammation – slight change in color, slight edema. No bleeding on probing; Score 2: Moderate inflammation – redness, edema, glazing. Bleeding on

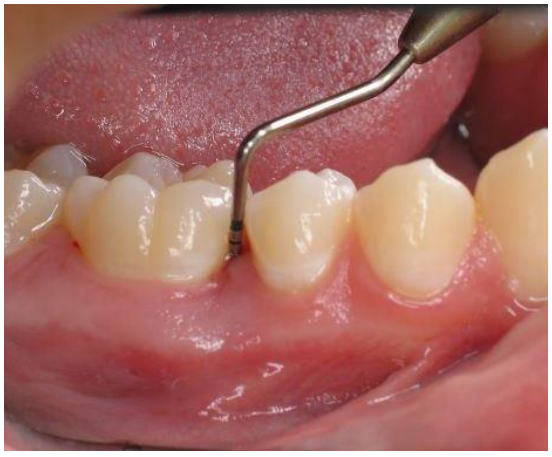

Figure 1. Measurement of clinical attachment level with a Williams Probe.

probing; Score 3: Severe inflammation – noticeable redness and edema, ulceration. A tendency toward spontaneous bleeding.

Silness & loe piaque index[11]

Score 0: Absence of microbial plaque; Score 1: Thin film of microbial plaque along the free gingival margin; Score 2: Moderate accumulation with plaque in the sulcus; Score 3: A large amount of plaque in sulcus or pocket along the free gingival margin.

Statistical analysis

Data were analyzed using the IBM Statistical Package for Social Sciences v15 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean±SD or median (interquartile range), as appropriate. To evaluate the differences between the two groups, The Student t-test was used for normally distributed variables and Mann-Whitney's U-test was performed for non-parametric variables. The Chi-square test was used for qualitative variables. All differences associated with a chance probability of .05 or less were considered statistically significant.

RESULTS

The median ages were 50.5 (11) and 47 (8.7) years for the test and control group, respectively. No significant difference was observed for age and gender between the study groups. There were no significant differences between groups as regards the frequencies of, hypertension, smoking (p>0.05) (Table 1).

As for the periodontal parameters of the study groups, PD, GI, PI, BOP, and CAL values were statistically higher in the test group compared to the control group (p<0.05) (Table 2).

DISCUSSION

Although the studies attributed the etiology of CSF to microvascular and endothelial dysfunction, widespread atherosclerosis, inflammation, and platelet dysfunction, its pathophysiology has not been clarified. We aimed to evaluate the periodontal health status of CSF patients and to detect underlying periodontal disease.

Table 1. General characteristics of the study groups.

Baseline characteristics	Group 1 (n=51) Test group	Group 2 (n=51) Control group	p-value
Age [median (IQR)]	50.5 (11)	47 (8.7)	0.080
HT [number (%)]	6 (13.3)	10 (19.5)	0.384
Sex (female/male)	18/33	21/30	0.627
Smoking [number (%)]	6 (11.7)	9 (17.6)	0.337
Number of slow flow coronary arteries (%)			
One vessel	27 (60)	–	
Two vessels	9 (20)	–	
Tree vessels	9 (20)	–	

HT: hypertension; IQR: interquartile range.

Table 2. Comparison of periodontal parameters of the study groups.

	Group 1 (n=51) Test group median (IQR)	Group 2 (n=51) Control group median (IQR)	p-value
PD	2.28 (0.8)	1.88 (0.4)	<0.001
CAL	2.27 (1.0)	2.0 (0.4)	0.001
GI	2.0 (0.6)	1.5 (0.5)	<0.001
PI	2.0 (0.5)	1.45 (1.0)	<0.001
BOP (%)	100 (59)	47 (9)	<0.001

PD: probing depth; CAL: clinical attachment level; GI: gingival index; PI: plaque index; BOP: bleeding on probing.

Periodontitis is a chronic multifactorial inflammatory disease and the systematic inflammatory response results in endothelial dysfunction, thus contributing to cardiovascular diseases[12].

In our study, we found PD, CAL, GI, PI, and BOP values that are periodontal disease markers statistically higher in the test group. Therefore, periodontitis may be one of the underlying causes of this disease. Indeed, previous papers revealed a link between inflammation and CSF too.

In a study, Xia and colleagues[13] found that hs-CRP levels were the most important predictor of coronary slow flow and suggested that this finding played an important role in inflammation in CSF. Likewise, Li et al.[14] argued that increased inflammatory markers might be an indicator of endothelial activation and inflammation in patients with CSF. Similarly in another study, Madak et al.[15] stated that the blood levels of Hs-CRP and N-terminal pro-B-type natriuretic peptide were higher in CSF patients than the control group, and suggested that inflammation was the main factor of many cardiovascular events, and was associated with different clinical coronary artery diseases.

Recent studies have shown a strong relationship between periodontitis and systemic diseases including cardiovascular diseases and diabetes[16,17]. Periodontal disease was previously associated with recurrent cardiovascular events in patients with a recent MI[6,18]. Likewise, in the study conducted by Gürkan et al.[8], 28 healthy individuals were compared with 32 isolated coronary artery disease patients. Patients with isolated coronary artery ectasia had significantly higher clinical periodontal parameters.

According to our results, the presence of periodontitis was significantly higher in the test group. In the English literature, periodontal treatment generally leads to an improvement in endothelial functions[19-21]. In a study, Li et al.[22] reported very low-quality evidence to support whether periodontal treatment can prevent the relapse of CVD in the long term in patients with periodontitis, but no significant indication on primary prevention was found. In a recent study, Lobo et al.[23] concluded that treatment of periodontal disease improved endothelial functions in patients with MI. Similarly, Teeuw et al.[24] reported that in patients with periodontitis and other comorbidities (CVD and metabolic syndrome), reductions in biomarkers of atherosclerotic disease such as CRP, interleukin – 6, total cholesterol, triglyceride, high-density lipoprotein, and also improvements in endothelial functions were detected after periodontal treatment.

Similarly, periodontal treatment has been shown to result in improved metabolic control in periodontitis patients with diabetes, as demonstrated by a significant decrease in HbA1c levels in a systematic review[25].

Limitations of our study; the present case-control study has a small population size. Correlation with inflammatory markers was not performed in patients with CSF. These inflammatory markers or cardiovascular events were not assessed after periodontal disease therapy.

CONCLUSIONS

Patients with CSF should be evaluated for underlying periodontal disease and treatment of periodontal disease can protect against future cardiovascular events. In addition, if we look back, patients with periodontitis should be carefully examined by dentists and well evaluated for cardiovascular diseases and other risk factors.

ACKNOWLEDGMENTS

This study was approved by the Clinical Research Ethics Committee of the XX University. All the procedures in this study were following the 1975 Declaration of Helsinki, updated in 2013.

AUTHORS' CONTRIBUTIONS

EE: Conceptualization, Project Administration, Resources, Supervision, Writing – Original Draft. **GU:** Conceptualization, Project Administration, Resources, Writing – Original Draft. **ZK:** Resources, Writing – Original Draft. **SB:** Supervision, Writing – Original Draft.

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Relationship between body mass index and waist-to-height ratio in childhood

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SUMMARY

OBJECTIVE: To evaluate whether there is an association between the body mass index z-score and waist-to-height ratio of children and adolescents.

METHODS: This was a cross-sectional study conducted in a school in Santo André, SP, between June and August 2019. Body mass index was measured for all participants, adopting the z-score cutoff of +2 recommended by the World Health Organization. The waist-to-height ratio was determined in children over two years of age and considered abnormal when ≥0.5. The qualitative variables are presented as absolute numbers and percentages. To compare qualitative data, we used the χ^2 test or Fisher's exact test. Pearson's test was applied to assess the correlation between BMI and waist-to-height ratio. The level of significance adopted was 5%.

RESULTS: The body mass index was calculated for 518 children and the waist-to-height ratio for 473 children. Regarding body mass index, 60.6% of the participants had normal weight, 3.1% were underweight, and 36.3% were overweight. overweight (24.7%) and obesity (22.7%) were more prevalent in adolescents. The waist-to-height ratio was abnormal in 50.5% of the sample. There was an increasing association between body mass index and waist-to-height ratio with age, according to the Pearson correlation coefficients for the age groups <5 years (r=0.459; p<0.001), 5 to 10 years (r=0.687; p<0.001) and >10 years (r=0.805; p<0.001).

CONCLUSION: There was a significant correlation between body mass index and waist-to-height ratio. This association was higher in adolescents. The waist-to-height ratio is easy to apply and may be useful as a predictor of cardiometabolic risk.

KEYWORDS: Body mass index. Waist-height ratio. Overweight. Child.

INTRODUCTION

The prevalence of overweight and obesity among children and adolescents has been growing significantly[1]. The percentage of obese children aged five to 19 years has increased ten-fold in the last 40 years[2]. In 2010, there were 35 million overweight children in the world, and that number was expected to double in 2020[3]. In Brazil, one-third of children between five and nine years of age were overweight in 2010[4].

Different anthropometric measures have been proposed to identify cardiometabolic risk (CMR) in children. Body mass index (BMI), although it does not provide any indication of the distribution of body fat, is the most commonly used parameter worldwide[5].

In adults, it is well established that increased abdominal fat, compared to BMI, is associated with greater CMR[6]. However, in children and adolescents, this association has not yet been fully established. A publication compiling data from five studies (n=4,255) showed that the magnitude of associations for BMI and waist-to-height ratio (WHtR) in children and adolescents were similar in relation to the clustered CMR factors (lipid profile, HOMA-IR, and blood pressure) and performed better at higher BMI values. However, the accuracy of these anthropometric variables to classify the increased risk was low[7]. On the other hand, a recent study (n=1,201) showed that the prevalence of individuals with metabolically unhealthy obesity in a pediatric population

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was high and that the WHtR was an independent predictor of this form of obesity[8].

Most authors correlate WHtR p≥0.5 with increased abdominal adiposity and CMR[9]. Because the cutoff value is the same for everyone, the WHtR is easy to interpret[10].

A national study with children aged four to seven years showed that when the normal weight group was evaluated using indicators of abdominal adiposity, the prevalence of abnormal nutritional status was higher than that when evaluated by BMI/age[11]. However, this association between BMI and WHtR in the identification of excess weight has not yet been fully elucidated for all age groups in Brazil. The objective of this study was to evaluate whether there is an association between the BMI z-score and WHtR of children and adolescents.

METHODS

This cross-sectional study was conducted at *Instituição Cidade dos Meninos Maria Imaculada* ("Cidade dos Meninos Maria Imaculada" Rehabilitation Center) in the municipality of Santo André, state of São Paulo (SP), Brazil, between June and August 2019.

All participants were invited to enroll in the study.

Children who did not want to participate, those whose family members did not sign the consent form and those with short stature were excluded. A systematic review suggested that there is a risk of misinterpretation of the WHtR in children with short stature[12].

Height was measured using a vertical stadiometer, graduated in centimeters and millimeters. Weight was measured using a digital scale with a precision of 10 grams. BMI was calculated by dividing the weight in kilograms by the height in meters squared.

Waist circumference was assessed for children aged two years or older, using a centimeter and millimeter graduated tape measure. The tape measure was placed at the midpoint between the iliac crest and the last rib. This anatomical point has the strongest correlation with abdominal adiposity[13]. The WHtR was obtained by dividing the waist circumference in centimeters by the height.

BMI was classified by BMI/age according to sex, adopting the z-score cutoff of +2 recommended by the World Health Organization[14]. Thus, children under five years old with overweight and obesity and children older than five with obesity and severe obesity were included in the group with excess weight. The WHtR was considered abnormal when the value was ≥0.5[10].

A total of 23 children were excluded from the analysis due to their short stature. The BMI was calculated for 518 children. The WHtR was calculated for children aged 24 months or older (n=473). Three children had no waist circumference recorded because they missed the days that the evaluators were at the school, and thus, the WHtR was not calculated for these children, only the BMI.

The data were entered into an Excel spreadsheet (Microsoft) and analyzed using Epi Info™, version 7.2.2.6. The qualitative variables are presented as absolute numbers and percentages. To compare the qualitative data, we used the $\chi 2$ test or Fisher's exact test. The level of significance was set at 5%. Pearson's test was used to correlate BMI with WHtR.

The study was approved by an ethics and research committee, opinion number 3.058.583, CAAE: 02670518.7.0000.0082

RESULTS

Table 1 shows the general characteristics of the children in the study. The mean age was 72.5±12.2 months (range 9 to 186 months), with a slight predominance of males (288; 53.2%).

Regarding nutritional status, 314 (60.6%), 188 (36.3%), and 16 (3.1%) were classified as normal weight, above the ideal weight (risk of overweight, overweight, obesity, and severe obesity) and thin, respectively. The WHtR was rated abnormally for 239 children (50.5%) (Table 1).

Overweight was present in 19% of children under the age of two years, 9.1% of children from two to five years, 15.3% of children from five to ten years and 22.7% of children older than 10 years. Among the adolescents, the prevalence of overweight was also high (24/24.7%). When comparing the prevalence of overweight and abnormal WHtR by sex in each age group, there was no significant difference (Table 2).

When correlating BMI, using the z-score +2 as the cutoff point, with the WHtR, using 0.5 as the cutoff point, the correlation between the two increased with age. The Pearson correlation coefficients were 0.459, 0.687, and 0.805 (p<0.001) in children younger than five years, children five to ten years old, and children older than 10 years, respectively (Figure 1). There was no difference between sexes (male: 0.535, p=0.000; female: 0.589, p=0.000).

DISCUSSION

The number of children above the ideal weight was ten times higher than the number of children classified as malnourished (36.3 vs. 3.1%). Studies have indicated an increase in the prevalence of excess weight in children, but the observed values in this study were above those described in Brazilian population studies[2,3].

Almost half of the adolescents evaluated were above the ideal weight. Data from the 2016 Brazilian Institute of Geography and Statistics showed that 23% of Brazilian adolescents had excess

Table 1. General characteristics and nutritional status of the children included in the study.

	n	%
Gender (n=518)		
Male	272	52.5
Female	246	47.5
Age (n=518)		
Children under 2 years old	42	8.1
2–5 years	209	40.3
5–10 years	170	32.8
10 years or more	97	18.7
Nutritional diagnosis body mass index z-score (n=518)		
Marked thinness	5	1
Thinness	11	2.1
Normal weight	314	60.6
Risk of overweight	57	11
Overweight	77	14.9
Obesity	41	7.9
Severe obesity	13	2.5
Waist-to-height ratio (n=473)		
<0.5	234	49.5
≥0.5	239	50.5

Table 2. Nutritional diagnosis by body mass index and classification of waist-to-height ratio by sex and age group.

Age	Classification	Male		Female		p-value
		n	%	n	%	
Body mass index (BMI)						
<2 years	No excess weight	20	47.6	14	33.3	0.68*
(n=42)	Excess weight	6	14.3	2	4.8	
2–5 years	No excess weight	97	45.9	93	43.1	0.74**
(n=209)	Excess weight	11	5.7	8	5.3	
5–10 years	No excess weight	82	48.2	62	36.5	0.42**
(n=170)	Excess weight	12	7.1	14	8.2	
≥10 years	No excess weight	35	36.1	40	41.2	0.81**
(n=97)	Excess weight	9	9.3	13	13.4	
Waist-to-height ratio (WHtR)						
2–5 years	<0.5	24	11.5	28	13.5	0.47**
(n=208)	≥0.5	83	39.9	73	35.1	
5–10 years	<0.5	72	42.6	51	30.2	0.28**
(n=169)	≥0.5	22	13	24	14.2	
≥10 years	<0.5	26	27.1	33	34.4	0.81**
(n=96)	≥0.5	18	18.7	19	19.8	

*p: significance level by Fisher's exact test; **p: significance level by the chi-squared test.

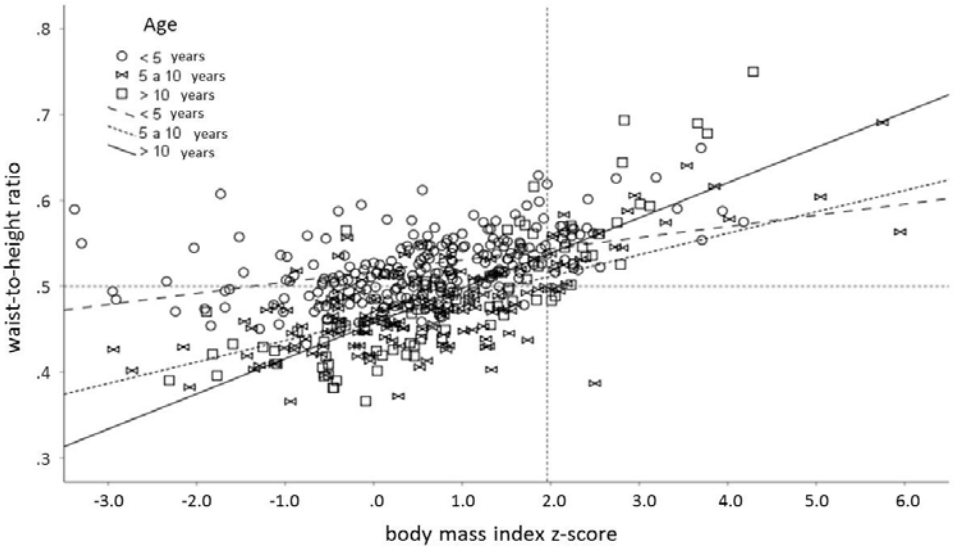

Figure 1. Correlation between body mass index z-score and waist-to-height ratio.

weight[15]. In 2015, a study conducted in Campinas, SP, found that 8.4 and 16.8% of adolescents were obese and overweight, respectively[16]. In the city of Ribeirão Preto, SP, in 2016, 30.9% of adolescents were overweight or obese[17]. Adolescents more frequently consume ultra-processed foods, favoring the emergence of obesity[18].

The correlation between BMI z-score and WHtR was stronger with increased age, ranging from r=0.459 for children under 5 years of age to r=0.805 for children over 10 years of age. A significant number of children and adolescents with a BMI z-score lower than +2 had a WHtR greater than 0.5 (Figure 1). These findings reinforce the importance of using both measurements in the pediatric age group, especially in adolescents.

Meta-analysis showed that both BMI and WHtR can be useful to define obesity in the pediatric age group when more sophisticated techniques, such as dual-energy X-ray absorptiometry, are not available[19].

The increase in WHtR is related to android obesity. We did not find differences between sexes based on WHtR. A study conducted in the state of Minas Gerais with prepubertal children found a higher prevalence of android obesity in girls[20]. Android obesity increases WHtR measurements and is associated with higher CMR[21]. In Brazil, female children and adolescents practice less physical activity than do boys, a factor that may contribute to the risk of obesity in this group[22].

A Brazilian study conducted with children aged six to ten years suggested that the WHtR could be used in conjunction with BMI to more reliably assess body fat distribution[23]. A survey conducted in Colombia with 1,919 adolescents showed that the use of BMI alone is not sufficient to predict CMR[24]. The introduction of WHtR in the pediatric routine would not increase the visit duration by much, as only an additional measurement of waist circumference would be required. In children and adolescents with excess weight, an abnormal WHtR contributes to the identification of CMR[25].

A limitation of this study was the evaluation of a single school in the municipality of Santo André, SP, thus not allowing us to extrapolate the data to the general population.

CONCLUSION

The prevalence of excess weight in the study population was higher than that described in population surveys, especially among adolescents. The WHtR is a simple, inexpensive, highly reproducible, and accurate tool for the prevention, control, and identification of childhood obesity and should be applied. The correlation between BMI z-score and WHtR increased with age, suggesting the importance of measuring both parameters, especially in adolescents.

AUTHORS' CONTRIBUITION

JCPF: Conceptualization, Data Curation, Formal Analysis, Project Administration, Writing – Original Draft. **CAV:** Data Curation, Formal Analysis. **LSS:** Data Curation, Formal Analysis. **SRC:** Data Curation. **FISS:** Formal Analysis, Writing – Original Draft. **ROSS:** Formal Analysis. Writing – Original Draft.

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Asthma, much more than a respiratory disease: influence of depression and anxiety

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SUMMARY

OBJECTIVE: The goals of this study are to compare self-reported depression and anxiety in subjects diagnosed of asthma and healthy controls.

METHODS: We designed a case-control study. Subjects were recruited using a consecutive sampling method from a single institution. Two groups were created: Asthma and healthy controls. Data of medical history and demographic background were collected from the medical record. Self-reported depression level was assessed using Beck´s depression inventory (BDI). Self-reported anxiety was measured with the "State-trait anxiety inventory" (STAI).

RESULTS: Fifty-one subjects with asthma, and fifty healthy patients were included in this study. BDI scores ($p<0.001$) were higher for asthma (10.22±7.3) than in the control group (5.2±6.56). STAI state ($p<0.001$) was higher in asthma (42.61±11.5) than in controls (34.88±9.25). STAI trait ($p<0.001$) showed higher scores in asthma (43.14±10.89) than in controls (34.62±9.19).

CONCLUSIONS: These study findings showed that BDI, and STAI trait and state scores are significantly higher in subjects who suffer from asthma than healthy controls.

KEYWORDS: Depression. Anxiety. Asthma.

INTRODUCTION

Depression and anxiety are considered "common mental disorders". Globally, the total number of people with depression was estimated to exceed 300 million in 2015 (4.5% of the world´s population). Up to nearly 40% of the population are affected at some point by an anxiety disorder[1]. The consequences of both conditions in terms of loss of health are substantial and have a considerable effect on a patient´s health-related quality of life (HRQL). Depression is considered the single largest contributor to global disability around the world. The most common underlying disorder in those who attempt suicide is depression.

Depressive disorders are characterized by symptoms as sadness, loss of interest or pleasure, feelings of worthlessness or guilt, sleep difficulties, fatigue, appetite or weight changes, feelings of tiredness, psychomotor disturbances, poor ability to concentrate, and even suicidality[2]. Depression can be long-lasting or recurrent, causing impairment to daily life activities.

Anxiety is associated with physical and psychological discomfort. All anxiety disorders share common symptoms, such

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as fear, anxiety, and avoidance. Other anxiety-related symptoms include fatigue, restlessness, irritability, sleep disturbances, reduced concentration, lack of memory, and muscle tension. Depression and anxiety often co-occur. Up to 90% of patients with anxiety develop symptoms of depression, and nearly 85% of patients with depression show some kind of anxiety symptom[3].

The prevalence of depression and anxiety is two to three times higher in people with chronic medical conditions than in healthy peopl[4]. People with a long-term condition and depression or anxiety have worse health status than people with depression or anxiety alone, and even than people with any combination of long-term conditions without depression[5].

Asthma is considered a chronic heterogeneous disease. Its pathogenesis involves various cells and mediators of inflammation, and it is characterized by airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness, and cough that vary over time and in intensity, together with variable expiratory airflow limitation, totally or partially reversible, either spontaneously or due to the action of the medication[6].

In 2017, the worldwide prevalence of asthma was around 3.5% with a reported incidence of 0.56%[7]. Asthma may disturb daily activities, as well as the patient's quality of life (QoL)[8]. It carries an economic burden, due to an often increment of the emergency care use, hospital admission, as well as work absenteeism. In addition, it can potentially cause early disability and premature death. The mean cost per patient per year is high, and can vary among countries, according to Nunes et al, in Europe is $USD 1,900, different from the USA, in which estimated mean is estimated around $USD 3,100[9]. People with asthma are more prone to emotional disorders, even when their symptoms are controlled[10]. Different authors, reported a high prevalence of anxiety and depression[11,12]. Labor et al. reported that 24.5% of asthma patients were depressed, and 44% had symptoms of anxiety[11].

Del Giacco et al, reported that anxiety may be a predisposing factor for developing asthma, and also, having asthma may predispose a higher risk of having an anxiety disorder, establishing the possibility of a bidirectional association between anxiety and asthma[13].

Besides, anxiety and depression have been associated with increased asthma exacerbations and emergency visits[14]. Depression acts as an independent co-morbid factor in asthma[15].

We hypothesized that people with asthma, had the worst levels of Self-perceived depression, anxiety trait, and state when compared to healthy people. So, the main objective of this research was to determine self-perceived depression and anxiety trait and state levels in a subset of asthma patients compared to healthy controls.

METHODS

Study design

A case-control study of a non-probability consecutive sampling method from a medical center was performed to compare self-assessed depression severity, anxiety trait, and anxiety state in patients diagnosed with asthma and healthy controls. All the data collection was supervised by the same researcher. The guidelines for reporting observational studies, stated by the "Strengthening the Reporting of Observational Studies in Epidemiology" (STROBE) were followed[16].

This study was approved by the ethics committee of clinic research of Galicia (Spain). The Helsinki declaration as well as all national and international ethical standards for human experimentation were respected[17]. Also, all participants signed the informed consent before their inclusion in the present research.

Analytic analysis

Sample size calculation

T-tests for differences between 2 different means were used to determine the sample size calculation by the G*Power 3.1.9.2 software and based on the BDI as the main outcome measurement of a pilot study (n=28 participants) with 2 groups (mean±SD), 14 patients with asthma (9.28±6.16 points) and 14 healthy controls (4.92±3.64 points). Furthermore, the followed parameters were used for the sample size calculation with an effect size of 0.86, α error of 0.01, and power (1-β error) of 0.90. Thus, the required total sample size was 84 participants, 42 for each group, necessary to achieve an actual power of 0.901. Finally, considering a possible 15% loss to follow-up, a total sample of 98 participants, 49 subjects for each group, was required for the present study.

Participants

A consecutive sampling method was used to recruit participants from the pneumology department of the Complexo Hospitalario de Ourense (Ourense, Spain). Two groups were established: Asthma (case group) and healthy patients (control group). Asthma patients were diagnosed and classified according to the GEMA (Spanish Group for Asthma Management) guidelines[6]. Healthy matched-paired participants were included, as the control group. The inclusion criteria were being older than 18 years old, agreement to sign informed consent, and lack of history of psychiatric. For the control group, healthy participants older than 18 years old were included, without chronic respiratory disease, or known psychiatric medical history and agreement to sign informed consent. Exclusion criteria: not complying with the inclusion criteria described above.

