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ORIGINAL ARTICLE

The Psychiatric, Psychological, and Psychotherapeutic Approach to Erectile Dysfunction – Between Good Practices and Clinical Challenges

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Abstract: *Erectile dysfunction (ED) has an intricate pathogenesis, with organic and psychosocial factors contributing to the shaping of its clinical manifestations and functional impairment. ED disrupts not only an individual's sexual life but may also contribute to impairments of self-esteem, social functioning, quality of life, overall well-being, mood, etc. The assessment process and therapeutic interventions should be adequate to the specific profile of each patient, therefore an interdisciplinary approach is usually recommended, in order to find the vulnerability factors, recent triggers, psychiatric and organic comorbidities or causes, and elements that contribute to maintaining the sexual dysfunction, such as lifestyle factors, interpersonal conflicts, or inadequate sexual education. This narrative review explores the evidence-based approaches to structured psychiatric, psychological, and therapeutical assessment in patients with ED, and the most validated psychosocial treatments available. The results support the use of recognized diagnostic criteria within DSM-5TR and ICD-11, together with structured instruments (scales, questionnaires, and inventories), in a multidisciplinary approach. The cognitive model and cognitive-emotional model of ED support the initiation of cognitive-behavioral therapy in these patients. Other interventions, like the PLISSIT model, sexual therapy, couple therapy, and sexual-focused psychoeducation are also supported by evidence. In conclusion, ED requires a combined, psychiatric, psychologic, psychotherapeutic, and organic-oriented assessment, with the purpose of tailoring the treatment to the patient's particularities.*

Keywords: *major depressive disorder, erectile dysfunction, antidepressants, antipsychotics, schizophrenia spectrum disorders, psychotherapy, psychoeducation*

INTRODUCTION

Erectile dysfunction (ED) is one of the most frequently reported sexual pathologies, with an estimated global prevalence varying from 3% to 76.5% [1]. The large variations in the reviewed reports regarding the prevalence of ED are explained by the method chosen during studies to define and quantify the manifestations of this disorder. When structured, validated clinical scales, questionnaires, or inventories were used, the prevalence of ED in young men was high [1]. Non-validated instruments have lower sensitivity, therefore the prevalence obtained using such tools is expected to be less accurate [1,2].

It is also important to highlight that ED was positively correlated with cardiovascular diseases, a high risk of all-cause mortality, mortality due to cardiovascular pathology, and a higher risk of developing dementia [1]. Further exploring this topic, ED is considered a "sentinel marker" for cardiovascular disease and could indicate a chronic and undetected coronary artery disease [2]. This correlation was supposed to be more significant in men with diabetes mellitus [2]. ED demonstrated significant value as an indicator of general health in men, irrespective of the cardiovascular risk factors [3].

Therefore, the importance of exploring the most adequate ways to diagnose and treat ED derives not only from the

relevance of this disorder on the quality of life and well-being of the patients but also from the potential comorbid conditions this diagnosis may reveal during the multidisciplinary assessment.

OBJECTIVES AND METHODOLOGY

The main objective of this review was to find the most evidence-based assessment methods for patients with ED from the perspective of mental health professionals. Secondary objectives were founding data on (a) the available psychosocial approaches to ED; (b) ways to integrate psychological, psychiatric, and psychotherapeutic evaluations for patients with ED; (c) finding the key aspects that can help the training of mental health specialists in the evaluation and treatment of patients with ED.

The identification of relevant articles involved searching three electronic databases (PubMed, Cochrane, and Clarivate/ Web of Science) as well as their list of references. There was no inferior time limit for the search, while the superior limit was June 2023.

All primary and secondary reports were allowed into the analysis. No language restrictions were applied.

The paradigm used for retrieving sources was “erectile dysfunction” OR “male sexual dysfunctions” AND “assessment” OR “psychotherapy” OR “counseling” OR “diagnosis” OR “cultural aspects” OR “skills training” OR “mental health specialists”.

RESULTS

The results of the literature search were distributed in three sections, according to their relevance for each category of mental health specialists involved in the assistance of patients with ED. The accent is placed on the need to conduct a multidisciplinary exploration of the ED (Figures 1 and 2) and to formulate a therapeutic plan that takes into account the main vulnerability factors identified during the initial assessment of the patient.

The psychiatric approach

The diagnosis of ED implies, according to the 5th edition (revised) of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5TR), significant difficulty in reaching or maintaining an erection, or a marked decrease in the penile rigidity during erection, that appears on almost all occasions of sexual activity (75-100%) [4]. There is also the requirement of a minimal duration of 6 months for these symptoms, and they should cause clinical distress, in order to complete the diagnosis criteria [4]. Like in the case of all sexual dysfunctions, ED should be distinguished from organic, toxic, stress-induced pathology, or other mental disorders [4]. ED may be lifelong (if the patient did not have any period of normal sexual functioning) or acquired (if the dysfunctions started after a period of normal sexual functioning) [4]. ED can also be mild, moderate, or severe (depending on the level of symptoms-induced distress), or it may be generalized or situational (depending on the types of stimulation, situations, or partners that may or may not trigger the symptoms) [4].

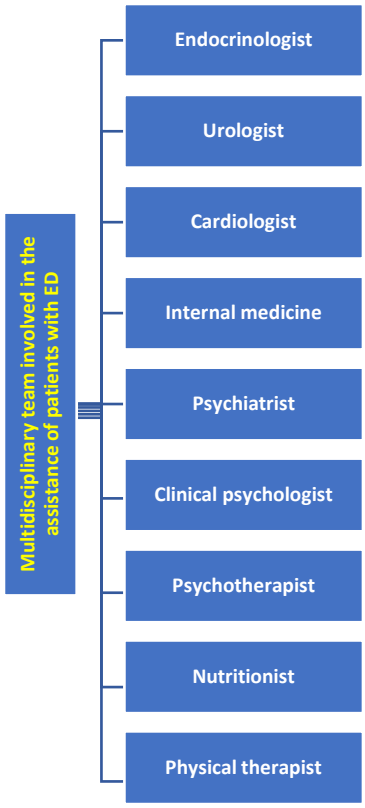

Figure 1: Multidisciplinary assessment of patients with ED

The diagnostic criteria in the DSM-5 and DSM-5TR changed from the previous versions (DSM-IV and DSM-IV TR) [4-7]. In these early editions, only two clinical criteria were mentioned, without any reference to the decrease of penile rigidity, as an independent criterion; also, no frequency of failed sexual acts was mentioned; no severity specifiers existed, but ED could have been classified as exclusively psychogenic or with