Descriptive data

Descriptive data such as sex, age, weight, height, body mass index (BMI; calculated by the Quetelet index as kg/m^2), and smoke habit were collected.

Outcome measurements

Self-perceived depression, anxiety trait, and state were considered as the primary outcomes.

Self-reported depression was assessed using the "Beck´s depression inventory (BDI-II)", which has become one of the most widely used instruments for assessing the severity of depression worldwide. It contains 21 items, scored by a 4-point Likert scale (0-3). The final results range from 0 to 63 points. Greater scores suggest increased depression severity. Scores between 0–10 indicate no signs of depression; 11–16 indicate mild depression; 17–20 indicate borderline depression; 21–30 indicate moderate depression; 31–40 indicate serious depression; and, 41 or higher indicate extreme depression. Anything over 17 points requires professional treatment[18]. This tool was validated in Spanish, with a Cronbach's α=0.87 and a high diagnostic validity (ROC=0.91) in the general population[19].

The State-Trait Anxiety Inventory (STAI) is a self-reported scale used for the assessment of anxiety state and trait in research and clinical practice. The STAI consists of two 20-item scales (S-Anxiety measuring anxiety as an emotional state, and T-Anxiety measuring anxiety as a personal characteristic or trait)[20]. Each item is scored by a 4-point Likert scale (1–4). Spanish version of the STAI was used. The final score was converted to a digit (0–80) as described for *Buela-Casal*, to be uniform with the original Y-version of the STAI[21]. Higher scores suggest higher levels of anxiety. This test has a good internal consistency, with a Cronbach´s α=0.92 for anxiety state (95%IC 0,91–0,93) and a Cronbach´s α=0.91 for anxiety trait (95%IC 0.90–0.92.)[22] and a Test-retest reliability coefficients on initial development ranged from 0.31 to 0.86[20].

Statistical analysis

Statistical analyses were performed by the SPSS 24.0 version (IBM–Windows; Armonk–NY: IBM Corp) using an α error of 0.01 in conjunction with a 99% confidence interval (CI).

Considering quantitative data, the test of Kolmogorov-Smirnov was applied to determine normality distribution. All data were described by the mean±standard deviation (SD) and range (minimum–maximum values). Regarding the parametric data (Kolmogorov-Smirnov p≥0.05), between-groups differences were compared by the Student *t*-tests for independent samples, and for the non-parametric data (Kolmogorov-Smirnov p<0.05), between-groups differences were compared by the Mann-Whitney *U* tests for independent samples.

For categorical data, frequencies were used to describe these data values and categorical between-groups differences were analyzed by the Fisher exact tests for the age dichotomous variable and the Chi-square (χ^2) test for the depression categories polytomous variable using bar graphs to show this distribution.

RESULTS

The study population included 101 participants, 37 men and 64 women. 11 participants (10.9%) were smokers, 21 (20.8%) former smokers, and 69 (68.3%) non-smokers. The control group was formed by 50 healthy participants, 64% female and 36% male, with a mean age of 42.84±15.69. They were not having chronic medication. In the asthma group, there were 51 patients, 62.7% female and 37.3% male, with a mean age of 59.76±17.7 years. According to Table 1, there were statistically

Table 1 Sociodemographic and clinical characteristics of the sample population.

	Sample	Control	Asthma	p-value
	Mean±SD (range) n=52	Mean±SD (range) n=50	Mean±SD (range) n=51	
Age (years)	51.39±18.45 (18–95)	42.84±15.69 (19–83)	59.76±17.17 (18–95)	<0.001*
Weight (Kg)	72.59±13.83 (43–115)	70.37±13.9 (47–115)	74.76±13.51 (43–109)	0.111†
Height (m)	1.66±0.1 (1.45–1.90)	1.69±0.92 (1.50–1.89)	1.63±0.10 (1.45–1.90)	0.003*
BMI (Kg/m^2)	26.31±4.79 (17.59–45.78)	24.48±3.42 (17.59–33.79)	28.09±5.27 (18.13–45.78)	<0.001*
Sex (female/male)	64/37	32/18	32/19	1.000‡
Smoker (no/yes/former)	69/11/21	38/7/5	31/4/16	0.026§

BMI: body mass index; SD: standard deviation; p<0.01 with a 99% confidence interval was considered statistically significant. *Mann-Whitney *U* test was used. †Student *t*-test for independent samples test was used. ‡Fisher exact test was used. §Chi-squared (χ^2) test was used.

Table 2. Depression and anxiety score differences between asthma and healthy participants.

	Sample	Control	Asthma	p-value
	Mean±SD (range) n=52	Mean±SD (range) n=50	Mean±SD (range) n=51	
STAI-State	38.78±11.10 (21–71)	34.88±9.25 (21–66)	42.60±1.50 (21–71)	<0.001*
STAI-Trait	38.92±10.91 (20–69)	34.62±9.19 (20–65)	47.13±10.89 (20–69)	<0.001†
BDI	7.73±7.35 (0–36)	5.20±6.56 (0–36)	10.21±7.30 (0–27)	<0.001†

BDI: beck depression inventory; SD: standard deviation; STAI: state-trait anxiety inventory; p<0.01 with a 99% confidence interval was considered statistically significant. *Student *t*-test for independent samples test was used. †Mann-Whitney *U* test was used.

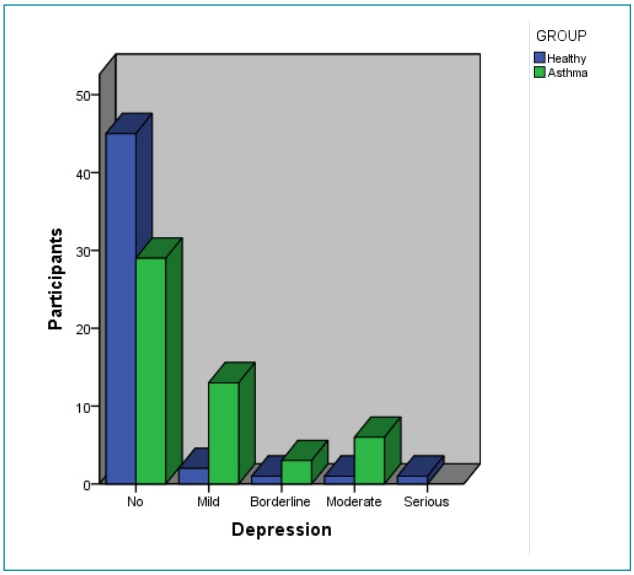

Figure 1. Depression categories between asthma and healthy subjects.

significant differences (p<0.01) between asthma and healthy participants for age, height and BMI, but not for weight, sex and smoker habits (p>0.01).

Regarding Table 2, state and trait anxiety as well as anxiety scores were greater in patients who suffer from asthma with respect to healthy participants, showing statistically significant differences (p<0.001). In addition, the Chi-squared (χ^2) test showed statistically significant differences (p=0.002) detailing more presence of mild, borderline, and moderate depression categories in patients with asthma versus healthy subjects according to Figure 1.

DISCUSSION

Psychological issues are very common in asthma and have a significant impact on quality of life, even if the asthma symptoms are under adequate clinical control[8,23].

In the present study, we found a higher self-reported anxiety state and trait scores in patients with asthma compared to healthy people, consistent with the data previously published[24,25]. Also found higher BDI scores in asthma patients, compared to controls and a direct correlation between the severity of depressive symptoms and asthma severity. The exact connection between asthma and anxiety/depression is still not fully explained. Regarding anxiety in asthma, even when disease control is reached, these patients could experience continuous distress, having presented the risk of future exacerbations, and fear of a crisis and reduced pulmonary function. Chronic psychological distress may cause a pro-inflammatory condition and be associated with increased superoxide and cytokine and leukocyte production. According to Veres et al., neurokinins and P substance (asthma pulmonary neurogenic inflammation byproducts) have a direct action on the central nervous system, inducing anxiety states[26].

Regarding depression and asthma, as a possible pathophysiological mechanism, Marini et al. described that the pro-inflammatory state noted in certain asthma phenotypes is similar to that induced by pro-inflammatory cytokine treatments in humans such as corticosteroids, long known to be associated with depressive symptoms[27]. Also, hypothalamic-pituitary-adrenal axis dysregulation, noted in major depression, have been described in asthma as a result of the inhaled corticosteroids[28,29].

This study had some limitations and should be taken into consideration in future studies. First, to recruit participants a consecutive sampling method was used and could be improved. Second, a larger sample size could be necessary to achieve more reliable results.

CONCLUSIONS

Asthma subjects showed higher scores of self-reported depression, anxiety state, and anxiety trait than controls.

AUTHORS' CONTRIBUTIONS

RHR: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. ÓÁCI: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **RDQ:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **RBBV:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **CCL:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **MSA:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **MELI:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **DLL:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

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ORIGINAL ARTICLE
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Examination of clinical data and semen analysis results of patients undergoing orchiectomy for testicular tumor

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SUMMARY

OBJECTIVE: Testicular tumor constitutes 1% of male neoplasms. Infertility can be determined in patients with testicular tumors before orchiectomy due to the deterioration of spermatogenesis. The aim of this study was to show the clinical, radiological, and pathological characteristics and spermiogram results of patients with testicular tumor and their relationship with each other.

METHODS: The data of patients who underwent orchiectomy due to testicular tumor between 2016 and 2019 were reviewed retrospectively. These data included sociodemographic data of the patients, pretreatment spermiogram characteristics, level of serum tumor markers, characteristics of the ultrasonography, type of orchiectomy, and histopathological examination.

RESULTS: This study included 53 male patients, with a mean age of 33.51±12.86 years. The mean levels of all tumor markers were above the reference levels. The mean tumor size was 34.68±23.32 mm. Multiple localizations and microlithiasis were detected in 11.3 and 13.2% of the tumors, respectively. The most common masses were hypoechoic (n=37; 69.8%) and hypervascular (n=47; 81%). Spermiogram and cryopreservation were performed in 29 (54.7%) of 53 patients preoperatively. The mean sperm concentration before orchiectomy was 24.21×10^6/mL and group A sperm motility 0.79%, group B sperm motility 39.10%, group C sperm motility 9.83%, and group D sperm motility 22.69% in testicular tumors.

CONCLUSION: Spermatogenesis adversely affected before the treatment due to local and systemic effects of testicular cancer. Fertility expectations can be increased in the subsequent years by semen analysis and referral to cryopreservation.

KEYWORDS: Cryopreservation. Infertility. Orchiectomy. Semen analysis. Testicular neoplasms.

INTRODUCTION

Testicular tumor constitutes 1% of male neoplasms and 5% of all urologic tumors[1]. In developed countries, the most common type of cancer in men aged 15–44 years is testicular tumor[2,3]. Alpha-fetoprotein (AFP), β-human chorionic gonadotropin (β-hCG), and lactate dehydrogenase (LDH) are used as serum tumor markers in testicular tumors and also contribute to diagnosis and staging[4]. The color Doppler ultrasonography is the main imaging method used to investigate the presence of a mass in the testis[5].

There is a 30% reduction in fertilization in patients with testicular cancer due to subsequent or spontaneous treatments[6]. Natural paternity rate for these patients is about 50% due to treatments such as orchiectomy, retroperitoneal lymph node dissection, radiotherapy, or chemotherapy continuously for 10 years[6,7]. Therefore, it is recommended that patients undergo

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semen analysis and cryopreservation before orchiectomy, radiotherapy, or gonadotoxic chemotherapy[4,6,8].

Decreased sperm parameters may be detected before orchiectomy due to the deterioration of spermatogenesis. The causes of this condition may include systemic effects, hormonal changes, autoimmune effects, changes in local blood flow, and increased intrascrotal temperature[8-10]. In our clinic, patients with testicular cancer are offered pretreatment semen analysis and cryopreservation to increase fertility expectancy parallel to the improvements in assisted reproductive technology[11,12].

We suppose that semen analysis of patients with testicular cancer should be evaluated preoperatively because of their predisposition to infertility. The rationale of this study was to investigate the factors that may cause the decreased infertility by using pretreatment clinical semen parameters and to examine the radiological, biochemical, and pathological data.

METHODS

Patients

This is a retrospective study. The data of patients who underwent partial or radical orchiectomy due to testicular tumor between 2016 and 2019 were reviewed. Sixty-five patients underwent orchiectomy in our clinic. Twelve patients who undergone orchiectomy except testicular tumor, such as torsion, atrophic testis, testicular abscess, and undescended testis, were excluded from the study. The data of 53 patients who underwent partial or radical orchiectomy due to testicular tumor were included in this study. All of these patients were 16 years or older.

Data collection

Serum AFP, β-hCG, and LDH levels were determined before orchiectomy. The Doppler ultrasonography data were used to determine the side, location, number, size, echogenicity, blood supply of the testicular mass, and testicular microlithiasis. The magnetic resonance imaging was performed in addition to ultrasonography for differential diagnosis of some masses. In some testicular masses, the intraoperative frozen examination was used. According to the results of the frozen examination, partial or radical orchiectomy was performed on the testicular mass. The histopathological results of the testicular mass were examined. Patients were offered to give spermiogram and to have cryopreservation by masturbation before orchiectomy. Both spermiogram and cryopreservation were evaluated by an experienced technician at the Assisted Reproductive Techniques Center in our hospital. In the spermiogram analysis, motility (i.e., total, progressive, and nonprogressive) and concentration were evaluated according to the World Health Organization (WHO) 2010 guidelines[13]. Sperm samples were obtained after 2–3 days of sexual abstinence. The semen was stored in the hospital in a sterile container and delivered to the laboratory within 1 h. The volume of seminal fluid was measured using a graded tube. The concentration was measured in an improved Neubauer chamber, with a magnification of 200×. A minimum of five power fields were examined, and for each field at least 200 spermatozoa were analyzed[14]. According to the WHO 2010 criteria and some studies, the minimum percentage of group A (rapidly progressive) + B (slowly progressive) (i.e., A+B=progressive motility) sperm motility should be 32%, and minimum percentage of group A (rapidly progressive) + B (slowly progressive) + C (non-progressive) (i.e., A+B+C=total motility) sperm motility should be 40% for the fertilization[13,15]. To better understand the results of semen analysis by urologists and patients, the sperm motility was expressed in both forms, i.e., A, B, C, D, and A+B and A+B+C. Semen samples were frozen in TEST-egg yolk buffer (TYB; Irvine Scientific, Santa Ana, CA, USA) in a 1:1 ratio and stored in liquid nitrogen at -196°C.

Statistical analysis

The sample size was calculated with the help of sample size calculator at www.calculator.net. Examining >51 patients was found to be sufficient for this study with 95% confidence level, 5% standard deviation expectancy, and estimated 110 general population size. The statistical analysis was performed using the SPSS version 22.0 software (IBM Corp., Armonk, NY, USA). The descriptive data were expressed in mean and standard deviation (SD), median (min–max), number, and frequency. The Kolmogorov–Smirnov test was used to check the normality of data for quantitative variables. The one-way ANOVA test was used to compare variables, and for all the statistical analyses the two-sided $p<0.05$ was considered as statistically significant.

RESULTS

This study included 53 male patients, with a mean age of 33.51±12.86 years. All patients were evaluated with tumor markers preoperatively. Sociodemographic data, levels of serum tumor marker, color Doppler ultrasonography data, and histopathological diagnosis of the patients are shown in Table 1. Preoperative magnetic resonance imaging was performed in 3 (5.7%) patients to support the diagnosis of testicular mass.

In 12 of 53 patients (22.6%), the intraoperative frozen examination was used. As a result, 4 (7.5%) of these patients underwent partial orchiectomy and 49 (92.5%) underwent radical orchiectomy. In the final histopathological examination, 1 (1.9%) patient had burned-out tumor, 7 (13.2%) patients had benign tumors, and 45 (84.9%) patients had malignant tumors.

Spermiogram and cryopreservation were performed in 29 (54.7%) of 53 patients preoperatively. The spermiogram results of the patients are shown in Table 2. There was no statistically significant difference than the normal criteria. In 29 patients who underwent spermiogram prior to orchiectomy, the percentage of group A motility was found to be 0% in 27, 6% in 1, and 17% in 1. According to the spermiogram results, 6 (20.6%) of 29 patients had sperm concentration $<1\times10^6$/mL with a few motile sperm, and 1 (3.44%) patient had azoospermia.

We aimed to investigate whether patient age and pathological subtype have an effect on other data. Therefore, the patients were divided into three groups according to age: Group 1 (10–30 years), Group 2 (31–50 years), and Group 3 (51–90 years). There was no statistically significant difference between age groups in terms of tumor markers, spermiogram results, tumor location, and tumor size parameters (Table 3). No statistically significant difference was found in terms of spermiogram results and tumor location parameters according to pathological diagnoses (Table 3).

Table 1. Sociodemographic, biochemical, ultrasound, and histopathological characteristics of patients.

Sociodemographic characteristics		
BMI (kg/m²)	Mean±SD	Min–Max
	24.73±4.22	18.44–34.28
	n	%
Comorbidity		
Diabetes mellitus	2	3.78
Arterial hypertension	1	1.89
Respiratory diseases	2	3.78
Hyperlipidemia	3	5.66
Arthritis	1	1.89
None	44	83
Cigarette smoking		
Yes	18	33.9
No	37	66.1
Education degree		
Elementary and secondary school	11	20.7
High school	24	45.2
University	18	34.1
Marital status		
Single	34	64.1
Married	19	35.9
Serum tumor markers		
	Mean±SD	Min–Max
AFP (normal range: 0–9 ng/mL)	351.47±2069.76	0.605–15002
β-hCG (normal range: 0.5–2.67 mIU/mL)	1086.27±6441.63	0.10–45945
LDH (normal range: 0–248 U/L)	258.75±244.31	115–1872
Color Doppler ultrasound		
Size (mm)	Mean±SD	Min–Max
	34.68±23.32	5–120
	n	%
Location		
Scrotal	52	98.1
Inguinal	1	1.9

Continue...

Table 1. Continuation.

Sociodemographic characteristics		
BMI (kg/m²)	Mean±SD	Min–Max
	24.73±4.22	18.44–34.28
	n	%
Laterality		
Right	28	52.8
Left	25	42.2
Microlithiasis		
Yes	7	13.2
No	46	86.8
Multifocality		
Single	47	88.7
Multiple	6	11.3
Localization		
Upper pole	13	24.5
Middle pole	12	22.6
Lower pole	10	18.9
Upper + Middle pole	2	3.8
Middle + Lower pole	4	7.5
Complete	12	22.6
Echogenicity		
Hyperechoic	1	1.9
Hypoechoic	37	69.8
Heterogeneous echo	10	18.9
Isoechoic	3	5.7
Missing	2	3.8
Blood flow		
None	3	5.7
Low	2	3.8
Mild	5	9.4
High	43	81.1
Histopathological evaluation		
	n	%
Seminoma (classic)	22	41.5
Mixt germ cell tumor	16	30.2
Embryonal carcinoma	2	3.8
Adenomatoid tumor	2	3.8
Teratocarcinoma	1	1.9
Lymphoma	1	1.9
Seminoma (spermatocytic)	1	1.9
Teratoma (postpubertal)	1	1.9
Retroperitoneal germ cell tumor	1	1.9
Leydig cell tumor	1	1.9

Continue...

Table 1. Continuation.

Sociodemographic characteristics		
BMI (kg/m²)	Mean±SD	Min–Max
	24.73±4.22	18.44–34.28
	n	%
Lipoma	1	1.9
Testicular fibroma	1	1.9
Leydig cell proliferation	1	1.9
Epidermoid cyst	1	1.9
Leiomyoma	1	1.9

BMI: body mass index; AFP, alpha-fetoprotein; β-hCG, β-human chorionic gonadotropin; LDH, lactate dehydrogenase.

Table 2. Summary of spermiogram results.

Spermiogram results		
	Mean±SD	Min–Max
Concentration (10^6/mL)	24.21±26.81	0–115
Group A motility (%)	0.79±3.31	0–17
Group B motility (%)	39.10±29.84	0–81
Group C motility (%)	9.83±12.20	0–50
Group D motility (%)	22.69±21.23	0–67

DISCUSSION

The aim of this study was to investigate the factors that may cause the decreased infertility by using pretreatment clinical semen parameters and to examine the radiological, biochemical, and pathological data.

In testicular cancer, the levels of serum tumor markers should be checked following history and physical examination in patients with suspected testicular cancer[16,17]. In our series, the mean levels of all tumor markers were above the reference levels, with AFP: 351.47±2069.76 ng/mL, β-hCG: 1086.27±6441.63 mIU/mL, and LDH: 258.75±244.31 U/L.

The present guidelines recommend Doppler ultrasonography to investigate all men with testicular mass before orchiectomy[18,19]. According to one of the earlier studies, among the total of 107 patients, 50 had right testicular mass, 56 had left testicular mass, and 1 had bilateral testicular mass[20]. It was stated that the mean patient age was 29 years, and the tumor size was 34.8 mm. The ultrasonographic evaluation of the masses revealed 18 (16.9%) multiple tumors, 32 (29.5%) microlithiasis, and 70 (63.8%) hypoechoic masses. In our study, testicular mass was detected more frequently on the right side (52.8%). The mean tumor size was similar to the earlier study. Unlike the earlier study, multiple localization and microlithiasis were detected in 11.3 and 13.2% of the tumors, respectively. Although the incidence of microlithiasis in testicular tumors reaches up to 48% in some studies, it is generally detected at a rate of 15–20%[21]. We detected the testicular microlithiasis at a rate similar to that in the literature (13.2%). The most common masses were hypoechoic (n=37; 69.8%) and heterogeneous echo (n=10; 18.9%). In a study evaluating the relationship between the histological subtype and sonographic images of the masses, the information of a total of 58 patients was reported[22]. In the Doppler examination of the masses, 81% (n=47) were reported as hypervascular, 14% (n=8) as hypovascular, and 5% (n=3) as avascular. Similarly, we found that the rate of hypervascular masses was approximately 81% (n=43). In fact, testicular masses usually show hypervascularity. The pathology of 3 (5.7%) patients with avascular mass diagnosed by using Doppler was reported as testicular lipoma, epidermoid cyst, and interestingly, a classical seminoma. In a study examining a rather large series of patients, the characteristics of a total of 325 testicular tumors were shared[23]. A total of 79.8% (n=197) hypoechogenic and 73.3% (n=181) hypervascular masses were detected. In this study, the rates of these two sonographic data were 69.8% and 81.1%, respectively, and they were quite high. Also in this study, the proportions of the

Table 3. Evaluation variables according to age groups and pathological types.

According to the age groups	
	p
AFP	0.655
B-hCG	0.530
LDH	0.784
A sperm motility (%)	0.472
B sperm motility (%)	0.563
C sperm motility (%)	0.896
D sperm motility (%)	0.387
Concentration	0.489
Location	0.649
Size	0.206
Pathology types	
Age group 1 vs. 2	>0.05
Age group 2 vs. 3	<0.01
Age group 1 vs. 3	<0.01
According to the pathology types	
	p
A sperm motility (%)	0.908
B sperm motility (%)	0.149
C sperm motility (%)	0.379
D sperm motility (%)	0.321
Motility	0.885
Concentration	0.099
Location	1
One-way ANOVA test results.	

AFP, alpha-fetoprotein; β-hCG, β-human chorionic gonadotropin; LDH, lactate dehydrogenase.

masses covering the upper pole, middle pole, and the entire testis were found to be similar (24.5, 22.6, and 22.6%, respectively). The magnetic resonance imaging was used to support differential diagnosis in 3 (5.7%) patients after the sonographic evaluation.

The most common pathological subtype was seminoma (n=23; 43.4%), as in other studies. Spermatocytic seminoma was detected in a 71-year-old patient. Twelve patients among 53 (22.6%) were evaluated intraoperatively by the frozen examination. The final pathology of the testicular masses using the frozen examination was reported as 4 benign and 8 malignant. The final pathology of 7 (13.2%) testicular masses was reported as benign. When the benign masses were examined retrospectively, frozen examination was performed in 4 of them, and partial orchiectomy was performed according to the results obtained from the frozen examination. The subgroup distribution of these benign masses was as follows: lipoma (n=1), epidermoid cyst (n=1), and adenomatoid tumor (n=2). According to the recent studies, partial orchiectomy with frozen examination and testis-sparing surgery is suggested for the small, non-palpable, incidentally detected testicular masses with no elevated tumor markers[24,25]. Our pathological results appear similar to those in the literature. In this study, it was determined that the spermiogram results did not differ according to histopathological diagnoses.

Spermatogenesis may be impaired in testicular tumors due to the destruction of the surrounding tissue, local secretion of β-hCG and some paracrine factors, increased intrascrotal temperature, autoimmune effects, and changes in local blood flow. Even in unilateral testicular cancer, spermatogenesis may be adversely affected in the contralateral testis. This situation reveals the tendency of the patients to infertility from the beginning. Negative changes in sexual life may occur in men with testicular cancer due to reduced sexual pleasure and desire. Testicular cancer can cause worse sperm density and motility, especially when compared with other cancers. In a study evaluating Danish males, those evaluated for infertility were 1.6 times more likely to develop testicular germ cell tumors following the infertility evaluations[26]. In another large-based study conducted in the United States, semen analysis showed that 24% of men had infertility and that these men were 3 times more likely to be diagnosed with testicular cancer than the general population[27]. All these studies show the relationship between testicular cancer and low semen quality. In a recent study, semen parameters between seminoma type testicular cancer and control group were compared according to WHO 2010 criteria[9,13]. A decrease in sperm motility (p=0.019), sperm concentration (p=0.003), total sperm count (p=0.001), and total motile sperm count (p=0.001) were found in the seminoma group. The mean sperm concentration was 46.72×10^6/mL in the tumor group. However, there was no information about the percentage of sperm in group A, B, C, and D motility. In our study, spermiogram and cryopreservation were performed before orchiectomy in more than half (n=29; 54.7%) of 53 patients treated for testicular mass. The reason why this procedure is not performed in the remaining patients is that some patients do not want to give semen and others are operated at night in emergency. It is remarkable that the percentage of group A motility (0.79%) was found to be quite low. However, the group A+B sperm motility ratio is above the reference value (32%) with 39.89%. In 29 patients who underwent spermiogram prior to orchiectomy, the percentage of group A motility was found to be 0% in 27, 6% in 1, and 17% in 1. Low percentage of forward motile sperm indicates a higher risk of infertility before orchiectomy. Even in the pretreatment period, approximately

50% oligozoospermia and 24% azoospermia may occur in testicular cancer[28]. According to our spermiogram results, 6 (20.6%) of 29 patients had sperm concentration <1×10^6/mL with a few motile sperm, and 1 patient (3.44%) had azoospermia. We found that the number of patients with oligozoospermia was slightly lower than those in the earlier study (n=12; 41.3%). The average sperm concentration of patients with oligozoospermia was 5.25×10^6/mL. In light of the data, it is noted that patients have a risk of infertility in the pre-orchiectomy period. It is certain that this risk will increase even more when chemotherapy or radiotherapy is added to the treatment after orchiectomy.

Although we provided important information about these patients, our study had some limitations. First, our patient population was not large. Second, we could not have spermiogram and cryopreservation in all patients before orchiectomy. Third, while examining the spermiograms of the patients, we could not evaluate them in terms of sperm morphology. Despite these limitations, it was important to show that patients may experience infertility risk, especially in the preoperative period.

CONCLUSION

Testicular cancer is more common in young men, and reproductive functions may be adversely affected in these patients. Currently, semen analysis and cryopreservation are recommended in patients with testicular cancer in the pretreatment period. Doctors who treat testicular cancer should be aware of fertility problems that may arise in patients in the future.

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AUTHORS' CONTRIBUTIONS

BT: Supervision, Writing – Original Draft. **SS:** Supervision, Writing – Original Draft. **CK:** Investigation. BB: Investigation. **EK:** Data Curation, Formal Analysis. **TE:** Data Curation, Formal Analysis. **MZ:** Data Curation. **SB:** Data Curation.

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Efficacy of High-Voltage Pulsed Radiofrequency for the Treatment of Elderly Patients with Acute Herpes Zoster Neuralgia

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SUMMARY

OBJECTIVE: The aim of this study was to evaluate the efficacy of high-voltage pulsed radiofrequency in comparison with standard-voltage pulsed radiofrequency for the treatment of elderly patients with acute herpes zoster neuralgia.

METHODS: Sixty-four elderly acute herpes zoster neuralgia patients were randomly assigned to the standard-voltage pulsed radiofrequency group (i.e., group S, 32 cases) and the high-voltage pulsed radiofrequency group (i.e., group H, 32 cases), which received the standard-voltage and high-voltage pulsed radiofrequency treatment, respectively. The doses of gabapentin and tramadol for analgesia were adjusted based on pain degree of patients. The therapeutic effectiveness were assessed using the numeric rating scale and the sleep quality scale. The doses of gabapentin and tramadol before pulsed radiofrequency and 1, 2, 4, 8, and 12 weeks after pulsed radiofrequency were measured. The incidence of clinically meaningful postherpetic neuralgia (pulsed radiofrequency) 12 weeks after pulsed radiofrequency was noted.

RESULTS: After pulsed radiofrequency, the numeric rating scale score and the doses of gabapentin and tramadol in group H were significantly lower than those in group S, respectively ($p<0.05$). The sleep quality scale score in group H was significantly higher than that in group S ($p<0.05$). The incidence of clinically meaningful pulsed radiofrequency in group H was significantly lower than that in group S ($p<0.05$).

CONCLUSION: For the treatment of elderly patients with acute herpes zoster neuralgia, when compared with the standard-voltage pulsed radiofrequency, the high-voltage pulsed radiofrequency can rapidly and steadily reduce the pain degree, improve the sleep quality, reduce the doses of anticonvulsants and analgesics, and decrease the incidence of clinically meaningful postherpetic neuralgia.

KEYWORDS: Pulsed radiofrequency treatment. Elderly. Herpes Zoster Oticus. Self-efficacy.

INTRODUCTION

The herpes zoster infection is caused by reactivation of the latent varicella zoster virus in the spinal or cranial nerve sensory ganglia, and it is characterized by a unilateral dermatomal rash and pain termed as "acute herpes zoster neuralgia (AHN)[1]." Moderate-to-severe AHN can cause physical disability and emotional distress[2]. Pain that persists for more than 3 months after the onset of acute zoster rash is generally considered to be postherpetic neuralgia (PHN)[3]. Moreover, the age and severity of AHN are the key risk factors for developing PHN[4]. The chronic pain in PHN can be difficult to alleviate despite the reported efficacy of many different treatments such as analgesics, topical lidocaine, topical capsaicin, nerve blocks, biofeedback, tricyclic antidepressants, gabapentin, and pregabalin[5], associated with a high economic burden on the individual and society.

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Therefore, much attention has been paid to improving the therapeutic effectiveness and alleviating pain quickly for patients with AHN.

Pulsed radiofrequency (PRF) can significantly improve the therapeutic effect of AHN and decrease the doses of analgesics[6]. Recent studies have demonstrated that the therapeutic effectiveness of PRF is affected by its parameters[7], and the high-voltage PRF has been demonstrated to enhance the clinical therapeutic effectiveness significantly for patients with neuralgia[8]. This study aimed to evaluate the efficacy of high-voltage PRF in comparison with standard-voltage PRF for the treatment of elderly patients with AHN.

METHODS

Patients and grouping

Sixty-four elderly AHN patients receiving PRF treatment in our hospital from February 2019 to June 2019 were enrolled. Based on the observer-blinded randomized controlled trial, 32 patients underwent high-voltage PRF (group H), and another 32 patients underwent standard-voltage PRF (group S). In group S, there were 13 males and 19 females, with the age of 71.42±5.43 years and the disease duration of 22.40±5.46 days. In group H, there were 15 males and 17 females, with the age of 72.81±5.92 years and the disease duration of 23.20±4.61 days. There was no significant difference in sex, age, or disease duration between two groups ($p>0.05$). This study was approved by the Ethics Committee of The Third People's Hospital of Hangzhou. The written informed consent was obtained from all participants.

Inclusion and exclusion criteria

The inclusion criteria were as follows: aged more than 65 years, exhibiting AHN located at the unilateral thoracolumbar section for less than 1 month, and the Numeric Rating Scale (NRS) score >6 points on a scale of 0–10 points. The exclusion criteria were as follows: inability to understand mandarin, inability to properly describe pain to investigator, relevant drug allergy, preexisting neuralgia, history of chronic pain, alcohol, or drug abuse, and contraindications to peripheral nerve block.

Treatment procedure

The patient was observed on the computed tomography (CT) in prone position; the electrocardiogram, blood pressure, and heart rate were continuously monitored, and the venous access was established. The nerve of the dorsal root ganglion (DRG) that needed to be therapeutically targeted was determined based on the region of skin pigmentation due to the herpes zoster infection, which is typically accompanied by hyperalgesia or allodynia. The upper-middle part of the intervertebral foramen corresponding to the target nerve was determined using a thin-slice (2-mm) CT guidance, and the puncture site, angle, and depth were assigned. After inducing local anesthesia, two PRF trocars (i.e., 20 gauge, 15 cm electrode with 10 mm active tip, PMF-20-150-10, Baylis Medical Inc., Montreal, QC, Canada) were carefully inserted until the needle tip reached the upper-middle part of the intervertebral foramen and underwent a three-dimensional CT reconstruction (Figure 1). The needle tip, which is connected to the PMG-230 pain treatment generator (Baylis Medical Inc., Montreal, QC, Canada), was slowly moved in a sensation-testing mode (50 Hz). When abnormal sensations (mainly soreness, numbness, thermally, and an occasional twitch-like or prickly sensation) were observed below 0.5V and no muscle movement was observed above 1.0V over the skin areas with hyperalgesia, we confirmed that the needle tip was appropriately positioned on the target nerve of the DRG. The settings that were subsequently used on the pain treatment generator were as follows: in group S, the output voltage was set at 50V and in group H, the output voltage was set at 50V and increased gradually to the maximum voltage (i.e., bearable without causing pain in conscious patients). The other parameters were as follows: pulse temperature, 42°C; pulse duration, 20 ms; pulse rate, 2 Hz; and pulse time, 480 s. All procedures were performed by the same physician (Bo Wang).

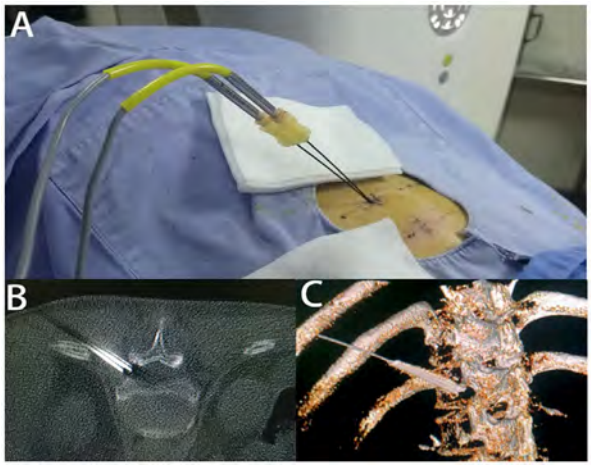

Figure 1. Images of PRF to the dorsal root ganglion. (A) PRF trocars image; (B) transverse CT image; and (C) three-dimensional CT reconstruction image. PRF: pulsed radiofrequency; CT: computed tomography.

Analgesia

Gabapentin was initiated at 300 mg/day at bedtime, and the dose increased by 300 mg/day up to a ceiling dose of 1,800 mg/day by day 7. The dose was increased regardless of whether efficacy was achieved at a lower dose, oral 25–100 mg of tramadol once or twice a day depend on the degree of pain and drug tolerance. Among patients who developed adverse effects, the dose was reduced to the previously tolerated level.

Observation indexes

The PRF parameters, such as sensation test voltage, resistance, and output voltage, in two groups were noted. The NRS and Sleep Quality Scale (SQS) scores and the doses of gabapentin and tramadol were noted in the morning before PRF and 1, 2, 4, 8, and 12 weeks after PRF, respectively.

Outcome measurement

In various studies, clinically meaningful PHN was defined as persistent pain with an intensity of three points or more on the NRS[9]. In this study, the incidence of clinically meaningful PHN 12 weeks after PRF was recorded.

Statistical analysis

The SPSS version 20.0 software (SPSS, Chicago, IL, USA) was used for the data analysis. The enumeration data were presented as number and rate and were compared using the χ^2 test. The measurement data were presented as mean±SD and were compared using the *t*-test. $p<0.05$ was considered statistically significant.

RESULTS

Comparison of PRF parameters between two groups

In group S and group H, the sensation test voltage was 0.38±0.07 V and 0.37±0.08 V, respectively, with PRF resistance of 249.02±17.34 Ω and 253.56±18.04 Ω, respectively. There was no significant difference of each index between two groups ($p>0.05$). PRF voltage in group H was 76.50±5.61 V, which was significantly higher than 47.73±2.45 V in group S ($p<0.01$).

Comparison of NRS and SQS scores between two groups

As shown in Figure 2, before PRF, there was no significant difference of NRS or SQS score between two groups ($p>0.05$). The NRS score in group H was significantly lower than that in group S at 1, 2, 4, 8, and 12 weeks after PRF, respectively ($p<0.05$). The SQS score in group H was significantly higher than that in group S at 1 and 2 weeks after PRF, respectively ($p<0.05$).

Comparison of Gabapentin and Tramadol doses between two groups

Before PRF, there was no significant difference of gabapentin or tramadol dose between two groups ($p>0.05$). The dose of gabapentin in group H was significantly lower than that in group S at 2, 4, 8, and 12 weeks after PRF, respectively ($p<0.05$). The dose of tramadol in group H was significantly lower than that in group S at 1, 2, 4, and 8 weeks after PRF, respectively ($p<0.05$) (Figures 3).

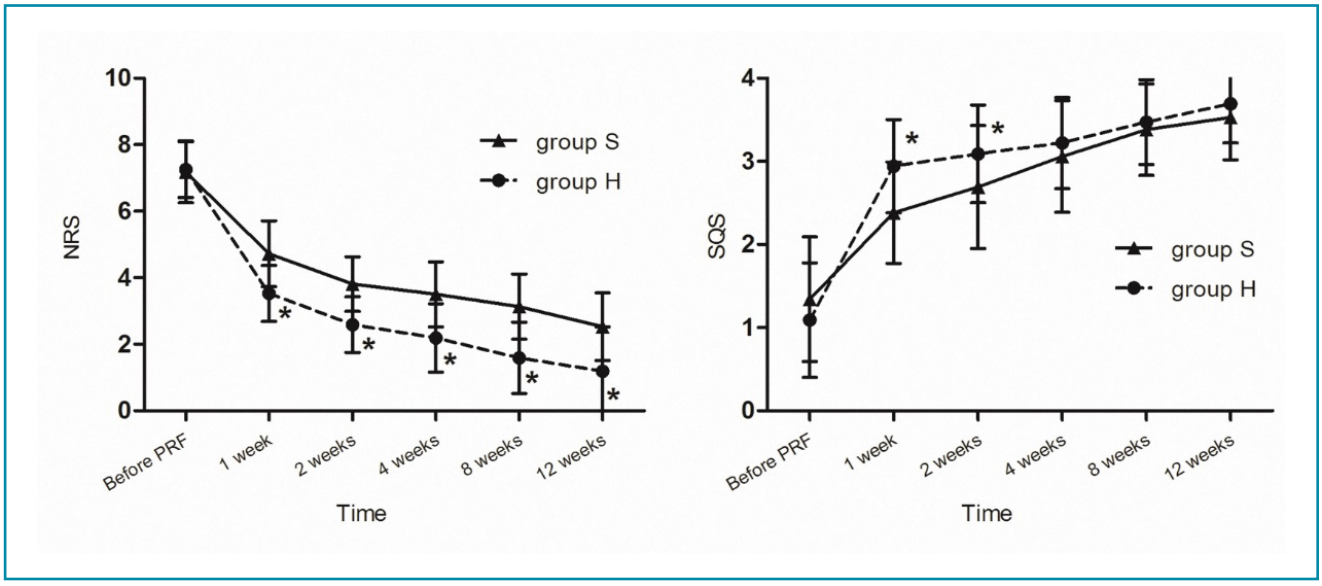

Figure 2. NRS and SQS scores between two groups. *$p<0.05$ compared with group S. NRS: numeric rating scale; SQS: sleep quality scale.

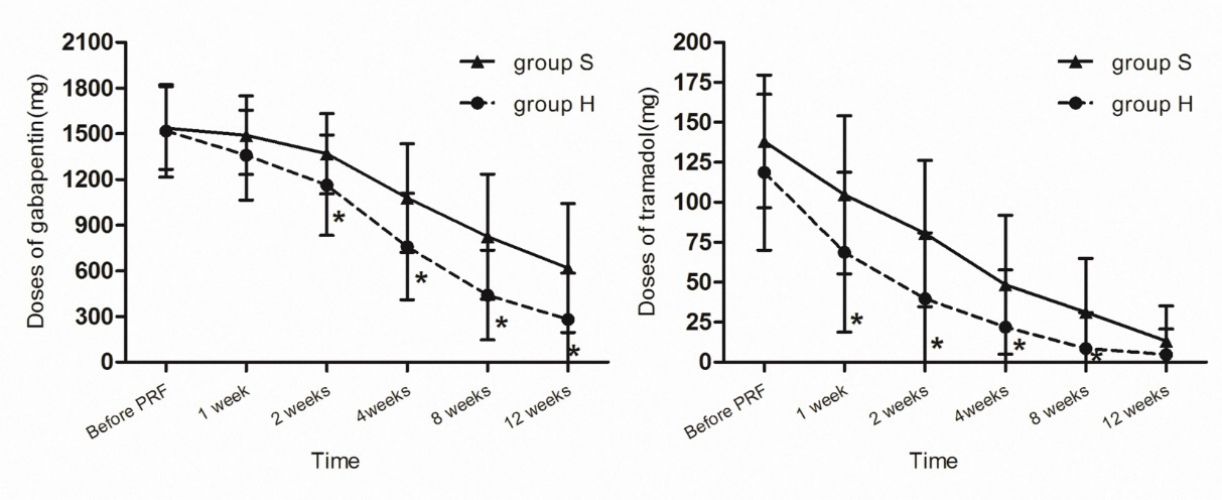

Figure 3. Doses of gabapentin and tramadol between two groups. *p<0.05 compared with group S.

Comparison of clinically meaningful PHN cases between two groups

After 12 weeks from PRF, there were 14 and 5 cases of clinically meaningful PHN in group S and group H, respectively. The incidence of clinically meaningful PHN in group H was 15.63%, which was significantly lower than 43.75% in group S (p<0.05).

DISCUSSION

Pulsed radiofrequency (PRF) is a new type of therapeutic technology. At present, the maximization of the effectiveness of PRF therapy has been a major problem for clinicians and scientists, and a large number of basic experiments and clinical studies have been conducted on parameters such as targets, time, waveform, temperature, and voltage[10-12]. This study compared the efficacy of high-voltage PRF and standard-voltage PRF for the treatment of elderly patients with AHN. The results showed that the NRS score in group H was significantly lower than group S 1, 2, 4, 8, and 12 weeks after PRF, indicating that the high-voltage PRF had significantly better therapeutic effect against AHN than the standard-voltage PRF. In addition, the SQS score in group H 1 and 2 weeks after PRF was significantly higher than that in group S. The rapid increase in SQS score was due to the dramatically rapid decrease in the degree of pain and improved the quality of life in group H, demonstrating that the high-voltage PRF had significantly better therapeutic effect against AHN than the standard-voltage PRF.

Gabapentin is a common drug for the treatment of neuropathic pain, mainly regulating voltage-gated calcium channel the alpha-2-delta subunits and achieving analgesic effects by reducing the release of glutamate, norepinephrine, and substance P[13]. The principal side effect is dose-dependent adverse reactions, such as dizziness and drowsiness; however, liver and kidney function may be impaired in severe cases. Therefore, the dosage should be monitored to reduce the discomfort due to medication in clinical practice, especially for elderly patients. In this study, the doses of gabapentin 2, 4, 8, and 12 weeks after PRF and tramadol 1, 2, 4, and 8 weeks after PRF were significantly lower in group H than in group S, demonstrating that the use of high-voltage PRF leads to the decrease in the dose of anticonvulsants and analgesics. The degree of pain in group H was stable and well controlled, and the patients could reduce the dose of oral medication at an earlier time point, reducing the possible adverse drug reactions.

In this study, 14 clinically meaningful PHN patients in group S and 5 clinically meaningful PHN patients in group H have occurred 12 weeks after PRF. The incidence of clinically meaningful PHN of the patients was lower in group H than in group S. Fortunately, all the NRS scores of PHN patients were not more than 5 points, and they had an acceptable quality of life. The incidence of PHN was less than that in the earlier literature report[14], demonstrating the effectiveness of PRF and early therapy.

This study has certain limitations. The sample size was relatively small, and the follow-up period was relatively short. In addition, we did not study the effect of different timings of PRF on the therapeutic effect of high-output voltage, and we did not investigate the dose-effect relationship between PRF output voltage and therapeutic effect. Ultrasound-guided punctured can be used to reduce the radiation exposure of patient. Such issues will be addressed in future studies. Moreover, multicentered, large-sample studies of high-voltage bipolar PRF for other neuropathic pains can be performed.

CONCLUSION

For the treatment of elderly patients with AHN, when compared with the standard-voltage PRF, the high-voltage PRF can rapidly and steadily reduce the degree of pain, improve the sleep quality, reduce the doses of anticonvulsants and analgesics, and decrease the incidence of clinically meaningful PHN.

AUTHORS' CONTRIBUTIONS

HZ: Conceptualization. BW: Data Curation, Formal Analysis. ZDL: Writing – Original Draft. JX: Writing – Review & Editing.

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Serum asprosin level in different subtypes of polycystic ovary syndrome: a cross-sectional study

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SUMMARY

OBJECTIVE: Polycystic ovary syndrome can be divided into different subtypes, including insulin resistance and hyperandrogenism. The aim of this study was to investigate the relationship between serum asprosin levels and polycystic ovary syndrome subtypes.

METHODS: A total of 93 women with polycystic ovary syndrome and 77 healthy women as controls were selected for this study. The clinical and laboratory data were compared between the Polycystic ovary syndrome group and the control group. The Polycystic ovary syndrome group was further divided into subgroups: (1) women with or without hyperandrogenism (polycystic ovary syndrome hyperandrogenism and Polycystic ovary syndrome none-hyperandrogenism, respectively) and (2) women with or without insulin resistance (polycystic ovary syndrome insulin resistance and Polycystic ovary syndrome none-insulin resistance, respectively). Serum asprosin was measured by using enenzyme-linked immunosorbent assay.

RESULTS: Serum asprosin levels showed no significant difference between the polycystic ovary syndrome and control groups. However, it was significantly lower in the Polycystic ovary syndrome HA and insulin resistance groups compared with the respective Polycystic ovary syndrome none-hyperandrogenism and none-insulin resistance groups ($p<0.05$). In the Polycystic ovary syndrome group, serum asprosin was negatively correlated with body mass index, luteinizing hormone, testosterone, basal antral follicles, fasting insulin, homeostatic model assessment of insulin resistance, and triglycerides. After adjusting for body mass index, the correlations were not significant, and asprosin was only positively correlated with prolactin (prolactin; $r=0.426$, $p<0.001$).

CONCLUSION: Our study shows that women with polycystic ovary syndrome hyperandrogenism or insulin resistance exhibit significantly lower serum asprosin levels compared with controls, and the lower asprosin level directly correlated with prolactin level.

KEYWORDS: Hyperandrogenism. Insulin resistance. Asprosin protein.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common and complex endocrine metabolic disease caused by genetic and environmental factors. PCOS is mainly characterized by menstrual abnormalities, infertility, hyperandrogenism (HA), polycystic ovarian morphology (PCOM), and metabolic abnormalities. Metabolic abnormalities are often manifested as obesity, insulin resistance (IR), and dyslipidemia[1,2]. PCOS increases the risk for type 2 diabetes mellitus (T2DM), gestational diabetes, as well as other pregnancy-related complications, cardiovascular events, and endometrial cancer[3]. IR is considered as the major risk factor for the onset of PCOS, and 70% of patients with PCOS have shown signs of IR[4]. It has also been demonstrated that aging affects the metabolic phenotype of PCOS, and therefore, age matching or correcting for age is important for PCOS studies[5].

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Asprosin, a recently identified hormone, is secreted by the white adipose tissue (WAT)[6]. It is a 140-amino-acid fragment from the C-terminal of profibrillin (encoded by FBN1) and induces the liver to increase the levels of plasma glucose. Earlier studies showed that asprosin was pathologically elevated in humans and mice with IR or obesity[6]. The olfactory receptor OLFR734 specifically binds with asprosin to modulate hepatic glucose production[7]. Several recent studies have shown that asprosin correlated with obesity in children and adults, T2DM and PCOS[8-10]. However, these results have been inconsistent. Thus, the aim of this study was to explore the potential relationship of asprosin with PCOS in women, especially those with HA or IR.

METHODS

Study subjects

This study retrieved 170 serum samples, including 93 from PCOS group and 77 from those without PCOS for the control group. The samples were obtained from the biobank affiliated to the Center for Reproductive Medicine of Shandong University. All serum samples were donated from infertility-related patients and were stored at -80°C. PCOS was diagnosed by following the Rotterdam diagnostic criteria[11]. Two of the following three criteria were positive, after the exclusion of other etiologies: (1) oligoovulation and/or anovulation, (2) clinical and/or biochemical signs of HA, and (3) polycystic ovaries on ultrasonography. The exclusion criteria included women having androgen-secreting tumors, hyperprolactinemia, 21-hydroxylase deficiency (21-OHD), Cushing's syndrome, congenital adrenal hyperplasia, thyroid disease, and abnormal intrauterine cavity. A history of recurrent spontaneous abortion, intake of medications, antidiabetic drugs, antiandrogens, oral contraceptives, insulin sensitizers, glucocorticoids, and ovulation induction agents were also excluded. The threshold for defining PCOM on ultrasound was the presence of 12 or more follicles measuring 2–9 mm in diameter or an increased ovarian volume (>10 mL) in at least one ovary. The controls were age-matched women who had infertility related to male factors or tube factors, during the same period in our *in vitro* fertilization program. PCOS was divided into subtypes according to testosterone (T) levels and homeostasis model of assessment for insulin resistance index (HOMA-IR): PCOS with HA (PCOS HA, T>60 ng/dL) and without HA (PCOS NHA) and PCOS with IR (PCOS IR, HOMA-IR≥2.5) and without IR (PCOS NIR)[12,13]. All the serum samples were collected in the follicular phase.

Clinical and laboratory data collection

The clinical and laboratory data were collected from electronic medical records (EMR) in our hospital. The anthropometric data included height, weight, body mass index (BMI), and menstrual cycle history. Serum hormones measured included follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), prolactin (PRL), testosterone (T), thyroid-stimulating hormone (TSH), dehydroepiandrosterone sulfate (DHEA-S), and anti-Müllerian hormone (AMH), and these were tested using electrochemiluminescence. Basal antral follicles were counted between the 3rd day and 5th day of menstruation by vaginal ultrasonic examination. Metabolic-related indicators including fasting glucose, fasting insulin, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides (TG) were measured. HOMA-IR was calculated as fasting glucose (in mmol/L) × fasting insulin (in mIU/L)/22.5.

Measurement of asprosin

Serum asprosin was measured using a commercial human asprosin ELISA Kit (Catalog No: E15190h, Wuhan EIAab Science Co. Ltd., China) according to the manufacturer's instructions. In brief, 100 µL serum covered with the plate sealer was incubated at 37°C for 2 h. First, the liquid was then removed, and 100 µL of detection reagent A was added and incubated at 37°C for 1 h. Second, the sample was washed 3 times, and 100 µL of detection reagent B was added. After 1-h incubation, the sample was washed 5 times, and 90 µL of substrate solution was added. Of note, 50 µL of stop solution was then added, and optical density of 450 nm was determined by automated microplate reader (PerkinElmer, Inc., Waltham, MA, USA). The acquired data were calculated by CurveExpert 1.4 (Hyams D.G., Starkville, MS, USA). The intra-assay coefficient of variation (CV) was ≤6.5%, and the inter-assay CV was ≤9.8%.

Statistical analysis

All the statistical analyses were conducted using the SPSS software (IBM, Armonk, NY, version 21.0) and GraphPad Prism 7 software (San Diego, CA, USA). Kolmogorov–Smirnov test were used to test the characteristics of participants' distribution. The data normally distributed were expressed as mean±SD, and the data with skewed distribution were shown as median (IQR, 25–75th). Independent samples *t*-test was used to compare the normally distributed variables, and Mann–Whitney *U* test was used to compare abnormally distributed variables. The Spearman's rank correlation coefficient analysis was performed to analyze the bivariate correlation between asprosin and other parameters. $p<0.05$ (two-sided) was considered as statistically significant.

RESULTS

The clinical characteristics of 170 subjects are described in Table 1. There were no significant differences in age, FSH, PRL, and TSH levels between the control and PCOS groups. PCOS patients had higher levels of different hormones and metabolic-associated parameters (i.e., LH, LH/FSH, E_2, T, AMH, fasting glucose, fasting insulin, HOMA-IR, DHEA-S, TC, HDL, LDL, and TG, p<0.05). Women in the PCOS group had significantly longer menstrual cycles than those in the control group (50.87±13.68 vs. 29.69±2.98; p<0.001). In the PCOS group, BMI was higher, and basal antral follicle numbers were more than in the control group (p<0.05).

As shown in Figure 1A, serum asprosin levels showed no significant difference between the PCOS and control groups [2.87 (2.18–4.47) vs. 3.24 (2.23–4.31) ng/mL, median (25–75th), p>0.05]. The asprosin levels were measured in different PCOS subtypes (Figures1B–D). The serum asprosin level in the PCOS HA group was notably lower than in the PCOS NHA group [2.52 (2.06–3.19) vs. 4.20 (2.35–5.79) ng/mL, median (25–75th), p<0.05] (Figure1B). The serum asprosin levels in the PCOS IR group were significantly lower than in the PCOS NIR group [2.46 (2.05–4.30) vs. 3.77 (2.47–7.18) ng/mL, median (25–75th), p<0.05] (Figure1C). In addition, this type of measurement was more pronounced in the PCOS IR&HA groups.

In the PCOS group, serum asprosin was negatively correlated with BMI, LH, T, basal antral follicles, fasting insulin, HOMA-IR, and TG. When adjusted for BMI, the correlations were not significant and asprosin was only positively correlated with PRL (r=0.426, p<0.001; Table 2). In addition, asprosin was still positively correlated with PRL (r=0.456, p=0.003) in PCOS NHA subjects. Moreover, there was no correlation between asprosin and other characteristics in PCOS HA subjects. These results indicate that obesity rather than PCOS might be responsible for the difference in asprosin levels.

Table 1. General clinical and laboratory characteristics of study participants.

	Total sample	Controls (n=77)	PCOS (n=93)	p-value
Age (years)[a]	28.48±3.47	28.34±3.06	28.60±3.78	0.615
BMI (kg/m²)[a]	23.75±4.10	22.30±3.69	24.95±4.05	<0.001
Menstrual cycles	41.33±14.79	50.87±13.68	29.69±2.98	<0.001
FSH (IU/L)[a]	6.15±1.51	6.32±1.13	6.02±1.76	0.183
LH (IU/L)[a]	7.84±4.54	5.55±1.63	9.74±5.26	<0.001
LH/FSH[a]	1.34±0.85	0.90±0.30	1.70±0.98	<0.001
E2 (pg/mL)[a]	40.87±17.70	35.03±12.80	45.71±19.70	<0.001
PRL (ng/mL)[a]	16.75±8.00	16.66±6.85	16.82±8.88	0.900
T (ng/dL)[b]	29.06 (22.27–62.84)	23.58 (18.06–28.67)	60.45 (27.82–68.35)	<0.001
TSH (μIU/mL)[a]	2.34±1.02	2.25±0.93	2.42±1.08	0.974
Basal antral follicles[a]	23.44±12.02	14.42±3.89	30.91±11.32	<0.001
AMH (ng/mL)[a]	7.85±4.96	5.52±3.21	10.12±5.32	<0.001
Fasting glucose (mmol/L) a	5.37±0.99	5.15±0.47	5.55±1.23	0.013
Fasting insulin (mIU/L)b	10.33 (7.38–18.97)	7.75 (6.35–10.20)	12.38 (8.76–25.30)	<0.001
HOMA-IR[b]	2.35 (1.71–4.81)	1.82 (1.49–2.27)	3.06 (2.12–6.62)	<0.001
DHEA-S (μg/dL)[a]	276.68±101.97	259.27±82.93	291.02±113.78	0.039
TC (mmol/L)[a]	4.47±0.85	4.29±0.87	4.61±0.82	0.023
HDL (mmol/L)[a]	1.35±0.28	1.43±0.24	1.29±0.30	0.004
LDL (mmol/L)[a]	3.01±0.72	2.80±0.70	3.17±0.70	0.002
TG (mmol/L)[b]	0.95 (0.68–1.33)	0.79 (0.63–1.09)	1.07 (0.81–1.45)	<0.001

BMI: body mass index; FSH: follicle stimulating hormone; LH: luteinizing hormone; E2: estradiol; PRL: prolactin; T: testosterone; TSH: thyroid-stimulating hormone; AMH: anti-Müllerian hormone; HOMA-IR: homeostasis model of assessment for insulin resistance index; DHEA-S: dehydroepiandrosterone sulfate; TC: total cholesterol; HDL: high-density lipoprotein; LDL: low-density lipoprotein; TG: triglycerides. [a]The data normally distributed are shown as mean ± SD. The independent sample *t*-test was performed. [b]The data distributed non-normally are shown as median (IQR, 25–75th). The Mann–Whitney *U* test was performed.

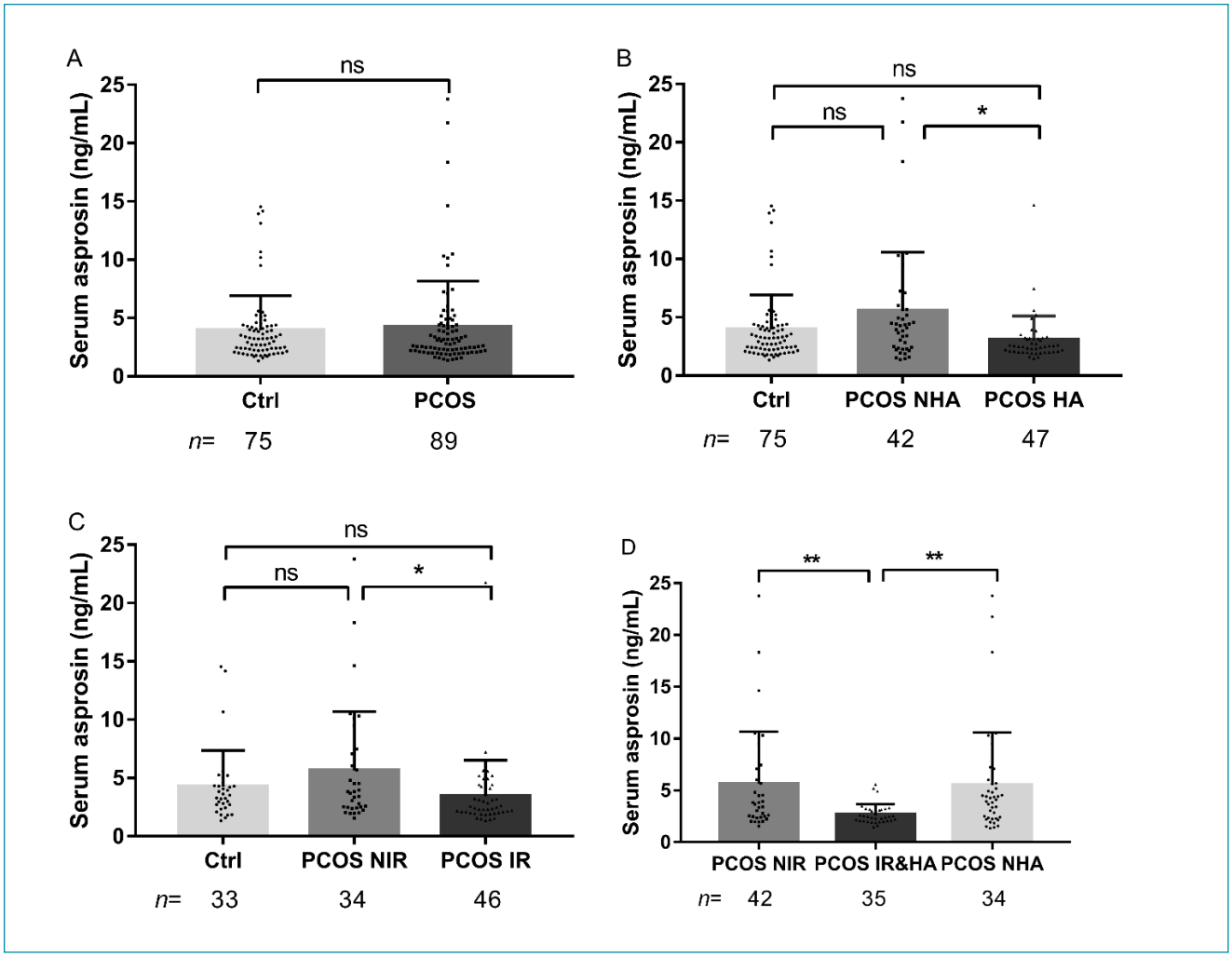

Figure 1. Serum asprosin levels between distinct groups. **(A)** Serum asprosin in PCOS group (n=89) had no statistically significant difference compared with the control group (n=75; p>0.05); **(B)** PCOS was subdivided into hyperandrogenism subtype (PCOS HA, n=47) and non-hyperandrogenism subtype (PCOS NHA, n=42), Serum asprosin in PCOS HA group were significantly lower than the PCOS NHA group (p<0.05); **(C)** PCOS patients were subdivided into insulin resistance subtype (PCOS IR, n=46) and non-insulin resistance subtype (PCOS NIR, n=34), Serum asprosin in PCOS IR group were significantly lower than the PCOS NIR group (p<0.05); **(D)** PCOS patients with both IR and HA (PCOS IR&HA, n=35). *indicates statistical significance at p<0.05 and **indicates statistical significance at p<0.01. PCOS: polycystic ovary syndrome.

DISCUSSION

This study showed that serum asprosin levels were similar between women in PCOS and control groups; however, lower levels were seen in PCOS HA and PCOS IR groups. Further analysis demonstrated that asprosin was positively correlated with PRL, i.e., independent of BMI.

Asprosin was first discovered by Romere et al. and was considered positively associated with IR[6]. Although IR was excluded from the diagnostic criteria for PCOS, it is a common physiological abnormality with metabolic dysfunctions in women with PCOS[14]. Adipose tissue can regulate the metabolism and balance the energy homeostasis through its role in endocrine regulation. Some small molecules secreted by the adipose tissue can either enhance or impair insulin action[15]. Recently, two other studies confirmed that asprosin was positively correlated with diabetes mellitus[8,16]. There are reports about the relationship between asprosin and PCOS; however, these results are inconsistent[10,17,18]. We would like to further explore the profiles of asprosin in PCOS subtypes.

We first compared the serum asprosin in women with PCOS and healthy controls in the concurrent period. Serum asprosin was comparable between women with or without PCOS, and it was slightly lower in the PCOS group. The PCOS group was then divided into different subgroups. Serum asprosin levels

Table 2. Bivariate correlations between serum asprosin level and other variables in the PCOS group and the subgroups categorized by testosterone.

	PCOS		PCOS (BMI-adjusted)		PCOS NHA (BMI-adjusted)		PCOS HA (BMI-adjusted)	
	r	p	r	p	r	p	r	p
Age (years)	0.111	0.299	0.039	0.717	0.015	0.924	0.147	0.329
BMI (kg/m^2)	-0.226	0.033	–	–	–	–	–	–
FSH (IU/L)	0.023	0.831	-0.006	0.952	0.021	0.895	-0.149	0.325
LH (IU/L)	-0.216	0.042	-0.164	0.127	0.054	0.739	-0.126	0.403
LH/FSH	-0.207	0.051	-0.137	0.204	0.010	0.952	-0.003	0.982
E2 (pg/mL)	-0.146	0.172	-0.048	0.654	0.153	0.341	-0.111	0.464
PRL (ng/mL)	0.170	0.110	0.426	<0.001	0.456	0.003	-0.058	0.704
T (ng/dL)	-0.299	0.004	-0.137	0.213	0.242	0.127	0.159	0.291
TSH (µIU/mL)	0.195	0.068	0.176	0.102	0.190	0.234	-0.083	0.582
Basal antral follicles	-0.255	0.016	-0.183	0.087	0.005	0.975	-0.080	0.596
AMH (ng/mL)	-0.207	0.079	-0.156	0.192	-0.099	0.545	0.012	0.948
Fasting glucose (mmol/L)	0.146	0.183	0.053	0.633	0.124	0.439	0.162	0.306
Fasting insulin (mIU/L)	-0.345	0.002	-0.195	0.083	-0.274	0.083	-0.070	0.677
HOMA-IR	-0.297	0.007	-0.158	0.164	-0.259	0.102	0.038	0.824
DHEA-S (µg/dL)	-0.072	0.505	0.016	0.885	0.148	0.361	0.252	0.094
TC (mmol/L)	-0.100	0.380	-0.075	0.514	-0.082	0.630	0.030	0.851
HDL (mmol/L)	0.209	0.062	0.189	0.093	0.180	0.287	0.142	0.371
LDL (mmol/L)	-0.144	0.199	-0.104	0.358	-0.135	0.425	0.063	0.691
TG (mmol/L)	-0.219	0.045	-0.075	0.499	0.036	0.826	-0.167	0.290

BMI: body mass index; FSH: follicle stimulating hormone; LH: luteinizing hormone; E2: estradiol; PRL: prolactin; T: testosterone; TSH: thyroid-stimulating hormone; AMH: anti-Müllerian hormone; HOMA-IR: homeostasis model of assessment for insulin resistance index; DHEA-S: dehydroepiandrosterone sulfate; TC: total cholesterol; HDL: high-density lipoprotein; LDL: low-density lipoprotein; TG: triglycerides. Correlations between variables were analyzed by using the Spearman's rank correlation coefficient analysis or the BMI-adjusted partial correlation test.

were lower in both PCOS HA and IR groups. We then analyzed the probable correlations between asprosin and PCOS. The results showed that asprosin was negatively correlated with IR and HOMA-IR, which contradicts earlier studies[6,8,16]. However, after adjusting for BMI, there was a positive correlation only between asprosin and PRL. Therefore, it is likely that obesity rather than PCOS might be responsible for the difference in asprosin levels. There might be some explanations for this. One possibility is that the serum asprosin might be influenced by confounding effects through sex hormones, specific population conditions, and repeated freeze-thaw cycles. Another possibility is the multiple interactions with some other adipokines such as irisin, visfatin, and adiponectin, which are also secreted by the WAT and associated with PCOS[19-21]. Meanwhile, we found that asprosin was positively correlated with PRL. Circulating PRL is mainly secreted by the lactotroph and mammosomatotroph cells in the pituitary gland. However, the adipose tissue can also produce PRL at extra-pituitary sites[22]. In both young healthy men and obese men, PRL was inversely associated with insulin sensitivity[23,24]. PRL produced by the adipose tissue was directly related to the PPARG, ADIPOQ, and GLUT4 levels in the human visceral and subcutaneous fat[24]. Considered together, PRL might influence asprosin levels through certain feedback mechanisms in women with PCOS, which also explains the first possibility.

Our study provides significant insights about the correlation between asprosin and PCOS. Three different studies about asprosin and PCOS were recently published[10,17,18]. Chia et al. reported that asprosin levels in women with PCOS were similar to those in corresponding controls[18]. However, Murat and Li found that circulating asprosin levels were elevated in women with PCOS compared with those in controls[10,17]. Our results were consistent

with the former results, but contrary to the latter. This diversity might be due to the different sample conditions and effects of PRL. Still, some limitations in this study should be acknowledged. For example, this study is based on EMR from a single hospital, and the sample size was relatively limited.

CONCLUSION

This study shows that women with PCOS HA or IR exhibit significantly lower levels of serum asprosin. The serum asprosin levels also correlated closely with various sex hormones and metabolic disorders, and the lower asprosin levels directly correlated with PRL levels.

AUTHORS' CONTRIBUTIONS

YJ: Data Curation, Project Administration, Validation, Writing – Original Draft. YL: Data Curation, Formal Analysis. ZY: Data Curation, Formal Analysis. PY: Data Curation, Formal Analysis. SZ: Funding Acquisition, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – Review & Editing.

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Evaluation of anti-SARS-CoV-2 antibody levels: two different methods

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SUMMARY

OBJECTIVE: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) disease (COVID-19) is still a major problem worldwide. Antibody response to SARS-CoV-2 has not yet been fully clarified, and clinical benefits of serological tests remain unclear. Despite the presence of numerous systems and methods used to analyze antibody levels, it is difficult to mention about standardization. This study aims to evaluate antibody levels of COVID-19 patients obtained by different methods.

METHODS: Specimens of 55 patients were included in this study. Patients underwent SARS-CoV-2 real-time polymerase chain reaction test, COVID-19 IgM/IgG antibody rapid test (Hotgen), and Roche SARS-CoV-2 antibody test.

RESULTS: In this study, the positive values of COVID-19 IgM/IgG antibody rapid test, Roche SARS-CoV-2 antibody test, and SARS-CoV-2 real-time polymerase chain reaction test were 37, 26, and 31, respectively, whereas the negative values were 18, 29, and 24, respectively. A comparison of the results using χ^2 test revealed a significant difference among SARS-CoV-2 real-time polymerase chain reaction, COVID-19 IgM/IgG antibody rapid test (Hotgen), and Roche SARS-CoV-2 antibody test.

CONCLUSIONS: We recommend antibody testing in close contact tracing as well as in real-time polymerase chain reaction negative symptomatic subjects. Standardization is important as positive values show significant variations among antibody tests.

KEYWORDS: SARS-CoV-2. Immunoassay. Serological tests. Immunoglobulins.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) disease (COVID-19) is still a major problem all over the world[1,2]. A substantial number of patients and deaths due to SARS-CoV-2 has been achieved worldwide, and unfortunately the number of cases continues to increase[3]. Accurate and rapid diagnosis of SARS-CoV-2 is essential for timely isolation of COVID-19 patients to stop the pandemic and to save the people's lives. The real-time polymerase chain reaction (RT-PCR) test, which has been developed and used for rapid detection of SARS-CoV-2, is used to detect viral nucleic acid and as the standard diagnostic test for COVID-19. Being time-consuming and troublesome and requiring specific equipment have restricted the use of RT-PCR test particularly in the areas with limited laboratory facilities[2,4].

Human antibody response against viral infection has been widely used to help with the diagnosis of viral infections. Comparing with the RT-PCR tests, the detection of antibody levels is more easily accessible as these tests are faster, cheaper, easy to use, and less frequently require laboratory expertise[2]. Antibody reactions against SARS-CoV-2 remain unclear, and the clinical benefits of serological tests are indefinite[5].

Long et al.[5] collected the serum samples from 164 subjects for antibody testing approximately after 30 days of exposure

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to the virus. Virus-specific IgG and/or IgM were found positive in all of 16 cases with positive RT-PCR result. In addition, virus-specific IgG and/or IgM were also found positive in seven out of 148 subjects with negative RT-PCR, indicating that 4.3% (7/164) of close contacts have been missed out by the RT-PCR test[5]. Seroconversion for IgM is achieved in five days after symptom onset, whereas seroconversion for IgG is achieved in 5–7 days after symptom onset[6,7]. Maximum seroconversion occurs in 2–3 weeks for IgM and in 3–6 weeks for IgG[5-7]. It is known that studies about this test are lacking or limited in number as this is a novel test.

The data from SARS pandemic indicate that serological responses including virus-specific IgM and IgG are adequate for making serological diagnosis[8,9]. The results of these studies revealed that the ELISA tests require less labor for serological diagnosis of SARS-CoV-2 pneumonia and are sensitive, more economic, and provide an advantage as they do not require viral cultivation[9].

The rapid test uses a double-antigen sandwich method to detect SARS-CoV-2 IgM/IgG antibodies and is a colloidal gold-based immunochromatographic assay[10]. The Roche Anti-SARS-CoV-2 assay is an immunologic test for *in vitro* quantitative detection of SARS-CoV-2 antibodies (including IgG) in human serum and plasma. It was aimed to use this test as an assistant in detecting immune response against SARS-CoV-2. The Roche Elecsys Anti-SARS-CoV-2 assay uses a recombinant protein for nucleocapsid antigen to detect SARS-CoV-2 antibodies[11].

Despite the presence of numerous antibody tests concerning COVID-19 pandemic, reliability or specificity comparisons have not been performed for many of them. The antibody testing will become more important with the increasing number of individuals affected by the pandemic. This study aimed to evaluate the results of the COVID-19 IgM/IgG rapid test (Pekin Hotgen Biotech Co., Ltd.) used in our hospital with the results of the Roche SARS-CoV-2 (Roche Diagnostics, Germany) assay studied in immunoassay system.

METHODS

Specimens from a total of 55 patients that have applied to our hospital between April and May 2020 were included in this study. All patients underwent the SARS-CoV-2 RT-PCR testing (Bioeksen, Turkey) of nasopharyngeal smear as well as the COVID-19 IgM/IgG rapid testing and the Roche SARS-CoV-2 antibody testing of blood samples at least once. For all the antibody testing procedures, the blood samples were collected into routine tubes, waited for 30 min for clotting to occur, centrifuged at 1500´ g and then stored at -70°C until the time of analysis. Completely resolved serum specimens were studied.

Of the 55 individuals from whom the study samples have been obtained, 32 were females and 23 were males. The age of these individuals ranged between 21 and 91 years, with a mean age of 33.96±11.849 years.

The Bio-Speedy Direct RT-qPCR SARS-CoV-2 nucleic acid detection kit (Bioeksen, Turkey) is designed for the qualitative detection of nucleic acid from SARS-CoV-2. The kit is a one-step reverse-transcription and real-time PCR assay targeting the SARS-CoV-2-specific N and ORF1ab gene region. Nasopharyngeal swab samples collected in viral nucleic acid–buffered tubes for SARS-CoV-2 RT-PCR were analyzed by using the Bio-Rad CFX96 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) device. RT-PCR was performed at 52°C for five min (1 cycle), 95°C for 10 sec (1 cycle) followed by 95°C for one sec and 55°C for 30 sec (40 cycles) steps[12]. The negative and positive controls for each study and the internal controls for each sample were evaluated, and the PCR result was interpreted as positive or negative result based on the appropriate controls.

All samples were analyzed by the COVID-19 IgM/IgG rapid testing, as well as with the original kits after the two-phase quality-control procedure in the Roche cobas e 601 device (Roche Diagnostics, Germany), which is routinely used in our laboratory.

The Roche SARS-CoV-2 antibody test is a test based on chemiluminescent immunoassay. The results are determined automatically by the software, comparing the electrochemiluminescence signal obtained from the reaction product with the signal of the threshold value previously obtained by calibration. The results of the samples analyzed in Roche cobas e 601 device are presented as reactive or unreactive and as cut-off index (COI). COI<1.0 nonreactive is interpreted as negative for Anti-SARS-CoV-2 antibody, COI≥1.0 reactive is interpreted as positive for Anti-SARS-CoV-2 antibody[11]. The data were compared using the χ^2 test.

RESULTS

Specimens from a total of 55 individuals were included in this study. All patients underwent the SARS-CoV-2 RT-PCR, the COVID-19 IgM/IgG rapid testing, and the Roche Anti-SARS-CoV-2 antibody testing procedures. The number of test-positive patients was 31, 37, and 26, respectively, and the number of test-negative patients was 24, 18, and 29, respectively, for each method (Table 1).

Among 37 patients with positive rapid antibody test result, seven patients had negative SARS-CoV-2 RT-PCR test result

Table 1. Number of patients.

Method	n=55	
	Positive	Negative
COVID-19 IgM/IgG rapid test	37	18
Roche Anti-SARS-CoV-2	26	29
SARS-CoV-2 RT-PCR	31	24

and 11 had negative Roche Anti-SARS-CoV-2 antibody test result. Only one out of the 11 patients with negative Roche Anti-SARS-CoV-2 antibody test result had negative SARS-CoV-2 RT-PCR test result.

While the Roche Anti-SARS-CoV-2 antibody test result was negative in 18 patients with positive rapid antibody test result, only one patient had positive SARS-CoV-2 RT-PCR test result. Overall, the Roche Anti-SARS-CoV-2 antibody test result was positive in 26 and negative in 29 patients.

The consistency between the SARS-CoV-2 RT-PCR test and the Roche SARS-CoV-2 assay was 76.9% for positive patients and 62.1% for negative patients, and the difference is significant (χ^2=8.47, p<0.004). The consistency between the SARS-CoV-2 RT-PCR test and the COVID-19 IgM/IgG rapid test was 81.1% for positive patients and 94.4% for negative patients, and the difference is significant (χ^2=28.08, p<0.001). The consistency between the COVID-19 IgM/IgG rapid test and the Roche SARS-CoV-2 assay was 70.3% for positive patients and 100% for negative patients, and the difference was significant (χ^2=23.99, p<0.001).

DISCUSSION

This study was carried out with the specimens obtained from 55 patients in our hospital. The RT-PCR test is used for rapid detection of SARS-CoV-2. The studies about the occurrence of antibody response following SARS-CoV-2 transmission and the stability of antibodies are limited. Although antibody testing using quick tests is older than antibody testing in immunoassay systems, comparative studies in the literature are lacking due to small number of the studies on this subject. Both the COVID-19 IgM/IgG rapid test and the Roche SARS-CoV-2 assay determine the total antibody level, but do not discriminate virus-specific IgM from IgG.

Zhao et al.[13] conducted a study in 173 patients and found seroconversion rate of 93.1% for overall antibodies, 82.7% for IgM, and 64.7% for IgG. They reported that antibody negativity determined in 12 patients might have been resulted from not analyzing the specimens in the advanced stages of the disease[13]. In this study, the antibody tests gave negative results but the PCR test gave positive result in a single case, although there were seven days between PCR and antibody testing procedures.

Among seven patients who were positive for the COVID-19 IgM/IgG rapid test but negative for the RT-PCR test, specimen for the RT-PCR testing was obtained on the same day from two patients, both on the same day and before 20, 38, and 40 days, respectively, from three patients, and before 42 and 60 days from two patients. The Roche SARS-CoV-2 antibody test result was positive in six of the seven patients with positive COVID-19 IgM/IgG rapid test result. Wang et al.[14] found the positivity rates for SARS-CoV-2 RNA of 63% in nasopharyngeal smear specimens and 32% in oropharyngeal smear specimens[14]. RT-PCR negativity in seven patients (12.72%) with antibody positive can be attributed to specimen-related positivity or time of sample collection. Accordingly, it can be concluded that antibody testing in symptomatic patients negative for RT-PCR is beneficial in identifying these patients. The lower number of RT-PCR positivity than antibody positivity in this study might be associated with all the specimens being nasopharyngeal smear because RT-PCR positivity is higher with the specimens obtained from lower respiratory tract such as bronchoalveolar lavage fluid and deep tracheal aspirate. Moreover, PCR positivity is associated with numerous factors including RT-PCR performance, quality and consistency of the PCR kits used, sample collection skills, and type of samples[13,14].

The detection of antibody levels is critical for making a diagnosis in the patients with negative RT-PCR test result; antibody-positive healthy individuals who are in quarantine period because of close contact should be considered as potential carriers and should undergo the RT-PCR testing more frequently, and antibodies detected in RT-PCR-positive patients indicate induction of specific antibodies in the individuals[13]. Detecting higher antibody positivity when compared with RT-PCR test positivity indicates that antibody testing can be used not to miss the cases and to take necessary isolation measures during pandemic. Besides, antibody testing may help with diagnosis because symptomatic and RT-PCR-negative patients with low viral burden might be overlooked. Long et al.[5] studied 16 specimens (i.e., three from asymptomatic patients) from 164 close contacts and determined RT-PCR positivity as well as virus-specific IgM and/or IgG seropositivity; however, they determined RT-PCR negativity and virus-specific IgM and/or IgG seropositivity in the specimens from seven of the remaining 148 asymptomatic patients[5]. In this study, there was only one patient (1.81%) with the specimen positive for RT-PCR and negative for antibody testing among 55 specimens, whereas seven (12.72%) specimens showed positivity for rapid antibody testing and negativity for RT-PCR. In immunoassay system, however, RT-PCR was negative in three patients with antibody positive. The rapid test was not negative in any of the Roche Anti-SARS-CoV-2 antibody-positive specimens analyzed by immunoassay system. Among the antibody

tests in this study, rapid test has higher antibody positivity rates (e.g., antibody positivity rate 67.27% for rapid test and 52.72% for immunoassay test). The rapid test and the immunoassay test differ significantly from each other in terms of detecting Anti-SARS-CoV-2 antibodies (χ^2=23.99, p<0.001).

In the study carried out using 208 plasma specimens (i.e., from 82 confirmed and 58 asymptomatic PCR-negative patients, 140 in total), Guo et al.[15] reported that the efficacy of IgM ELISA is higher than PCR after 5.5 days of symptom onset and that the positivity rate increases significantly with IgM ELISA plus PCR (98.6%) when compared with PCR alone (519%)[15]. Many studies have emphasized that serological tests can increase the positivity rate and that they should be used in subclinical patients and, in future, epidemiological studies[16-18]. Also, in this study, considering the consistency between the tests in terms of both positivity and negativity rates, we concluded that antibody testing is important in the diagnosis and patient monitoring.

We reached to seven patients (hospital staff) with antibody positive and RT-PCR negative determined in this study; these patients confirmed that they have had COVID-19 and have undergone antibody testing accordingly. Therefore, it can be concluded that rapid test positivity is not a false positivity. In addition, the significant difference between the Anti-SARS-CoV-2 antibody test and the SARS-CoV-2 RT-PCR test indicates the importance of antibody testing. In general, although immunoassay systems show better sensitivity and specificity, one of the striking outcomes of this study is higher antibody positivity rate with rapid test when compared with the immunoassay system. Therefore, studies on antibody levels gain importance in determining the seroprevalence among population and in detecting antibody levels for both diagnostic and therapeutic purposes.

CONCLUSIONS

The antibody testing might be important for close contact tracing. Moreover, we believed that antibody testing should be performed in RT-PCR-negative symptomatic patients. In addition, since the positivity rate shows significant difference among antibody tests as mentioned in this study, studies on this subject and standardization are of importance.

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ETHICAL APPROVAL

This study was approved by the Ethics Committee of Health Sciences University Diyarbakır Gazi Yasargil Training and Research Hospital (dated October 16, 2020, Decision No. 601, including reference number).

AUTHORS' CONTRIBUTIONS

OA: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Software, Supervision, Validation, Visualization, Writing – Original Draft, Writing – Review & Editing. **AM:** Data Curation, Investigation, Supervision, Visualization, Writing – Review & Editing. **GSA:** Formal Analysis, Methodology, Resources, Supervision, Validation, Visualization, Writing – Review & Editing. **ARO:** Data Curation, supervision, Supervision, Visualization, Writing – Review & Editing.

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Attitude and knowledge of medical students toward donation after circulatory death

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SUMMARY

OBJECTIVE: A survey among medical students in a Brazilian public university was performed to investigate the acceptance of organ donation in Brazil, particularly donation after circulatory death (DCD).

METHODS: A questionnaire including 26 objectives and Likert scale questions was validated and sent to all medical students of our institution. The answers were analyzed considering the whole set of individuals as well as by dividing the medical students into two groups: less graduated students and more graduated students.

RESULTS: From 1050 students, 103 spontaneous answers (9.8%) were retrieved after 3 weeks. A total of 89.3% agreed totally with deceased donor organ donation and 8.7% agreed partially. However, only 50.5% of the students agreed totally and 31.1% agreed partially to living donation. Students revealed that 82.6% know the concept of brain death. On the other hand, 71.8% of them declared not knowing the concept of planned withdrawal of life-sustaining therapy, mainly cardiorespiratory support. A total of 85.4% of students agreed totally with donation after brain death and 11.7% agreed partially. However, when questioned about donation in awaiting circulatory death after a planned withdrawal of life-sustaining therapy, only 18.4% agreed totally and 32% agreed partially. Both groups of less and more graduated students showed similar results.

CONCLUSIONS: Our study found a clear lack of information and consequently in acceptance of DCD. Education in the field of end-of-life management may improve not only the acceptance of DCD donation but also the whole understanding of planned withdrawal of life-sustaining therapy.

KEYWORDS: Death, sudden, cardiac. Health knowledge, attitudes, practice. Students, medical. Surveys and questionnaires. Organ transplantation.

INTRODUCTION

In the 90s, the Maastricht's group established the four categories for DCD donors, and it had a great impulse[1]. Nowadays, this practice is legitimate in the United States, many European countries, Canada, Australia, Japan, China, but still not accepted in Brazil[2].

The current organ shortage for transplantation and the increased graft demand for patients in waiting list prospect DCD as an effective source of grafts, expanding the potential donor pool. Over the last decades, DCD donors have been responsible for a significant increase in numbers of deceased

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organ donors, while donors after brain death (DBD) has remained broadly stable, particularly in some countries such as the United Kingdom[3,4].

In Brazil, despite the advances in last decade, the rate of effective donation was only 17.0 per million population in 2018[5]; therefore, far to supply the needs of those in the waiting list. These data clearly show the importance of discussing the acceptance of DCD in the Brazil.

In general, the Brazilian society is quite conservative, and the lack of appropriate education remains an ordeal especially in the poorer areas of the country. These aspects surely impair the debate about DCD in our context. However, medical students supposedly represent a well-educated part of the society.

The attitude of medical students toward DCD has not been investigated in Brazil so far. To address this issue, we conducted a survey among medical students from our institution in order to better understand the acceptance of DCD along the graduation course in medicine.

METHODS

In an analytical-descriptive study, medical students from the first to the sixth year from the Faculty of Medicine of the University of São Paulo (FMUSP) voluntarily responded to an anonymously questionnaire designed to determine their knowledge and opinion on organ donation, particularly on DCD. This study was approved by the local Ethical Committee (CAPPESQ 3.291.655).

The questionnaire was validated by six referees: two senior physicians who worked in a liver transplant program for more than 10 years, two medical students, one statistician, and one chief nurse coordinator from our liver transplantation program. The enquiry included 26 objectives and Likert scale questions, allowing the student to choose from the following alternatives: "agree partially," "agree totally," "disagree partially," "disagree totally," or to be "indifferent" to the situation proposed by the question. The questionnaire was sent by e-mail to all students from the FMUSP.

The answers to the questionnaire were analyzed considering the whole set of individuals as well as by dividing the medical students into two groups: less graduated students (from first to third graduation year) and more graduated students (from fourth to sixth graduation year), who have been more exposed to clinical practice. The students' sex, self-determined religion, and origin of the state were also evaluated.

The Mann-Whitney *U* test was used to compare the answers between the two groups, and the chi-square test was used to analyze the answers among the different religious beliefs. The statistical significance was set at 0.05. All statistical analyses were performed using the R software with packages "stats" and "rstatix" (https://www.r-project.org/).

RESULTS

We retrieved 103 spontaneous answers after 3 weeks. Considering that the total number of students of the school is 1050, we observed a 9.8% of spontaneous answers to thequestionnaire. The higher number of responses was in the third year (27.2%), followed by the second (22.3%), first (19.4%), fourth (13.6%), fifth (9.7%) and only 7.8% from the sixth year of the medical school.

A total of 50.5% of the enquire was answered by women and 49.5% by men. The majority of the responses were from students from São Paulo state (82.5%). Regarding religion, 31.1% of the students classified themselves as agnostic, 23.3% as atheist, 19.4% as catholic, 7.8% as Kardecist spiritists, 4.9% as having no religion, 3.9% as protestant, and 8.7% as others.

Concerning student's statement regarding organ donation and transplantation, 78.6% answered that it would be an issue for medical graduation program and 81.6% for postgraduation course. Moreover, 36.9% have evaluated their knowledge about this issue as regular, 35% as inadequate, and only 18.4% as adequate. In fact, only 44.7% declared to have studied this subject during medical school.

The collected results showed that students are steadily aware about donor/recipient imbalance in organ transplantation waiting list, as 88.4% agreed completely or partially with the affirmation of not having enough donors for transplantation in Brazil. At the same time, 95.2% of the students seem to trust in transplantation as a life-saving procedure, as when they agreed in undergoing this treatment if necessary. In fact, 89.3% agreed totally with deceased donor organ recovery and 8.7% agreed partially. When confronted to a living donation, 50.5% of the students agreed totally and 31.1% agreed partially. Despite this decrease in agreement, 73.8% of the students agreed totally to donate part of their livers for a loved one and 19.4% agreed partially.

On the one hand, students revealed that 82.6% have some knowledge on the concept of brain death; however, only 31.1% answered, and they completely comprehended this issue. On the other hand, 71.8% of them declared not knowing the concept of planned withdrawal of life-sustaining therapy, mainly cardiorespiratory support. When asked about the concept of unsuccessful resuscitation after cardiac arrest, 68.9% responded to fully or partially comprehend.

When the enquire targeted the acceptance of donation after brain death, 85.4% of the students agreed totally and 11.7% agreed partially. However, when questioned about donation in awaiting cardiac or circulatory death after a planned withdrawal of life-sustaining therapy, only 18.4% agreed totally and 32% agreed partially (Table 1). When the question was about acceptance of donation in case of unsuccessful resuscitation after cardiac arrest, 39.8% agreed totally and 27.2% agreed partially (Table 1), even though 95.1% agreed in being an organ donor in case of this type of death.

Concerning the best donation criteria, 57.3% of the students preferred presumed donation and 23.3% chose consented donation. A total of 76.7% agreed totally for donation in case of a family member having a brain death diagnosis and 16.5% agreed partially. However, when asked about authorization for donation in case of a family member having an unsuccessful resuscitation after cardiac arrest, 50.5% students agreed totally and 27.2% agreed partially.

Table 1. Students demonstrated greater acceptance of organ donation after brain death but less acceptance of donation after circulatory death.

	n	%	95%CI	
			Lower	Upper
Do you agree with organ donation in cases of brain death?				
Indifferent	3	2.9	0.0	9.5
Partially agree	12	11.7	5.8	18.3
Totally agree	88	85.4	79.6	92.0
Do you agree with organ donation after cardiac arrest, when the patient has an unfavorable neurological prognosis, but is not yet diagnosed with brain death?				
Totally disagree	8	7.8	0.0	17.6
Partially disagree	15	14.6	4.9	24.4
Indifferent	28	27.2	17.5	37.0
Partially agree	33	32.0	22.3	41.9
Totally agree	19	18.4	8.7	28.3
Do you agree with organ donation in cases of cardiac arrest in emergency care which did not respond to resuscitation maneuvers?				
Totally disagree	5	4.9	0.0	15.1
Partially disagree	8	7.8	0.0	18.0
Indifferent	21	20.4	10.7	30.6
Partially agree	28	27.2	17.5	37.4
Totally agree	41	39.8	30.1	50.0

CI: confidence interval.

We also investigated whether self-determined religion could influence the students' answers regarding acceptance of donation after brain death, in DCD Maastricht's groups II and III. In none of these situations, religion had no impact on their answers (p=0.343, p=0.741, and p=0.695, respectively).

Finally, we investigated if the acceptance of organ donation was different between the two groups of students according to their graduation year. We could not find any influence of the students' training process in the acceptance of organ donation, particularly regarding DCD donation (Table 2).

DISCUSSION

In order to understand the view and the acceptance of organ donation in Brazil, particularly DCD donation, this survey was conducted in a selected portion of our society: students from a medical school. Even though we have retrieved only 9.8% of the enquiries sent to the students, we obtained enough answers to study their view. Despite the use of social media, the current adherence index was lower than a previous survey investigating the attitude about organ transplantation of medical students from this same faculty (33.04%)[6].

Our results have showed that medical students are steadily aware about the insufficient rate of donors for patients on waiting lists for organ transplantation in their countries, and they clearly responded in favor to donation after brain death. However, we could observe some reluctance toward living donation. Chen et al., in an investigation with university students in China, observed higher willingness to donate a living kidney to a relative (94%) than to donate after death (61.3%)[7]. In contrast, 89.3% of our students totally agreed with deceased donor organ donation and only 50.5% with living donation. However, 73.8% of the students totally agreed to donate part of their livers for someone next to them. Comparing these two different results, we can clearly observe the cultural differences of these two societies. Despite the well-educated individuals investigated in both enquires, these conflicting results suggest that East countries have difficult in accepting deceased donation, while West countries struggle to proceed with living donation.

The perspectives of medical students and nurses toward transplantation and donation have been investigated earlier[7-10]. Ríos et al., studying the acceptance of living liver donation among medical students in Spain, could not find any influence of the religion of the students[9], similar to the results of this study. On the other hand, Dutra et al. found a higher willing to donate organs among spiritual students than in Catholics and Protestants[8]. Xie et al. found an unfavorable view of nurses in China regarding this issue and advocated for a better training for this important group of professionals[10]. The view of patients

Table 2. Students were divided between the first 3 and the last 3 years of medical course.

	First 3 years of medical school		Last 3 years of medical school		p-value*
	n	95%CI	n	95%CI	
Do you agree with organ donation in cases of brain death?					
Indifferent	–	–	3	4.23(0–13.59)	0.1044
Partially agree	2	6.00(0.00–13.00)	10	14.08(7.04–23.45)	
Totally agree	30	94.00(88.00–100.00)	58	81.69(74.65–91.05)	
Do you agree with organ donation after cardiac arrest, when the patient has an unfavorable neurological prognosis, but is not yet diagnosed with brain death?					
Totally disagree	4	13.00(0.00–31.00)	4	5.63(0.00–17.89)	0.5709
Partially disagree	2	6.00(0.00–25.00)	13	18.31(7.04–30.57)	
Indifferent	11	34.00(19.00–53.00)	17	23.94(12.68–36.20)	
Partially agree	11	34.00(19.00–53.00)	22	30.99(19.72–43.24)	
Totally agree	4	13.00(0.00–31.00)	15	21.13(9.86–33.38)	
Do you agree with organ donation in cases of cardiac arrest in emergency care which did not respond to resuscitation maneuvers?					
Totally disagree	2	6.00(0.00–25.00)	3	4.23(0.00–17.12)	0.9344
Partially disagree	5	16.00(0.00–34.00)	3	4.23(0.00–17.12)	
Indifferent	3	9.00(0.00–28.00)	18	25.35(14.08–38.25)	
Partially agree	8	25.00(9.00–43.00)	20	28.17(16.90–41.07)	
Totally agree	14	44.00(28.00–62.00)	27	38.03(26.76–50.93)	

Both groups presented similar responses, showing no significant influence of the level of medical training at the acceptance of organ donation.
CI: confidence interval.
*p-value calculated by the Mann-Whitney *U* method.

has also been investigated[11], showing the lack of education as a major problem and advocated for better public information to improve organ donation acceptance. In this study, we found a decent level of knowledge of the concept of brain death among students (82.6%), which translated into a high acceptance of donation with brain death. However, the knowledge and acceptance of DCD donation was lower.

Regarding DCD donation, which is the main goal of this study, we already expected that it would be a difficult discussion in our society. For example, despite still a matter of debate in developed countries, end-of-life interventions are much well accepted in Europe[12]. Different from other countries, removing life support in terminal patients in Brazil is not welcome in general. In our results, 71.8% of the students do not know the concept of planned withdrawal of life-sustaining therapy, mainly cardiorespiratory support. They are quite used to not fully invest in patients with critical prognosis; however, planning withdraw of life-sustaining therapy is still highly controversial in Brazil, and this was revealed in this study.

This controversy clearly impacted the student's responses regarding DCD donation. While 85.4% answered to completely agree with brain death donation, only 18.4% agreed totally and 32% agreed partially with DCD Maastricht category III, and 39.8% agreed totally and 27.2% agreed partially with DCD Maastricht category II. Indeed, this was again revealed when the students were asked about consenting donation in case of a loved one's brain death, and 76.7% were totally in agreement; whereas, when consenting donation in case of a family member having an unsuccessful resuscitation after cardiac arrest, only 50.5% of the students agreed totally. Interestingly, these points-of-view do not change during the course of medical school, as both groups of less and more graduated students showed similar responses. It seems therefore that acceptance of DCD donation goes beyond of education in our society. Wu et al. found a higher acceptance rate of DBD donation (69.7%) than DCD donation (30.3%) among students of traditional Chinese Medicine[13], showing that the organ transplant education for medical students and the public is warranted.

CONCLUSIONS

This study showed that medical students in Brazil are well informed about organ donation, particularly regarding donation after brain death, even though there is still room for improvement. However, there is a clear lack of information and consequently in acceptance of DCD. Education in the field of end-of-life management may improve not only the acceptance of DCD donation, but the whole understanding of planned withdrawal of life-sustaining therapy.

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AUTHORS' CONTRIBUTIONS

RBM: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft. **DVG:** Funding acquisition, Investigation, Writing – original draft. **DRW:** Data curation, Methodology, Writing – original draft. AGS: Formal analysis, Resources. **AJFC:** Formal analysis, resources. **RMA:** Methodology, Writing – review and editing. LBH: Data curation, Investigation. **FHG:** Conceptualization, Writing – review and editing. **LACDA:** Supervision, Writing – review and editing.

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REVIEW ARTICLE
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Micronutrient deficiency in premature infants after hospital discharge: what is the evidence of free access in the last five years?

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INTRODUCTION

The preterm population is at increased risk for micronutrient deficiency, a situation that requires adequate supplementation, due to the risk of causing sensorimotor, cognitive, behavioral and somatic growth changes[1,2]. There are recommendations from scientific institutions about supplementation with iron, multivitamin and zinc for premature children[3,4].

The imbalance between the supply and demand of nutrients, associated with low reserve, are conditions that justify the need to supplement micronutrients for premature children. As approximately 80% of the iron reserve is made in the third trimester of pregnancy, premature babies are born with reduced iron deposits[5]. The zinc deposit in hepatocytes is also relatively smaller due to the smaller size of the liver[6]. In addition, the deficiency in micronutrient intake may be associated with low availability of breast milk, making the children unable to meet the demands of rapid growth in the first months of life[7]. Several other factors are related to micronutrient deficiency and prematurity. Family income and maternal schooling are relevant socioeconomic risk factors. In addition, low birth weight stands out as a biological risk factor[8].

Many studies assess micronutrient deficiencies and supplementation in premature infants during the hospitalization period, however, there is a shortage of these analyses in the course of the first years of life, after discharge from hospital. In this context, this systematic review aims to analyze the scientific evidence, published in the last five years, regarding the association between prematurity and micronutrient deficiency in the period after hospital discharge.

METHODS

The systematic review was based on the recommendations of the Preferred Reporting Items for Systematic Reviews (PRISMA) at all stages of design, implementation and reporting[9].

Database and search strategy

The bibliographic survey was carried out between June and July, 2019, in the following databases: Publisher Medline (PubMed) and Virtual Health Library (VHL). Manual searches of the references in the selected studies were also carried out in order to identify articles that were not found in the databases, which were submitted to the same analysis protocol. As a search engine, we used the term "premature infant" combined with the following descriptors: "micronutrients", "anemia", "iron deficiency", "zinc", "vitamin A" and Vitamin D". The following filters were selected: humans; free full text; past five years; English, Portuguese or Spanish.

Eligibility criteria and study selection

The inclusion criteria for the review were: any original article that assessed micronutrient deficiency in premature infants after

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hospital discharge; and that was published in the past five years. The exclusion criteria were: studies that did not assess premature infants; studies that evaluated only the period of hospitalization in the neonatal intensive care unit, as well as reviews, congress publications, theses and dissertations. Two independent reviewers carried out the selection of studies in two phases, including analysis of the title, summary and reading of the full text. A third reviewer resolved the disagreements. The steps included identification of articles in the databases, exclusion of duplicate files, initial selection by title and reading of the abstract, and complete analysis of the manuscripts that met the eligibility criteria. Duplicate articles in different databases were identified using the Mendeley Desktop software.

SYSTEMATIZATION OF RESULTS

The extraction and systematization of the results were performed in a Microsoft Excel® document and following the specific purposes of knowing the prevalence and factors associated with anemia or deficiencies of iron, zinc and vitamins A and D.

The initial screening identified 987 titles. After removing the duplicate files, 491 articles were analyzed. Of these, 460 were excluded after reading the title and summary, totaling 31 remaining articles. After the complete reading to assess the pre-established eligibility criteria, four original articles were included in this review. The details of the selection process are shown in Figure 1. The main characteristics of the studies are shown in Table 1. The results were organized according to the following themes: a) anemia and iron deficiency in preterm children; b) zinc in preterm children; c) vitamins and prematurity.

Anemia and iron deficiency in preterm children

The three studies, all carried out in Brazil, assume different criteria for the biochemical definition of iron deficiency and anemia. The work of Ferri et al. (2014)[8] defines iron deficiency and anemia by ferritin <10 ng/mL and hemoglobin <10 g/mL. Yamada & Leone (2014)[5] characterize anemia by hemoglobin levels <10.5 g/mL, not specifying which ferritin levels define iron deficiency. Freitas et al. (2016)[7] characterize iron deficiency by ferritin levels <30 ng/mL and use more detailed criteria for anemia, which varies according to the corrected gestational age (CGA) and the child's weight: (a) Hb <8, 5 g/dL (weight ≤1500g) and Hb <9.0 g/dL (weight between 1500–2000 g) for IGC between six to eight weeks; (b) Hb <9.0 (weight ≤1500 g) and Hb <9.5 (weight between 1500–2000g) for CGA from eight to ten weeks; (c) Hb <10 g/dL for 6-month CGA; (d) Hb <11 g/dL for CGA beyond 6 months. These divergences in the used criteria make it difficult to compare results between studies.

The hematological evolution of preterm infants in the first months is significantly different from children born at term, even with the corrected gestational age. The Yamada & Leone (2014)[5] cohort compared the biochemical characteristics between two groups of children (late preterm and full term), during the first two months of life, and considering the CGA. The study points out that the levels of hemoglobin, hematocrit and reticulocytes decrease in both groups with advancing age; however, these values are significantly lower in the group of late preterm infants. In addition, the mean ferritin levels were similar between groups. Thus, this inference shows a type II error of 42% due to sample size.

The prevalence of anemia and iron deficiency tends to increase with the advancement of CGA in the first months of life[7]. In premature infants, micronutrients are rapidly depleted due to the rapid expansion of erythrocytes, which accompanies the growth process in the first weeks after birth. In the cohort of Freitas et al. (2016)[7], the prevalence of anemia and iron deficiency was, respectively, 36.7% and 25.7%, with one month of CGA, and 38.3% and 68.9% at six months of CGA, with a margin error of 12% due to sample size. In contrast, in the cohort of Ferri et al. (2014)[8], the prevalence of anemia and iron deficiency was 26.5% and 48%, respectively, with a margin of error of 6%, with one year of CGA. It is worth mentioning the different methodologies of the studies in the characterization of anemia and iron deficiency, affecting the observed prevalence rates.

Socioeconomic factors have an impact on the prevalence of anemia and iron deficiency[8]. In the cohort of Ferri et al. (2014)[8], anemia was associated with the level of maternal schooling, number of pregnancies, family income and family history of alcoholism. Freitas et al. (2016)[7] corroborate these associations when verifying that maternal schooling is a predictor of low adherence to the use of recommended supplements, and that anemia in groups of premature infants who have low adherence to supplementation tends to be 2.5 times more prevalent. However, anemia is observed in 34% of premature infants at six months of CGA (margin of error of 15%), even when there is adequate adherence to micronutrient supplements, a relevant fact that signals a multifactorial etiology[7].

Ferri et al. (2014)[8] relate the consumption of cow's milk at six months of CGA to the 1.7-fold increase in the risk of anemia at twelve months of CGA. Cow's milk, in addition to having low iron bioavailability, generates an environment rich in proteins that are difficult to digest, such as casein, and minerals, such as calcium, which impair iron absorption[8].

As adverse effects of ferrous sulphate supplementation, nausea, vomiting, abdominal discomfort and constipation were reported in 19% of premature infants participating in the study by Freitas et al. (2016)[7], solved after replacement with other iron compounds.

The studies converge on the aspects that anemia is prevalent in the premature population, with emphasis on families that have socioeconomic vulnerability, and that iron supplementation should be performed. The early insertion of cow's milk is highlighted as a factor associated with iron deficiency and anemia.

Zinc in preterm children

Two studies, both Brazilian, assess zinc in premature children, and one of them describes the doses of zinc supplementation and the levels of serum zinc that characterize their deficiency[7,8]. Both converge on the appropriate supplementation period, between 36 weeks and six months of CGA. Freitas et al. (2016)[7] used a daily dose of 0.5 mg/kg and considered a serum level of serum zinc below 70 µg/dL as a deficiency.

Ferri et al. (2014)[8] found no association between zinc supplementation before 6 months of CGA and the presence of anemia at 12 months of CGA; however, in this study, only 20% of premature infants received zinc supplementation before 6 months of CGA.

On the other hand, Freitas et al. (2016)[7], when assessing preterm infants at six months of CGA, showed that 36% had zinc deficiency (margin of error of 14%), and found associations between low adherence to micronutrient supplementation,

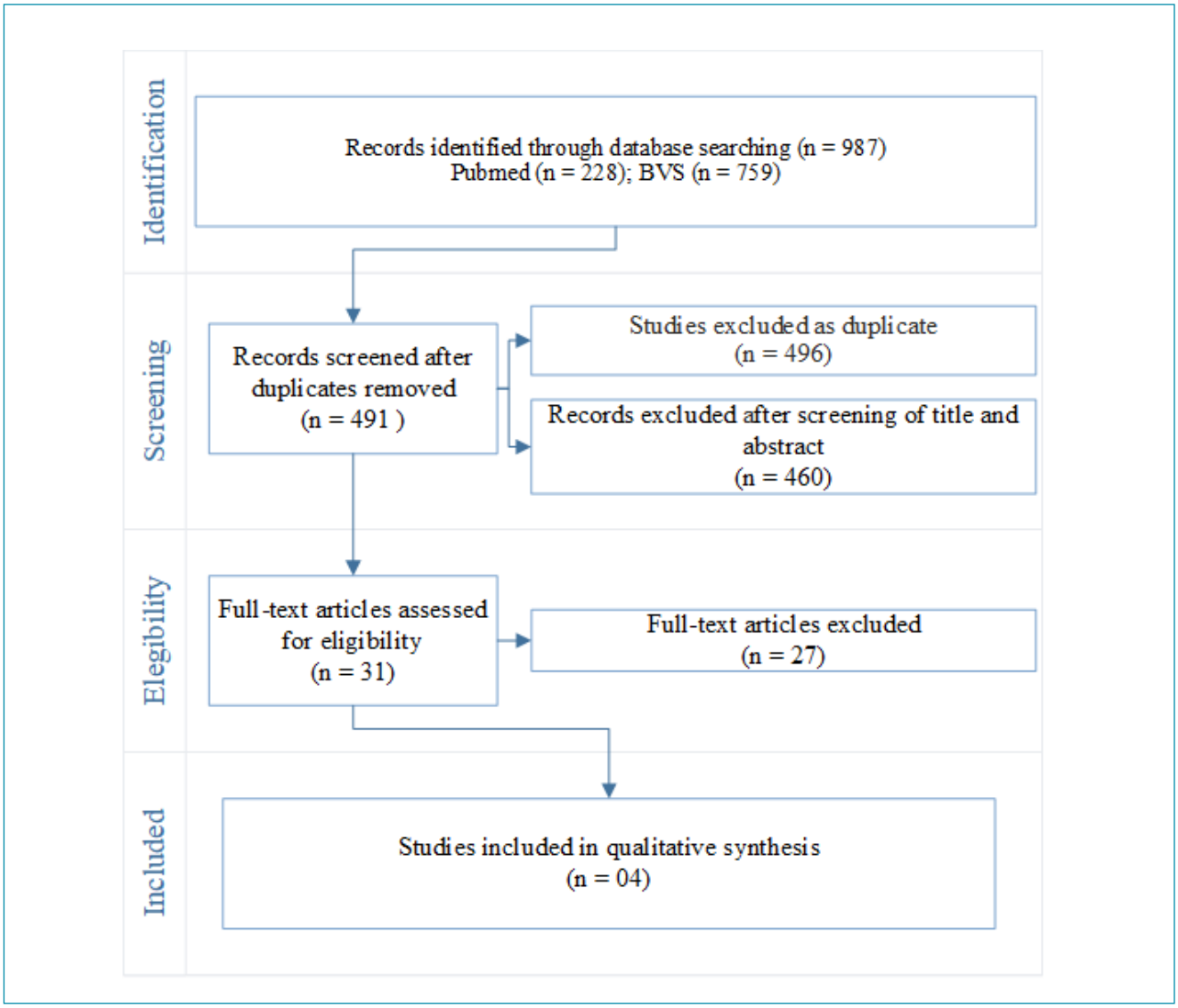

Figure 1. Flowchart of bibliographic research and study selection procedures, as recommended by PRISMA.

lower serum zinc levels and higher frequency of their deficiency. They also observed that 75% of premature infants with low adherence to supplementation presented with zinc deficiency, and this condition was 3.1 times more prevalent among the low adherence group when compared to the group with adequate adherence.

Zinc supplementation is recommended for premature children in the first six months of CGA, and inadequate zinc supplementation is associated with a greater chance of developing their disability[3,7].

Vitamins and prematurity

Two studies looked at serum vitamin levels in preterm infants, one Brazilian and one Polish. The study by Freitas et al. (2016)[7] evaluated vitamin A levels, and its deficiency was defined by serum retinol levels below 0.2 mg/L. Czech-Kowalska et al. (2016)[10] evaluated the levels of 25-hydroxyvitamin D (25-OHD) on a continuous scale in premature children at 40 weeks and 3 months of CGA.

In the study by Freitas et al. (2016)[7], 24% of children with one month of CGA showed vitamin A deficiency,

Author (year)	Country	Study Type	Sample Size	Studied Population	Age	Main methodological aspects	Outcomes	Results
Yamada et al., 2014[5]	Brazil	Prospective Cohort	25/21	Late preterm and term	First 2 months	serial blood iron deficiency markers of preterm and term comparison	Comparison of hematological parameters and systemic iron content between late term and term	Iron deficiency anemia markers significantly increased in the past.
Ferri et al., 2014[8]	Brazil	Prospective Cohort	310	Preterm <34 weeks and <1500 g	First 12 months	Anthropometric and dietary data in the neonatal period and at six and 12 months of corrected age. Laboratory tests at 12 months.	Iron deficiency and anemia's associated factors prevalence	Prevalence of anemia and iron deficiency: 26.5% and 48%, respectively. Associated factors: consumption of cow's milk, low maternal age, multiparity and being born small for gestational age.
Freitas et al., 2016[7]	Brazil	Prospective Cohort	58	Preterm	First 6 months	Laboratory tests performed at one and six months of corrected age. Monthly verification of adherence to the use of prescribed supplements.	Prevalence of iron, zinc and vitamin A deficiencies and associated factors. Analysis of adherence to the use of prescribed supplements.	Prevalence of anemia, iron and zinc deficiency are higher in those with less adherence to supplements, which was associated with low maternal education.
Czech-Kowalska et al., 2016[10]	Poland	Prospective Cohort	290	Preterm	First 3 months	Anthropometry, biochemical and bone mass analysis at 40 weeks and at three months corrected.	Verification of clinical and biochemical parameters, including bone metabolism markers, as potential predictors of bone mineral content.	Serum PTH can be a simple predictor of bone mineral content in premature infants at term, but urinary phosphate and serum osteocalcin excretion can predict reduced bone mineral content at 3 months of age.

however, at 6 months of CGA, none of them was deficient[7]. A significant portion of the population, 58%, still did not use multivitamins in the first consultation after hospital discharge. The main adverse effects from the use of multivitamins reported in this study were nausea and vomiting, representing 7% of the sample. However, the adverse effects ceased after the trademark of these supplements was changed[7].

According to the study by de Czech-Kowalska et al. (2016)[10], serum levels of 25-hydroxyvitamin-D (25-OHD) are not predictors of bone mass variation in premature infants. The mean serum 25-OHD levels did not differ between groups of premature infants with low or high bone mass density, with 40 weeks and six months of CGA. Furthermore, the mean serum 25-OHD values were significantly similar between the two periods[10].

LIMITATIONS AND PROSPECTS

As limitations, the use of samples restricted to a single health center and the short time of clinical follow-up can be observed. The works of Yamada & Leone (2014)[5] and Freitas et al. (2016)[7] have limitations in terms of sample size, with a margin of error above 10% in estimating prevalence. Ferri et al. (2014)[8] presented a considerable loss percentage and exclusion rate of participants. Czech-Kowalska et al. (2016)[10] showed a bias in selecting their population, coming from a tertiary neonatal care unit that receives only external deliveries. In addition, all studies present a cohort design, so we did not find randomized clinical trials with this approach.

In contrast to the large number of studies carried out during the neonatal period and during hospitalization, there is a lack of robust evidence about micronutrient deficiencies, their supplementation and their long-term effects on the health of children born prematurely after hospital discharge. Thus, this is a fertile field for future research, mainly based on randomized clinical trials.

CONCLUSION

Anemia and iron and zinc deficiencies are prevalent among premature infants. Socioeconomic factors and low adherence to the use of supplements are associated with micronutrient deficiencies. Thus, micronutrient supplementation and monitoring of adherence are strategies for preventing micronutrient deficiencies; in addition, laboratory monitoring of hematological parameters and serum iron and zinc levels among premature infants is essential.

AUTHORS' CONTRIBUTIONS

BACF: Conceptualization, Methodology. **KOR:** Conceptualization, Methodology, Writing – Original Draft, Writing – Review & Editing. **LFGF:** Conceptualization, Methodology**. DRM:** Conceptualization, Methodology. **RDLA:** Writing – Original Draft, Writing – Review & Editing. **FOM:** Writing – Review & Editing. **FGC:** Writing – Review & Editing.

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Complementary feeding of premature infants: a challenge

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INTRODUCTION

Complementary feeding is of high risk for premature children due to feeding difficulties, not recommended foods, food contamination risks, and the fact that nutritional quality may not meet or exceed the needs for nutrients and energy[1,2].

These factors are a cause for concern, since premature children are at greater risk of postnatal growth deviation, both in terms of acceleration and failure. There is also evidence that postnatal growth has significant long-term consequences and can be influenced by the time and type of solid foods offered[3-5]. For this reason, correct guidance offered to caregivers plays a fundamental role and should be offered by health professionals. However, surprisingly, there are no evidence-based guidelines for preterm infants, which leads to different and contrasting approaches on the part of caregivers and health professionals[1,2,6].

Considering the absence of guidelines for premature children, this review aims to analyze the scientific evidence regarding complementary feeding practices in premature children published over the past five years.

METHODOLOGY

This integrative review was based on the recommendations of the Preferred Reporting Items for Systematic Reviews (PRISMA) at all stages of design, implementation, and reporting[7].

A bibliographic survey was carried out from June to July 2019, in the following databases: Publisher Medline (Pubmed), Latin-American and Caribbean Center on Health Sciences Information (Lilacs), Scientific Electronic Library Online (Scielo), and Library Cochrane. Manual searches of references were also carried out in the selected studies to identify papers that had not been found in the databases, which were handled according to the same analysis protocol. As a search engine, we used "premature infant" AND "complementary feeding". The following filters were selected: humans, free full text, last five years, English language, infant age group.

The inclusion criteria for the review were: any original article that investigated the introduction of complementary feeding in premature infants that had been published over the past five years. Complementary feeding was defined as the introduction of semi-solid, soft, or solid foods other than breast milk, formula, or animal milk. The exclusion criteria were: studies that did not assess premature infants; those in which the term "weaning" had been used to indicate transition from breast-feeding to formula or animal milk, instead of semi-solid, soft or solid foods; those evaluating infants less than six months after hospital discharge; review studies; congress publications; theses and dissertations.

Two independent reviewers, including analysis of the title, abstract, and reading of the full text, selected the studies in two phases. A third reviewer resolved any disagreements. The steps included identification of papers in the databases, exclusion of duplicate files, initial selection by title and abstract, and complete analysis of the manuscripts that met the eligibility criteria. Duplicate papers in different databases were identified using Mendeley Desktop program.

The results were extracted and systematized using a Microsoft Excel® document. The results were organized according to the

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specific purposes of knowing the following evidence about complementary feeding of premature infants: (1) Studies on the timing of food introduction and its composition; (2) Difficulties encountered and parents' perceptions; (3) Conduct of health professionals.

RESULTS

The initial screening identified 113 titles. No duplicate files were found. Of these, 86 were excluded by reading the title and abstract, totaling 27 remaining papers. Once the entire paper had been read to assess the pre-established eligibility criteria, six original papers were included in this review. The details of the selection process are shown in Figure 1. The main characteristics of the studies are shown in Table 1.

Time of introduction and composition of complementary feeding

Three studies were analyzed from this perspective.

The study by Gupta et al.[8], carried out in India, is a randomized multicenter trial with children born at less than 34 gestational weeks, compared using two groups, according to the introduction of complementary feeding at four or six months of corrected gestational age (CGA), and followed up to 12 months of CGA. The type of food varied, from semi-solid to solid, based on WHO recommendations. Parents were advised to continue offering breast milk, be aware of food safety during food preparation, quantity, consistency, frequency, and composition. From two weeks onwards, iron and vitamin D supplementation was recommended. Upon completing 12 months of corrected age, patients were evaluated under the following aspects: height, weight, and body mass index by age (z-score), bone mineral density, lipid profile, HOMA-IR index, blood pressure, serum ferritin, psychomotor development, hospital admission rate, and disease recurrence.

The most striking result of Gupta et al.[8] when comparing both groups was regarding the hospital admission rate. In the study, those who started complementary feeding after four months of CGA had a 52% increased risk of hospital admission due to various complications, with more episodes of diarrhea and of lower airway infections up to the age of 12 months of CGA. The authors believe these are due to complementary food being potentially contaminated and to the decreased immunological benefit of breast milk. Thus, they recommend that complementary feeding of premature children born before 34 gestational weeks starts at six months of CGA, depending on the risks and benefits.

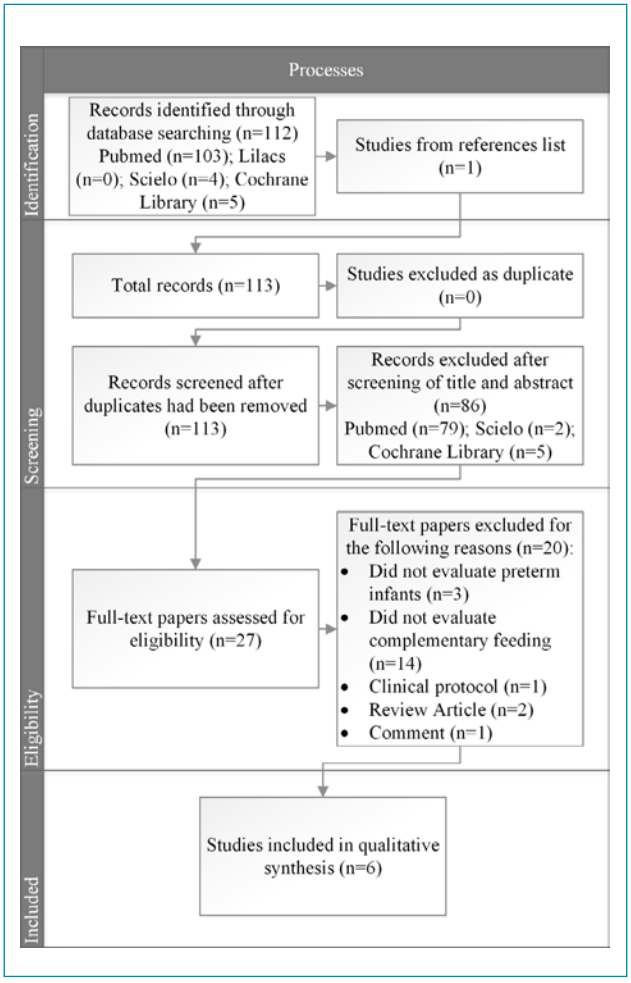

Figure 1. Flowchart of bibliographic research and study selection procedures in accordance with PRISMA recommendations[7].

Brusco & Delgado[9], in a cross-sectional study carried out in Brazil with preterm infants between three and twelve months of age, observed that the introduction of mushy food occurred at an appropriate time, but liquids and solids were introduced early. The study detected the consumption of milk other than breast milk, water, teas, juices, fruits, vegetables, meat, and crackers. The authors also noted that the children's parents had a doctor, a speech therapist, and family members as their main sources of information for the introduction of complementary food, and a dietitian and the child's health book were also mentioned.

Gianni et al.[10] developed a prospective study in Italy to investigate the timing and content of complementary feeding in a cohort of late preterm infants (born between 34 and 36 weeks), by contacting the mothers over the telephone in the first twelve months after delivery. Mothers started complementary feeding following pediatrician's advice in

Table 1. Main characteristics and results of interest for the articles included in the review.

Author (year)	Country	Study design	Sample size (sample calculation)	Age range	Study population	Main methodological aspects	Outcomes	Results
Menezes et al. (2018)[11]	Brazil	Transversal	38 (no)	6 to 24 months CGA	Premature	Premature infants who started complementary feeding; interview during routine consultations; two hospitals.	Difficulties in introducing complementary feeding.	Refusal to open mouth (42.1%), food selectivity (28.9%), and refusal to eat (26.3%) were the most frequent defensive behaviors.
Gianni et al. (2018)[10]	Italy	Prospective, observational cohort	57 (no)	1, 3 and 6 months after discharge	Late preterm infants (34 to 36 weeks)	Telephone contact with mothers and questionnaire about the time of introduction and composition of solid food.	Time of introduction and composition of complementary feeding.	The average postnatal age for complementary food introduction was 5.7+0.7 months. The foods offered contained low protein and/or energy content. Fruit as the first type of solid food was associated with complementary feeding being introduced 1.6 month earlier.
Baldassarre et al. (2018)[13]	Italy	Transversal	347 (no)	Not applicable	Pediatricians	Internet questionnaire with pediatricians, considering the time of introduction and composition of complementary foods.	Pediatricians' individual attitudes towards the introduction of complementary food for premature infants.	Time of introduction of solid foods: 44% were based exclusively on age, 18% on neuropsychomotor development and 4% on body weight; the remaining 34% were based on two or more of these aspects. Food composition was not based on evidence. Pediatricians' conducts were heterogeneous.
Gupta et al. (2017)[8]	India	Randomized, unblinded, multicenter	403 (yes)	Birth to 12 months CGA	Premature children under 34 weeks	Two groups: complementary food at 4 months (n=206) and at 6 months (n=107); three public health facilities.	Effect of starting complementary feeding at 4 months versus 6 months of CGA on weight for 12 months of CGA.	No evidence of effect for the weight-for-age Z-score at 12 months of CGA. Due to the higher rate of hospitalization in the 4 month group, it is recommended to start complementary feeding at 6 months of CGA.
Johnson et al. (2016)[12]	United Kingdom	Population-based cohort	2385 (yes)	Birth to 24 months CGA	Premature infants from 32 to 36 weeks; terms (controls)	Moderate and late preterm infants (n=1130) and controls born at term (n=1255); validated questionnaire assessed eating behaviors.	Prevalence of feeding difficulties in premature infants with 2 years of corrected age and the impact of neonatal and neurodevelopmental factors.	Moderate and late preterm infants are at increased risk of experiencing eating difficulties due to oral motricity and selective pattern at 2 years of CGA. However, these are mediated by other neurobehavioral sequelae in this population.
Brusco & Delgado (2014)[9]	Brazil	Transversal	32 (no)	3 to 12 months chronological age	Premature	Premature infants who attended follow-up consultations at a hospital. Study considered chronological age.	Time of introduction of complementary feeding, harmful oral habits, guidelines received, and eating difficulties.	The time of introduction was adequate for mushy food, but early for liquids and solids. 53.1% had eating difficulties. Extreme prematurity was associated with refusal to eat and sagging lips, tongue, and cheeks.

CGA: corrected gestational age.

88% of the cases but decided on their own in 12% of the cases. The average CGA for the introduction of complementary foods was 5.7± 0.7 months. The introduction of food started with low-calorie and/or protein foods in most cases. Fruit as the first type of complementary food was associated with complementary food being introduced 1.6 month earlier.

Difficulties encountered and parents' perceptions of complementary feeding

Three studies address the difficulties encountered in introducing complementary feeding. Both are transversal and use questionnaires that assess the perception of those responsible for the attitudes and behaviors of premature infants.

The two Brazilian studies involved preterm infants with a gestational age below 37 weeks: Menezes et al.[11] evaluated children who started complementary feeding between six and 24 months of CGA; Brusco & Delgado[9] evaluated chronological children aged between three and 12 months. A United Kingdom study by Johnson et al.[12], analyzed children born between 32 and 36 gestational weeks.

In general, the following aspects were evaluated: refusal to eat (low appetite, rejecting food, food selectivity); problems related to motor skills (biting, chewing, swallowing, choking, coughing); behavioral problems during mealtime (crying, tantrums, making a mess); gastrointestinal manifestations (nausea, vomiting, reflux) during or after meals. Complaints of nausea or vomiting were prevalent in both Brazilian studies and the three studies corroborate the association of prematurity with refusal to eat and behavioral problems.

Brusco & Delgado[9] associate refusal to eat with extreme prematurity and extremely low birth weight due to admission to a neonatal intensive care unit and potential traumatic sequelae due to the use of tubes during treatment.

Menezes et al.[11] associate refusal to eat with the use of formulas during a period that was supposed to include breastfeeding only, justifying difficulties in introducing new foods to multifactorial.

Brusco & Delgado[9] and Menezes et al.[11] converge in the hypothesis of low perception of parents about the manifestations of defensive behavior of children during complementary food introduction.

Johnson et al.[12] concluded that children born between 32 and 36 gestational weeks have a greater chance of refusing food, and having motor and behavioral problems during the introduction of complementary feeding, when compared to those born at term. However, the authors attribute the findings to other neurobehavioral sequelae rather than to those assessed in the study.

Conduct of health professionals towards complementary feeding

From the perspective of health professionals, Baldassarre et al.[13], in Italy, analyzed the conduct of pediatricians regarding complementary feeding of premature infants. This was a cross-sectional study carried out through a questionnaire sent to pediatricians in primary care through "Google Forms" platform. Of an estimated population of one thousand pediatricians, 347 participated. The study addressed the timing and composition of complementary feeding introduced to premature infants.

The authors found heterogeneity in relation to pediatricians' behavior when introducing complementary foods to premature children. Pediatricians were based on corrected gestational age, chronological age, weight or neurological development or, still, on the combination of two or more of these variables to indicate the time of food introduction. This means this topic is quite divergent. Regarding the initial composition of complementary feeding, there are also differences between the recommendations of these health professionals, with some of them not being in line with international recommendations.

DISCUSSION

Premature children had a higher nutritional requirement when compared to those born at term and, thus, the introduction of complementary feeding in a timely manner can supply this energy demand, preventing restricted postnatal growth, low weight, and neuropsychomotor deficit in the short term. It must be considered that preterm infants are a very heterogeneous population, since their gestational age at birth can vary between 23 and 36 weeks. Thus, the guidelines for the introduction of solid foods to children born at term cannot be used for premature infants, and the optimal time of introduction should consider motor development, higher nutritional needs, organ immaturity, increased intestinal permeability, and increased risk of hospitalization due to infections[1,2].

As a brief history of the available recommendations, in 2001, the World Health Organization (WHO)[14] started to recommend the introduction of complementary feeding at six months of age as a strategy to encourage breastfeeding. In 2008, the Committee of the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN)[15] reviewed current knowledge and practices and concluded that, in industrialized countries in Europe, the introduction of complementary feeding should not occur before 17 weeks (four months) and no later than 26 weeks (six months) of age. It should be noted that these two recommendations are aimed at children born at term. There is a recommendation for premature babies, published

in 1994 in the United Kingdom[16], saying the introduction of solid foods should start when a premature infant reaches a weight of 5 kg, has lost the extrusion reflex, and is able to eat with a spoon. Unlike complementary feeding guidelines for term infants, this guideline does not include an age recommendation.

Based on limited evidence, a review study published in 2012 concludes that the corrected age of 3 months (13 weeks) may be appropriate for introducing solid foods for most preterm infants, considering good head control as an important milestone for safety[1]. However, some authors find acceptance difficulties related to motor skills with the introduction of complementary feeding at 3 months[6,17].

The only randomized clinical trial found in the present review, carried out by Gupta et al[8], recommends introducing complementary feeding for premature children up to six months of CGA, and weighing the risks and benefits of its introduction atfour months. The cross-sectional studies by Brusco & Delgado[9] and Gianni et al.[10] reveal how inadequate complementary feeding of premature children is, both in terms of type of food and time of introduction, and also demonstrate how guidelines offered by health professionals influence parents' practices. The main limitations of these studies are: (a) Gupta et al.[8] evaluated a vulnerable socioeconomic population in a developing country, (b) Brusco & Delgado[9] and Gianni et al.[10] studied small- size groups, with no sampling; and (c) Brusco & Delgado[9] analyzed children considering their chronological age.

The strategies followed in a nutritional intervention carried out with very-low-birth-weight preterm infants included: breast milk supplemented with a multi-component supplement, guidance on the introduction of semi-solid foods between four and six months of CGA, and monthly monitoring by a health team at home[18]. The intervention group was observed to have higher weight and head circumference at six months of CGA, indicating that the proposed nutritional intervention can potentially reduce growth restriction of premature infants in the period assessed[18]. Ensuring an ideal nutritional intake from birth and introducing solid foods in a timely manner and with an adequate composition of proteins, energy, and nutrients promote the adequate growth of premature children[1,2].

Based on these considerations, the results found provide insight into dietary practices and highlight the need for multicenter randomized controlled trials specifically designed to assess when and how solid foods are introduced for premature infants and their benefits and risks.

Cross-sectional studies by Menezes et al.[11], Brusco & Delgado[9], and Johnson et al.[12] point out that difficulties related to complementary feeding are prevalent in premature children, especially behavioral issues and food refusal. Menezes et al.[11] and Brusco & Delgado[9] raise the hypothesis that parents have little knowledge about the manifestations of their children's defensive behaviors during complementary feeding introduction. However, the following limitations should be considered: (a) Menezes et al.[11] and Brusco & Delgado[9] used small samples and did not calculate the sample, which makes it difficult to infer data on other populations, and (b) the use of chronological age in the analysis performed by Brusco & Delgado[9] may have generated bias in the association found between food refusal and extreme prematurity.

The period of complementary feeding is marked by exposure to new foods, tastes, and eating experiences. It coincides with the phase of children's rapid growth and development, in which they are susceptible to deficiencies and excesses of nutrients[19]. During the introduction of complementary feeding, brain and intestinal maturation is taking place, eating experiences help shape the connections involved in food sensations and the development of food preferences is already beginning[20].

Premature children with lower gestational age or very low birth weight are more likely to have eating difficulties in the first year of life, manifested by oral-motor disorders and refusal to eat. Therefore, it is recommended that health professionals monitor the eating practices after hospital discharge to optimize the development of children's eating skills[21,22]. Prospective research and experimental design are necessary to verify the causal relationships between prematurity and eating difficulties, as well as to analyze the perception of those responsible for the children in this process.

According to the results of Baldassarre et al.[13], a disparity in conduct from the perspective of health professionals can be attributed both to the lack of scientific evidence and to the lack of information dissemination, updating, training, and qualification of these professionals. These divergences regarding the use of parameters to guide the introduction of complementary food and its composition can have deleterious consequences. The child's neuromuscular development, with good head control for the safe intake of solid foods, should be assessed. There is no evidence to support the recommendation to introduce solid foods when children reach a bodyweight of 5 kg, since children with postnatal growth restriction can benefit from solid foods before reaching this weight[1,2,16,23]. Thus, this is a vast field of research in the sense of providing evidence-based guidelines for health professionals to act in a standardized way in the care of premature children.

The present review highlights many gaps in the knowledge about complementary feeding of premature children. Little is known about what is considered ideal or what health professionals and caregivers are doing in practice. A situational diagnosis is required simultaneously with prospective multicenter

experimental studies aimed at premature children. Such guidelines should include: (a) ideal time of introduction, such as difficulties and the appropriate nutritional composition of complementary food, (b) dissemination of this information to health professionals and caregivers, and (c) monitoring of these guidelines within the scope of health professionals and caregivers. In addition, we suggest developing further studies to investigate the immediate and long-term effects of healthy food introduction patterns and timing on the health of premature infants.

AUTHORS' CONTRIBUTIONS

BACF: Conceptualization; Data Curation; Formal Analysis; Investigation; Methodology; Project Administration; Writing – Original Draft; Writing – Review & Editing. **LML:** Formal Analysis; Methodology; Writing – Original Draft; Writing – Review & Editing. **EEFP:** Formal Analysis; Writing – Original Draft. **TCS:** Formal Analysis; Writing – Original Draft. **KOR:** Formal Analysis; Writing – Original Draft. **FOM:** Writing – Original Draft. **FGC:** Writing – Original Draft.

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The glymphatic system and its relation with neurological diseases

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INTRODUCTION

The glymphatic system (GS) is a paravascular pathway located between the vascular adventitia and vascular astrocytic end-feet. It is responsible for clearing toxic peptides from the cerebral parenchyma[1,2]. This paravascular clearance system was named glymphatic system due to the fusion of the words "glial" and "lymphatic" since its very existence depends on glial cells and also because of its functional similarity with the peripheral lymphatic system[1].

Jessen et al.[2] have demonstrated a relationship between a dysfunctional GS and the emergence of neurodegenerative diseases, as well as situations of excessive sleep deprivation and natural aging[2,3]. Several neurodegenerative diseases have been associated with these conditions. Alzheimer's dementia (AD) is the main target of current research[2,3]. Despite the literature limitations on the study of the GS, new correlations with neurodegenerative diseases are constantly emerging.

This article aims to review how the glymphatic system functions, the factors that interfere with it, and its correlation with neurological pathologies.

METHODOLOGY

This is a descriptive study based on the literature available in the MEDLINE/Pubmed database. The terms searched were the following: "glymphatic system," AND "sleep," "cognitive decline," "aging," "neurodegenerative disease," "Alzheimer's disease", all in English. All articles considered relevant were included in this review, as were the studies referenced therein, in order to raise awareness about the method. Duplicate items were discarded.

DISCUSSION

Approximately 68% of the central nervous system's total volume of water is in the intracellular space, whereas the remaining 32% is in the extracellular space[1]. The extracellular space is distributed in the interstitial fluid (ISF), cerebrospinal fluid (CSF), and cerebral blood circulation[1,2]. The CSF accounts for approximately 10% of the total volume of intracranial fluid[4], being produced by the choroid plexuses and playing an important role in the distribution of nutrients and removal of toxic interstitial metabolites[2]. The CSF, after circulating through the ventricular system and subarachnoid space of the cortex and spinal cord[2], penetrates into a perivascular space called the Virchow-Robin space[2].

The Virchow-Robin spaces are filled with CSF and bounded by a leptomeningeal cell layer on the inner wall – facing the vessel, and on the outer wall, facing the perivascular astrocytic end-feet[2,5]. The central nervous system (CNS) has all its blood vessels surrounded by vascular astrocytic end-feet[2]. These vascular end- feet create the outer wall in the perivascular space, resembling a tunnel that surrounds the vasculature[2].

From the subarachnoid space, the CSF is directed to the Virchow-Robin spaces by a combination of arterial pulsatility, breathing, and pressure gradients[2]. The CSF and ISF exchange continuously due to the continuous influx of CSF into the perivascular spaces[2]. The subsequent distribution of the interstitial fluid to the brain parenchyma is facilitated by aquaporin-4 (AQP4) water channels[2], with this protein being expressed in the polarized portion of astrocytic end-feet. In these, the CSF penetrates into the parenchyma along the paravascular spaces

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that surround the penetrating arteries, and the interstitial cerebral fluid is drained along the paravenous pathways[1,2] (Figure 1).

This elimination pathway and the role of AQP4 channels in the clearance of neurotoxic metabolites were demonstrated in 2012 by Iliff et al.[6], in a study based on an *in vivo* two-photon imaging technique with small fluorescent markers[5]. Animals without astrocytic AQP4 channels have been shown to exhibit a 70% reduction in interstitial solute clearance[5]. Thus, one can infer that substances involved in neurodegenerative pathologies could accumulate inadequately in the glymphatic pathway[3,5].

Even though the CSF and ISF that drain into the subarachnoid space exit the skull through unidirectional arachnoid granulations, Lee et al.[7] have suggested that part of that CSF follows along the internal carotid artery through the perivascular spaces, as well as within the perineural spaces of the cranial nerves, notably the olfactory and vagus nerves[4,7]. Noteworthy are the extensions that follow the olfactory tracts across the cribriform plate and protrude into the nasal submucosa. They have been shown to account for 15–30% of CSF solute removal[4]. The nasal submucosa has a dense lymphatic network that drains the CSF and solutes into the deep cervical lymph nodes and is of special importance in the removal of molecules with a large molecular weight[2,4].

In addition to the aforementioned physiological variations, some other factors interfere with the glymphatic influx, such as arterial pulsatility, sleep, and the natural aging process.

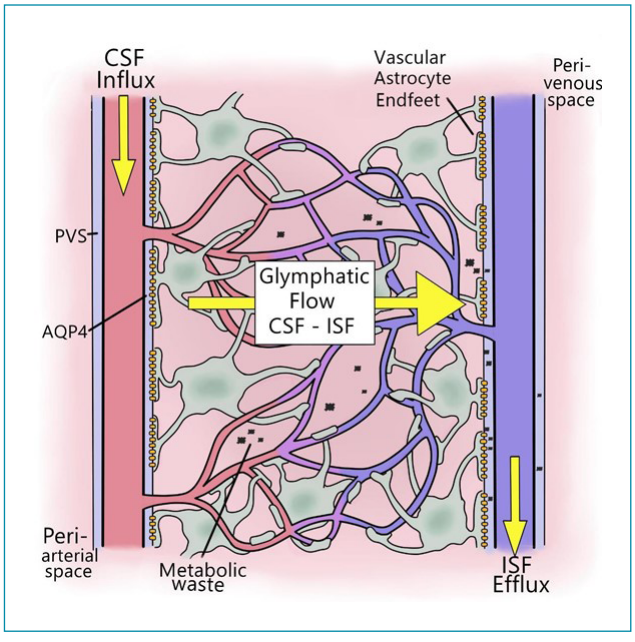

Figure 1. Schematic representation of the encephalic lymphatic flow

Arterial pulsatility

The constant production of CSF by the choroid plexus creates a pressure that determines the direction of fluid flow through the ventricular system into the subarachnoid space[2]. The CSF entering the perivascular space is essential for facilitating both lymphatic exchange and clearance[2]. The CSF has already been shown to follow the course of cerebral arteries, especially due to the pulsatility generated by smooth muscle cells that create pulse waves along the entire length of penetrating pial arteries, which explains why the perivascular influx occurs preferentially around the arteries rather than in brain veins[2,4].

With the aid of ultra-fast magnetic resonance imaging (MRI) encephalography, it was possible to reveal that pulsations related to the respiratory cycle and changes in the vasomotor tone also propagate through the human brain and, therefore, can potentially contribute to the glymphatic flow[2,3]. On the other hand, the CSF flow within the ventricular compartments can be largely driven by breathing, which is also considered an important factor in the glymphatic flow[2].

Sleep

Sleep has been identified as one of the main factors interfering with the dynamics of glymphatic influx. Recent studies have shown that sleep is the only state during which glymphatic activity is active, suggesting that it is suppressed during wakefulness[2,8]. Such findings indicate that the sleep state is particularly conducive to the convective flow of fluids and, therefore, to the release of metabolites[2,8]. In this way, sleep seems to increase glymphatic activity and, consequently, the removal of neurotoxic waste produced during wakefulness[2,8].

This feature was demonstrated in 2013 by Xie et al.[8], by comparing *in vivo* two-photon images of the CSF flow in the cortex of awake, anesthetized, and sleeping mice[8]. Anesthetized and sleeping mice had a 60% increase in the interstitial volume and rate of β-amyloid clearance during sleep[8]. Conversely, while the mice were awake, there was a reduction in the volume of the interstitial space and, consequently, an increase in convective fluid resistance and CSF flow suppression[2,8]. The activity of neuromodulators, such as glutamate and noradrenaline, has been linked to a reduction in interstitial space, especially noradrenergic signaling originating from the locus ceruleus[8]. These modify the cell volume and thus reduce the size of the interstitial space, which prevents the CSF influx observed during sleep[8].

Aging

Aging is a major contributor to decreased glymphatic activity[2]. Jessen et al.[2] evaluated the glymphatic function of young and elderly mice and demonstrated the occurrence of a significant reduction in this function in approximately 80–90% of

the older mice. This is mainly due to a change in the location of the AQP4 enzymes in young animals, which remain in the astrocytic end-feet and play a fundamental role in interstitial solute clearance through the perivascular drainage pathways[2,9]. In elderly animals, these enzymes lose their perivascular polarization and are concentrated in the astrocytic parenchymal processes, thus impairing the exchange of CSF and ISF, leading to a decline in glymphatic activity as age advances[9].

Other factors that can contribute to a decreased activity of the GS are a decline in CSF production (66%) and pressure (27%)[2], in addition to arterial stiffening resulting from aging, which reduces arterial pulsatility and, consequently, decreases glymphatic influx[2]. The decline in glymphatic activity associated with advancing age is a fundamental condition for the emergence of neurological disorders[2,4]. It should be noted that most neurodegenerative diseases have aging as a common risk factor[2]. A failure in the lymphatic system with progressive aging can lead to the accumulation of aggregated and hyperphosphorylated proteins, which makes the senile brain more vulnerable to the development of neurodegenerative pathologies and even to cognitive dysfunction[2,9].

The Glymphatic System and neurodegenerative diseases

Most neurodegenerative diseases are characterized by the accumulation of aggregated proteins in the CNS[2] (Table 1). These protein aggregates are present in both the ISF and CSF, as are β-amyloid and tau folded fibrillary tangles in AD, and folded α-synuclein in Parkinson's disease[2].

AD is characterized by the accumulation of β-amyloid protein and the formation of tau substance tangles in various brain regions[2,4]. Impaired clearance of these substances by the GS favors the accumulation of β-amyloid in the cerebral parenchyma and disease progression[1,2]. This deregulation is a consequence of changes in the expression of AQP4 that occur in aging and the breakdown of the blood-brain barrier, which contributes to the accumulation of β-amyloid in the cerebral vasculature[1,3], resulting in cerebral amyloid angiopathy[10], which consequently will culminate in arterial stiffening and impaired glymphatic flow[10].

Rassmussen et al.[9] and Reddy et al.[11] demonstrated in an animal model with AD that this change in the expression of AQP4, both in its erratic location and in the loss of polarization,

Table 1. Some diseases and their respective mechanisms of glymphatic system dysfunction.

Authors	Disease associated with a dysfunction in the glymphatic system	Clinical and pathological impairment
Peng et al.[12] (2016).	Alzheimer's disease	Aging associated with a dysfunctional blood-brain barrier and dysfunctional AQP4 enzymes implies glymphatic dysfunction, which promotes the accumulation of protein aggregates (β-amyloid and tau substance) with a more accelerated disease progression.
Prasad et al.[14] (2014).	Diabetes	Hyperglycemia, formation of ROS, blood-brain barrier dysfunction, and abnormal neovascularization lead to losses in the drainage of neurotoxic solutes from the interstitial fluid by the glymphatic pathway, favoring the development of neurodegenerative diseases.
Iliff et al.[3] (2013).	Vascular dementia	Structural changes in the cerebral blood vessels resulting mainly from atherosclerosis impair the drainage of neurotoxic solutes through the glymphatic route, which favors the appearance of dementia.
Iliff et al.[15] (2014).	Traumatic injury	The formation of astroglial scars, neuroinflammation, and the impaired expression of AQP4 disrupt glymphatic function, which contributes to an increase in neuronal damage secondary to traumatic injury and the emergence of neurodegenerative pathologies.
Zou et at.[16] 2019.	Parkinson's disease	The perivascular accumulation of α-synuclein aggregates and a change in AQP4 expression in the substantia nigra and in the autophagy of this substance caused neuroinflammation, neuronal damage, and errors in glymphatic function, which demonstrated progression of PD.
Jessen et al.[2] (2015).	Amyotrophic lateral sclerosis and frontotemporal dementia	An increase in the levels of noradrenaline in the CSF may lead to a reduction in glymphatic activity and AQP4 channels, impairing this neurotransmitter's glymphatic function and worsening the clinical progression of the pathologies.

AQP4: aquaporin 4; ROS: reactive oxygen species; PD: Parkinson's disease; CSF: cerebrospinal fluid.

modified the glymphatic clearance, favoring the accumulation of β-amyloid in the brain[9,11].

Peng et al.[12], in a study published in 2016, demonstrated the erratic deposit of β-amyloid in the perivascular spaces of transgenic mice based on the expression of the human amyloid precursor protein. Changes in the clearance of this substance have become evident due to reduced receptors in the blood-brain barrier[12]. Accordingly, it is important to note that significant deposits of β-amyloid were preceded by lymphatic failure, which therefore can be an early biomarker of AD[12]. Jessen et al.[2] also demonstrated that the APOE gene, responsible for expressing apolipoproteins for cerebral lipid transport, is related to the elimination of β-amyloid[2,13]. Thus, defects in its gene expression can be considered relevant genetic risk factors for AD and impaired glymphatic function[2,13].

Similar to AD, vascular dementias are also influenced by the GS. Changes in the structure of blood vessels due to high blood pressure and atherosclerosis may damage the perivascular space and impair drainage of toxic solutes by the GS[1,3]. It is speculated that an abnormal increase in the perivascular space in these diseases may have an impact on solute elimination flows, resulting in interstitial fluid obstruction, favoring the deposition of neurotoxic substances and, consequently, the appearance of degenerative pathologies[1,3].

Recently, diabetes has been linked to vascular complications and neurodegenerative diseases, such as AD[1,14]. Neuronal damage caused by hyperglycemia and the formation of reactive oxygen species affect the blood-brain barrier function, in addition to causing abnormal vascular remodeling[14]. These changes promote inappropriate clearance of the interstitial solute and other substances, such as β-amyloid, through the glymphatic pathway, favoring the accumulation of these toxic compounds and the development of pathologies in the CNS[1,14].

Like aging, traumatic brain injury can lead to progressive neurodegeneration and induce the release of peptides, such as C-tau[15]. This is a biomarker of brain injury and correlates with the severity of traumatic injury[15]. This pathology is linked to the formation of astroglial scars and persistently activated neuroinflammation[4,9]. As a result, there is a marked decrease in glymphatic function and changes in the expression of AQP4. This reinforces the important role the GS plays in eliminating toxic solutes into the interstitial fluid[4,9], an association between dysfunction in this system and progressive neurological involvement in patients with traumatic brain injury.

In 2014, Iliff et al.[15] tracked the pathway of human tau release with the aid of intracortical injections in *in vivo* models. It was seen that large amounts of human tau were deposited around blood vessels, which hindered the removal of this protein by the glymphatic pathway, thereby aggravating neuronal damage secondary to traumatic injury[15].

Another neurodegenerative disease involving dysfunctional processes in the GS is Parkinson's disease (PD)[2,16]. PD is characterized by the progressive loss of dopaminergic neurons in the midbrain (substantia nigra) and the formation of Lewy bodies[2,16]. The pathophysiological mechanism of PD involves an imbalance between the production and release of α-synuclein in the brain due to mutations in the α-syn gene and a decreased removal rate of this compound due to pathogenesis mechanisms still poorly elucidated in the literature[16]. Zou et al.[16] demonstrated the perivascular accumulation of α-synuclein aggregates and changes in the expression of AQP4 in the black substance by blocking the lymphatic drainage in young mice, which resulted in neuroinflammation with dopaminergic neuronal loss and motor deficits[16]. Furthermore, it became evident that changes in the autophagy process of α-synuclein favor the formation of aggregates of the substance and impair the flow of lymphatic clearance, worsening the progression of the pathology in the mice studied[16].

In addition to the aforementioned conditions, amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are related to a dysfunctional GS and progression of the neurological scenario[17,18]. ALS and FTD are multisystem neurodegenerative diseases with progressive neurological deterioration[17]. Approximately 50% of individuals with ALS have FTD[18]. Recently, the GS has been shown to participate in the pathophysiology of ALS and DFT, having been associated with inefficient clearance of neurotoxic substances[17]. Brooks et al.[19] found that individuals with ALS and DFT have high noradrenaline levels in their CSF and neuronal tissues[17,79]. Jessen et al.[2], in 2015, hypothesized that increased noradrenaline in the CSF might lead to a reduction in glymphatic activity, thereby impairing AQP4 channels and thus creating a turbulent interstitial flow. All this would in turn cause harm to the glymphatic function of this neurotransmitter, worsening the dementia process involved in these pathologies[3,17] (Table 1).

Limitations of the study

The main limitation of this study is its narrative nature, therefore, not following systematic evidence-based criteria.

CONCLUSION

The glymphatic system plays an important role in the elimination of neurotoxic peptides and is closely related to the development of neurodegenerative diseases, most of which are secondary to the erratic accumulation of those substances. However, further studies are still needed to find out how the glymphatic flow functions, especially through radiological techniques, such as magnetic resonance imaging, and biomarkers, for the early

detection of cellular changes and also to propose appropriate therapeutic interventions. In the future, a complete understanding of the GS may help in the prevention of degenerative diseases of the central nervous system.

AUTHORS' CONTRIBUTIONS

KHPN: Conceptualization, Data Curation, Writing – Original Draft. **GBA:** Conceptualization, Writing – Original Draft. **MACSV:** Conceptualization, Writing – Review & Editing.

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REVIEW ARTICLE
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Vaccines Developed against COVID-19: a narrative review

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INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic calls for a quick evaluation of the multiple competence approaches to obtain protective immunity and safety, thus diminishing the undesired immune potentiation, which plays an important role in the pathogenesis of the virus[1,2].

The clinical manifestations change the disease from mild to serious, possibly leading to death. Other symptoms include rhinorrhea, productive expectoration, headache, and sore throat. Also, some people can have rare symptoms, such as gastrointestinal ones, including diarrhea and vomit. Other symptoms may also manifest themselves, such as hyposmia (impaired smelling capacity) and hypogeusia (impaired taste capacity)[3].

Hence, pharmaceutical companies and research institutions have been competing to develop severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccines—from conventional viral ones, based on proteins, to the most advanced, based on the DNA and mRNA[1]. Each current vaccine strategy has different advantages and disadvantages. Therefore, it is essential to quickly advance various strategies and then evaluate their safety and effectiveness. One of the main obstacles in the initial development of the coronavirus vaccine against SARS was the discovery that whole-virus or protein vaccines increased infectiousness[4].

Given the above, the main and guiding objective of this research was to verify the possible compositions of the vaccines being developed and produced against COVID-19, aiming to answer the following research question: What are the possible vaccine compositions being produced against COVID-19?

METHODS

Protocol and registry

This narrative review complied with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) recommendations[5], aiming at the most rigorous scientific evidence protocol criteria. Two independent researchers searched for the scientific articles in the MEDLINE (PubMed), LILACS, SciELO, Scopus, Web of Science, and BIREME databases, without restriction of language and place of publication, encompassing the period from 2015–2020. The research was structured and organized in the PICOS framework, an acronym that stands for target population of interest or health problem (P) correspond to humans of both sexes with no age restriction; intervention (I): vaccine; comparison (C), composition; outcome (O): COVID-19; cross-sectional studies (S), observational studies, case reports, case-control studies, controlled clinical trials, and randomized controlled (Table 1).

Research strategy

The descriptors were chosen from the dictionary in Health Sciences Descriptors (DeCS) and Medical Subject Headings (MeSH). The search in the other databases was adjusted based on the descriptors. At first, the following Boolean operators were proposed for the search: (((COVID* vaccine* hesitancy[Title/Abstract]) OR (COVID* vaccine acceptance[Title/Abstract])) OR (COVID* vaccin* hesitanc*[Title/Abstract])) OR (COVID* intention to vaccin* [Title/Abstract]) OR (COVID* vaccin* accept*[Title/Abstract]) AND (2020:2020[pdat]). The

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search was concentrated in January 2021. To complement it and avoid the risk of bias, the gray literature was searched in Google Scholar.

Eligibility criteria

The studies were included with no restriction of language, date, and place of publication. The inclusion and exclusion criteria, developed specifically for this research, are shown in Table 2. The study scored 12 in the modified protocol by Pithon et al.[6], which evaluates their quality.

Risk of bias

The quality of the methods used in this study was independently evaluated by the reviewer (PH), following the PRISMA recommendation[5]. The evaluation gave priority to the clearly described information. In this stage, the review was blind, masking the names of authors and journals to avoid any potential bias and conflict of interest.

Exclusion criteria

Studies published as letters to the editor, guidelines, literature reviews, systematic reviews, meta-analyses, and abstracts were excluded. Studies with absent or unclear descriptions or not fully available were also excluded (Table 2).

Data analysis

The data were extracted for the study eligibility process using an appropriate spreadsheet for narrative reviews, developed by two researchers in Excel®. The extracted data were entered in the spreadsheet by one of the researchers and then checked by another one. The studies were selected at first by their title; then, the abstracts were analyzed, and only the potentially eligible ones were selected. Based on their abstracts, the articles were selected to be fully read.

Study selection process

Those whose title was within the context, but the abstract was unavailable, were also retrieved and analyzed in full. Studies not within the context, case reports, letters to the editor and/or editorials, literature reviews, indexes, abstracts, and studies on animals, were excluded.

Collected data

After the screening, the text of the selected article was reviewed, and its data were extracted in a standard manner by an author (LFG) supervised by PH. The year of publication, place of the research, language of publication, type of study, sample, method, result, and conclusion of the study were identified.

Clinical result

The clinical result of interest consisted of investigating possible compositions of the vaccines against COVID-19 that are being developed and produced. Those that did not follow the predefined approach were not included in the sample of the narrative review.

Table 1. Description of the PICOS components.

Acronym	Definition
P	Humans of both sexes without age restriction
I	Vaccine
C	Composition
O	COVID-19
S	Cross-sectional study Observational study Case reports Case-control studies Clinical trials Cohort studies

Source: Developed by the authors.

Table 2. Summary of the inclusion/exclusion criteria.

Inclusion criteria	
Design	Case reports Case and control studies Controlled clinical trials Cohort studies Screening studies Observational studies
Place	No restriction
Language	No restriction
Exclusion criteria	
Design	Letters to the editor Guidelines Literature reviews Systematic reviews Meta-analyses
Studies	Unclear, poorly described, or inadequate studies
Form of publication	Abstract alone

Source: Developed by the authors.

RESULTS

Initially, 56 articles were selected, narrowed down to 53 after excluding the repeated ones; then, the titles and abstracts were analyzed, and 51 papers were excluded for not being in the scope proposed for the research. Hence, two articles[7,8] were included in the final analysis of the present research (Figure 1). The selected article was designed as a randomized controlled study.

The databases were consulted based on the selected descriptors, obtaining the results presented in Table 3.

The main characteristics of the research selected for this study—such as the number of recruited patients, methods, results, and conclusion—are shown in Table 4[7,8].

Study design

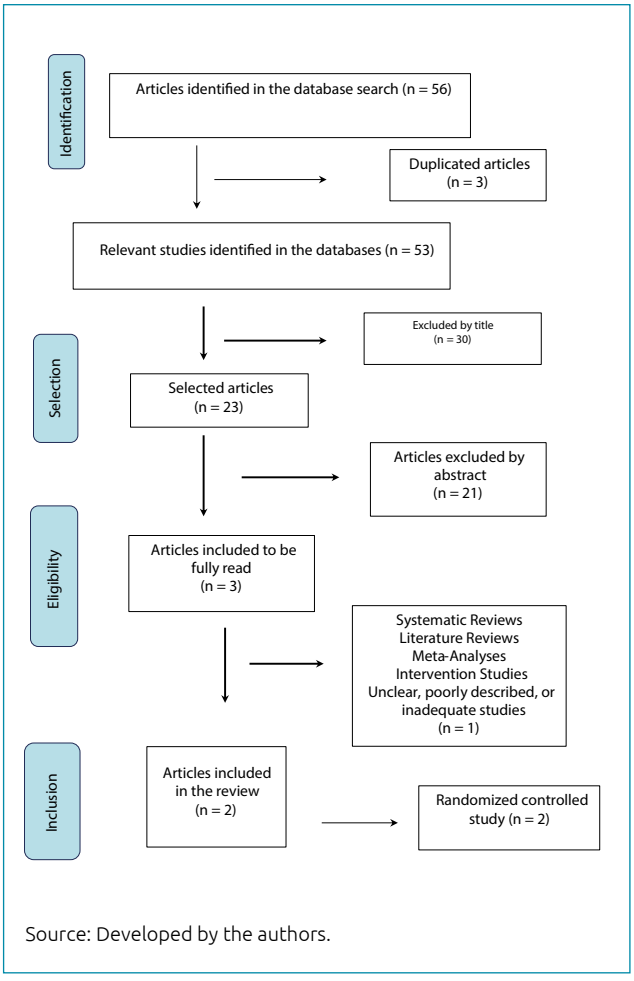
The first study[7] was carried out between April 23 and November 4, 2020, with 23,848 recruited participants vaccinated—n=1,077 in COV001 (the United Kingdom), n=10,673 in COV002 (the United Kingdom), n=10,002 in COV003 (Brazil), and n=2,096 in COV005 (South Africa). Approximately, 11,636 participants in COV002 and COV003 met the inclusion criteria for the primary analysis, of whom 5,807 received two doses of ChAdOx1 nCoV-19 and 5,829 received two doses of the control product. Most of the participants in COV002 and COV003 included in the primary effectiveness analysis were 18–55 years old [n=6,542 (86.7%) of the 7.548 in the United Kingdom and 3,676 (89.9%) of the 4,088 in Brazil]. Participants 56 years old or more were recruited later and contributed with 12.2% of the total in the current analysis [n=1,006 (13.3%) in the United Kingdom and 412 (10.1%) in Brazil].

In the second research[8], conducted from April 23 to May 21, 2020, approximately, 1,077 participants were included and vaccinated with either ChAdOx1 nCoV-19 (n=543) or MenACWY (n=534). The mean age of the participants was 35 years.

Vaccine and effectiveness

One participant had an asymptomatic infection 3 weeks after the first dose of ChAdOx1 nCoV-19. Another two participants in the control group had symptomatic infections 8 weeks and 21 weeks, respectively, after the initial sample collection. There were 131 symptomatic cases of COVID-19 eligible to be included in the primary effectiveness analysis more than 14 days after the second dose of the vaccine[7].

There were 30 (0.5%) cases out of the 5,807 participants in the vaccine group and 101 (1.7%) cases out of the 5,829 participants in the control group, resulting in a 70.4% vaccine effectiveness. In participants who received two doses, the vaccine



Source: Developed by the authors.

Figure 1. Flowchart of the search for and analysis of articles.

effectiveness was 62.1%; whereas, in those who received the first dose with a decreased amount of the vaccine and later a standard dose, the effectiveness was 90%[7].

Two doses of the vaccine are obtained from the United Kingdom and Brazil, and the vaccine effectiveness was similar when analyzed in subgroups according to the duration between vaccines—53.4% in participants with an interval shorter than 6 weeks between the doses and 65.4% in participants with an interval of at least 6 weeks. For the secondary analysis of cases that occurred more than 21 days after the first standard dose in participants who received only standard doses, 192 cases were included with a 64.1% vaccine effectiveness[7].

Vaccine and adverse events

More than 21 days after the first dose, 10 participants were hospitalized due to COVID-19, two of them with severe COVID-19, one of which was fatal. All these 10 cases were in the control group. Severe adverse events occurred in

Table 3. Classification of the references obtained from the PubMed, SciELO, LILACS, Web of Science, and Scopus databases.

Descriptors	Total number of articles	Number of excluded references	Reason for excluding	Number of selected articles	Database
(covid-19) and (SARS-CoV-2) and (vaccines) and (CITE-seq) and (immune response) and (infection) and (multi-omics) and (proteomics) or (single-cell RNA-seq) or (single-cell TCR-seq) or (single-cell) or (secretome)	0	–	–	–	SciELO
(covid-19) and (SARS-CoV-2) and (vaccines) and (CITE-seq) and (immune response) and (infection) and (multi-omics) and (proteomics) or (single-cell RNA-seq) or (single-cell TCR-seq) or (single-cell) or (secretome)	0	–	–	–	LILACS
(covid-19) and (SARS-CoV-2) and (vaccines) and (CITE-seq) and (immune response) and (infection) and (multi-omics) and (proteomics) or (single-cell RNA-seq) or (single-cell TCR-seq) or (single-cell) or (secretome)	0	–	–	–	Web of Science
(covid-19) and (SARS-CoV-2) and (vaccines) and (CITE-seq) and (immune response) and (infection) and (multi-omics) and (proteomics) or (single-cell RNA-seq) or (single-cell TCR-seq) or (single-cell) or (secretome)	0	–	–	–	Scopus
(covid-19) and (SARS-CoV-2) and (vaccines) and (CITE-seq) and (immune response) and (infection) and (multi-omics) and (proteomics) or (single-cell RNA-seq) or (single-cell TCR-seq) or (single-cell) or (secretome)	0	–	–	–	BIREME
(covid-19) and (SARS-CoV-2) and (vaccines) and (CITE-seq) and (immune response) and (infection) and (multi-omics) and (proteomics) or (single-cell RNA-seq) or (single-cell TCR-seq) or (single-cell) or (secretome)	56	53	Excluded by title (30); excluded by abstract (21); duplicated (3)	3	PubMed
Total	56	54		2	PubMed

Source: Developed by the authors.

168 participants, of which 79 received ChAdOx1 nCoV-19, while 89 received MenACWY. There were 175 events (84 in the ChAdOx1 nCoV-19 group and 91 in the control group), of which 3 were considered possibly related to the experimental or control vaccine[7]. Unsolicited adverse events in the 28 days after the vaccination considered possibly, probably, or definitely related to the ChAdOx1 nCoV-19 were predominantly mild and moderate and solved during follow-up[8].

Vaccine and post-vaccination effects

Approximately 56 participants in the ChAdOx1 nCoV-19 group and 57 in the MenACWY group received prophylactic paracetamol. Of those who did not receive prophylactic paracetamol, 328 (67%) out of the 487 participants in the ChAdOx1 nCoV-19 group and 180 (38%) out of the 477 participants in the MenACWY group reported pain after the vaccination, mostly in mild-to-moderate intensity. With the prophylactic paracetamol, the pain was reported in fewer participants—28 (50%) in the ChAdOx1 nCoV-19 group and 18 (32%) in the MenACWY group[8].

Fatigue and headache were the most reported systemic reactions. Fatigue was reported in the ChAdOx1 nCoV-19 group by 340 (70%) participants without paracetamol and 40 (71%) with paracetamol, and in the MenACWY group by 227 (48%) participants without paracetamol and 26 (46%) with paracetamol. Headaches were reported in the ChAdOx1 nCoV-19 group by 331 (68%) participants without paracetamol and 34 (61%) with paracetamol, and in the MenACWY group by 195 (41%) participants without paracetamol and 21 (37%) participants with paracetamol. Other systemic adverse reactions were common in the ChAdOx1 nCoV-19 group, such as muscle pain [294 (60%) participants without paracetamol and 27 (48%) with paracetamol], malaise [296 (61%) and 27

Table 4. Summary of the included articles

Author/ year/ place of publication	Objective	n	Method	Results	Conclusion
Voysey et al., 2020[7]	To evaluate the safety and effectiveness of vaccine ChAdOx1 nCoV-19 in a combined interim analysis of four trials.	11,636	This analysis includes data of four ongoing blind randomized controlled studies, conducted in the United Kingdom, Brazil, and South Africa. The participants in the ChAdOx1 nCoV-19 group received two doses containing 5 × 1010 viral particles (standard dose); a subgroup in the United Kingdom study received half a dose as the first one, with a decreased amount, and a standard dose as the second one. The participants were analyzed according to the treatment received. The data cutoff date was November 4, 2020.	In participants that received two standard doses, the vaccine effectiveness was 62.1% in the ChAdOx1 nCoV-19 group, and in participants that received a decreased dose followed by a standard dose, the effectiveness was 90.0%. The general vaccine effectiveness in both groups was 70.4%. Twenty-one days after the first dose, there were 10 cases of hospitalization due to COVID-19—two classified as severe COVID-19, one of whom died. Three events were classified as possibly related to a vaccine.	The ChAdOx1 nCoV-19 has an acceptable safety profile and was considered effective against symptomatic COVID-19 in this provisional analysis of ongoing clinical trials.
Falegatti et al., 2020[8]	To evaluate the safety, reactogenicity, and immunogenicity of a vector coronavirus vaccine expressing the spike protein of SARS-CoV-2.	1,077	The co-primary outcomes evaluate its effectiveness (measured by virologically confirmed symptomatic COVID-19 cases) and safety (measured by the occurrence of severe adverse events). The analyses were conducted with group allocation in participants that received the vaccine. The safety was evaluated throughout 28 days after the vaccination.	Local and systemic reactions were more common in the ChAdOx1 nCoV-19 group (including pain, feverishness, chills, muscle pain, headaches, and malaise), many of which were diminished with prophylactic paracetamol. There were no severe adverse events related to the ChAdOx1 nCoV-19. After one booster dose, all the participants had neutralizing activity.	The ChAdOx1 nCoV-19 revealed an acceptable safety profile and antibody response. These results, along with the induced humoral and cellular immune responses, support the large-scale evaluation of this candidate vaccine in an ongoing phase 3 program.

(48%)], chills [272 (56%) and 15 (27%)]; and feverishness [250 (51%) and 20 (36%)][8].

In the ChAdOx1 nCoV-19 group, 87 (18%) participants without paracetamol and 9 (16%) with paracetamol reported a temperature of at least 38°C, while 8 (2%) patients without paracetamol had a temperature of at least 39°C. The severity and intensity of local and systemic reactions were greater in the first post-vaccination day. The adjusted analysis of the effects of prophylactic paracetamol on the adverse reactions of any severity on the first two days of post-vaccination with ChAdOx1 nCoV-19 revealed a significant decrease in pain, feverishness, chills, muscle pain, headache, and malaise[8].

DISCUSSION

Due to the quick worldwide dissemination of SARS-CoV-2 infection and the high mortality rate, the development of a vaccine is an urgent commitment of public health, as the vaccination can restrain the propagation of COVID-19 and

reduce mortality. Intense research and vaccine development are currently underway, especially in China, Russia, the United Kingdom, the United States, besides other participating countries[9].

Collaborative efforts are taking place to ensure unprecedented large-scale and quick production, which is necessary to immunize billions of people. It is also essential that the implementation be equitable all over the world. The different types of vaccines employ a variety of strategies (vector, DNA, mRNA, inactivated, and so on). Currently, the objective is to prove that they are safe and immunogenic in humans (studies in phases 1/2), advancing to phases 2 and 3 to demonstrate their effectiveness and collect comprehensive data on safety[10]. The first stage in vaccine development is the preclinical one, to establish its safety profile. The last phase in pharmacovigilance monitors the adverse event of the vaccine. This phase involves strict monitoring of the vaccines to detect, analyze, understand, prevent, and communicate any adverse events after immunization, or any other aspects related to the vaccination or immunization[11].

In one of the studies[8], severe adverse events occurred in 168 participants, of which 79 had received ChAdOx1 nCoV-19 and 89 had received MenACWY. In the other study[7], there were 175 events (84 in the ChAdOx1 nCoV-19 group and 91 in the control group), 3 of which were considered possibly related to the experimental or control vaccine. Unsolicited adverse events in the 28 days after the vaccination considered possibly, probably, or definitely related to the ChAdOx1 nCoV-19 were predominantly mild, moderate, and solved during follow-up. Other adverse systemic reactions were common in the ChAdOx1 nCoV-19 group, such as muscle pain, malaise, chills, and feverishness[8].

There are still many unanswered questions that need to be clarified regarding SARS-COV-2 to elucidate how the presence of antibodies will affect the clinical course and severity of the disease. It needs to be found whether the infection will protect from future ones, and if so, for how long the protection will last and what are the correlations of this protection[3]. The authors of this study[7] point out approximately 30 (0.5%) cases out of the 5,807 participants in the vaccine group and 101 (1.7%) cases out of the 5,829 participants in the control group, resulting in a 70.4% vaccine effectiveness. In participants who received two doses, the vaccine effectiveness was 62.1%, while in those who received the first dose with a decreased amount of the vaccine and later a standard dose, the effectiveness was 90%. However, the usefulness of the COVID-19 vaccination campaigns does not depend only on the vaccine effectiveness and safety[12].

CONCLUSIONS

Such a need is grounded on scientific knowledge, which makes it easier to develop an ideal COVID-19 vaccine in a short time, using new ways to facilitate its development, testing, and large-scale production. However, the challenge to researchers and health professionals consists of validating, confirming, and increasing the effectiveness of the vaccine. It will be essential to identify the vaccine components that induce protective immunity to protect the vulnerable population. Hence, the studies included in this review demonstrate that the developed and applied vaccines had significant results regarding their effectiveness and protection against COVID-19.

AUTHORS' CONTRIBUTIONS

LFG: Formal analysis, Investigation, Methodology. **PH:** Conceptualization, Data curation, Resources, Supervision. **JVS:** Validation, Visualization.

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COMMENTARY
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Commentary: "Sleep quality is inversely related to body mass index among university students"

Tingting Li[1], Xu Zhang[1], Baohong Xue[1]*

Dear Editor,

We read with great interest the study of Jun Wang et al.[1] in which they demonstrated the relationship between sleep quality and BMI among university students. The authors indicate that the prevalence of obesity is higher in males than in females, and the sleep quality of female students may be related to body mass index.

Firstly, it is of great significance[2] for college students to research the relationship between sleep quality and body mass index. Studies had shown that the poor sleep quality among female students may be related to multiple factors[3]. The relationship between sleep quality and BMI in females needs to be further explored. And there is a small sample size of different groups in the present study. Thus, more samples should be recruited in a future study.

Secondly, the authors used a cross-sectional study and were unable to explore causality further. Therefore, further longitudinal studies are needed to investigate the relationship between sleep quality and body mass index in college students and to develop appropriate interventions for high-risk groups.

AUTHORS' CONTRIBUTION

TL: Conceptualization, Data curation, Writing-original draft. **XZ:** Formal analysis, Writing-original draft. **BX:** Supervision, Validation, Visualization, Writing-review & editing.

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COMMENTARY
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Comment on "Is ionizing radiation a risk factor for anxiety in employees?"

Xu zhang[1], Tingting Li[1], Baohong Xue[1]*

Dear Editor,

We read with great interest the study by Asli Kurtar Mansiroglu et al.[1], in which they demonstrated that radiation is likely to be a risk factor for anxiety among hospital staff, although, in our opinion, the author should combine more clinical cases to confirm this view.

First, although the two groups of research objects selected by the researchers come from the same working environment, there are still some differences, which will affect the accuracy of the research results; the author should choose a more similar working environment to reduce the interference of relevant influencing factors and research conclusions.

Second, the author's conclusion is only to prove that radiation is one of the risk factors affecting employee anxiety. However, the further influencing mechanism is not determined.

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COMMENTARY
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Deciphering SARS-CoV-2 mortality: H1N1 as an aid

Georgios T. Stathopoulos[1]*

To the Editor:

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has globally disrupted contemporary life[1]. It has caused 73,996,237 confirmed cases and 1,663,474 deaths since December 19, 2020, with a global mortality rate of 2.25%[2]. While efforts to understand, cure, and prevent the disease are ongoing, the mortality rate and disease burden imposed by SARS-CoV-2, as well as the appropriateness of pandemic containment measures, are also being discussed[3-5]. To this end, geographic distributions of disease burden and disease-specific mortality are often key to understand disease vulnerabilities, formulate policies, and aid in opinion-making[6].

In this issue of Revista da Associação Médica Brasileira, Kant and colleagues report on their multi-center retrospective experience with 143 patients with H1N1 and 309 patients with SARS-CoV-2 from seven centers in Turkey[7]. Among their pertinent findings related to the clinical profiling of the two diseases, one result is striking: in their hands, H1N1 was more lethal than SARS-CoV-2! In more detail, Kant et al[7]. report that although H1N1 patients required fewer hospitalization days compared with SARS-CoV-2 patients (mean±SD: 4.4±5.7 *versus* 10.9±7.6 days; p<0.001; Mann-Whitney U test), they actually required more intensive care support (H1N1 *versus* SARS-CoV-2: 41 *versus* 18%; p<0.001; χ^2 test), more mechanical ventilatory support (H1N1 *versus* SARS-CoV-2: 28 *versus* 9%; p=0.004; χ^2 test), and succumbed more frequently (H1N1 *versus* SARS-CoV-2 mortality: 8.4 *versus* 3.2%; p=0.004; χ^2 test). Data from Turkey have been properly reported, are plausible, and in accordance with data from the United States reported earlier this year[8].

To put the work of Kant et al.[7] into perspective, the author analyzed current SARS-CoV-2 data and compared them with global H1N1 data obtained after the end of the H1N1 pandemic, in the 27 most heavily affected countries (Table 1)[2,9]. According to the multi-center results provided by Kant et al.[7], nationwide, Turkish H1N1 death rates exceeded by far SARS-CoV-2 death rates, and this is also applicable to 12 other countries including the United States, Spain, and Brazil. However, the opposite was true for 15 other countries that experienced far higher death rates from SARS-CoV-2, such as Mexico, Egypt, China, and Italy. Overall, when the 27 countries that were most affected from both outbreaks were examined, death rates from SARS-CoV-2 and H1N1 were not statistically significantly different (Figure 1A). These data show that the study by Kant et al.[7] is accurate in reflecting the Turkish experience from the two viral outbreaks, and that both pandemics cause comparable mortality, as anticipated for severe viral pneumonias.

But how can the astonishing fact that SARS-CoV-2 is less lethal than H1N1 in at least 12 countries be associated with a response that has been disproportionately greater, with stricter measures, and economic stagnation worldwide and in these countries (including in Brazil and Turkey) due to SARS-CoV-2 as compared to twhe H1N1 outbreak 11 years ago? Table 1 and Figure 1B illustrate the answer, which is the disproportional size of both outbreaks in terms of number of cases and deaths. To this end, H1N1 caused 6.14 million (16 thousand on average) cases and 227 thousand (593 on average) deaths in the 27 countries examined, while SARS-CoV-2 has already caused 56.7 million (1.3 million on average) cases and 2.1 million (47 thousand on average) deaths in the same countries, while the pandemic is still at large. Thus, one can argue that the SARS-CoV-2 outbreak has already taken a ten-fold higher toll than the H1N1 outbreak 11 years ago, underpinning its societal and financial impacts[10].

But what are the determinants of these strikingly different death rates? At a global scale, we have shown earlier this year that SARS-CoV-2 incidence and mortality are linked with economic growth, while H1N1 rates were rather associated with overpopulation

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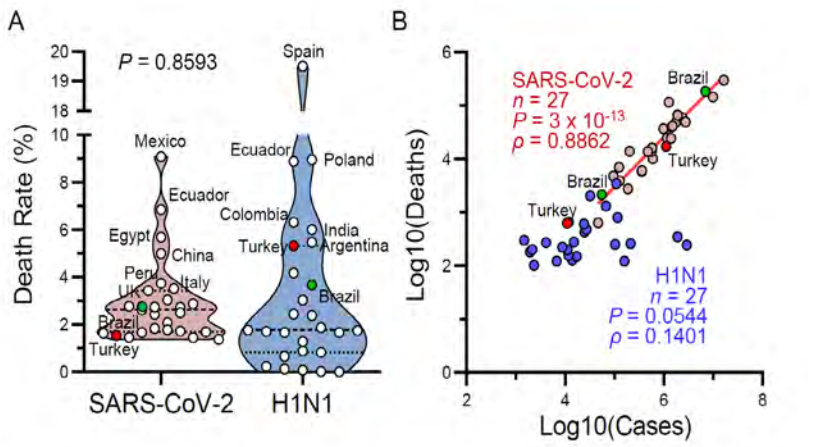
and urbanization, like a true airborne disease[10]. In an elegant recent prospective two-center case-control study, Sesé et al. showed the correlation between poor socioeconomic status and SARS-CoV-2 severity in terms of disease presentation and outcome[11]. It is also well known that co-morbidities largely define SARS-CoV-2 death rates, implying increased vulnerability of high-income countries with elderly populations to SARS-CoV-2[12]. To this end, biomarkers of SARS-CoV-2 susceptibility in elderly patients and populations with cardiovascular, cerebral, and pulmonary co-morbidities have been proposed, including low circulating CD3+CD8+T-cells and cardiac troponin[13]. One cannot overemphasize the importance of such clinical, cellular, and molecular biomarkers of risk, as well as of clinical studies such as that by Kant et al.[7], which will hopefully be considered by policy-makers and their health care advisors during future infectious outbreaks to prevent lockdown measures and economic recession, and to enforce viral containment measures that are more focused and effective.

Table 1: Raw data used for plots shown in Figure 1, expressed as person numbers (n) or percentages (%).

County	WHO Region	SARS-CoV-2 Cases (n)	SARS-CoV-2 Deaths (n)	H1N1 Cases (n)	H1N1 Deaths (n)	SARS-CoV-2 Death Rate (%)	H1N1 Death Rate (%)
Czechia	Europe	602404	10036	2445	102	1.67	4.17
Romania	Europe	571749	13862	7006	122	2.42	1.74
Portugal	Europe	358296	5815	166922	122	1.62	0.07
Saudi Arabia	Eastern Mediterranean	360335	6080	14500	128	1.69	0.88
Greece	Europe	127557	3870	17977	149	3.03	0.83
Chile	Americas	576731	15959	12258	156	2.77	1.27
Poland	Europe	1171854	24345	2024	181	2.08	8.94
Japan	Western Pacific	187103	2739	11636	198	1.46	1.70
Ecuador	Americas	203461	13915	2251	200	6.84	8.89
Peru	Americas	987675	36817	9165	223	3.73	2.43
Italy	Europe	1888144	66537	3064933	244	3.52	0.01
Republic of Korea	Western Pacific	46453	634	107939	250	1.36	0.23
Germany	Europe	1406161	24125	222360	258	1.72	0.12
Colombia	Americas	1444646	39356	4310	272	2.72	6.31
Egypt	Eastern Mediterranean	123153	6990	15812	278	5.68	1.76
Spain	Europe	1773290	48596	1538	300	2.74	19.51
France	Europe	2367648	58989	1980000	344	2.49	0.02
Canada	Americas	475214	13659	25828	429	2.87	1.66
United Kingdom	Europe	1913281	65520	28456	474	3.42	1.67
Russian Federation	Europe	2762668	49151	25339	604	1.78	2.38
Argentina	Americas	1510203	41204	11458	626	2.73	5.46
Turkey	Europe	1113827	17121	12316	656	1.54	5.33
China	Western Pacific	95375	4764	120940	800	5.00	0.66
Mexico	Americas	1267202	115099	70715	1316	9.08	1.86
India	South-East Asia	9956557	144451	33783	2024	1.45	5.99
Brazil	Americas	6970034	182799	58178	2135	2.62	3.67
United States of America	Americas	16446844	301536	113690	3433	1.83	3.02



Stathopoulos GT. Deciphering SARS-CoV-2 mortality: H1N1 as an aid. **Figure 1**

Raw data were from references WHO[2] and Tang et al.[8] and are summarized in Table 1. (A) Case fatality rate (%). Each circle denotes one country (Brazil in green and Turkey in red). Data are shown as rotated kernel density plots (violins) with medians (dashed lines) and quartiles (dotted lines), names of top-affected counties, Turkey and Brazil, and probability (p) by Wilcoxon's matched-pairs signed rank test. (B) Dot plot of number of deaths ***versus*** number of cases. Each circle denotes one country (Brazil in green and Turkey in red). Raw data for SARS-CoV-2 (blue circles and regression line) and H1N1 (red circles) are shown together with Spearman's correlation probabilities (p) and coefficients (ρ). Note the strong correlation between SARS-CoV-2 deaths and cases, which is not evident for H1N1.

Figure 1. Cases and deaths from SARS-CoV-2 and H1N1 in 27 countries.

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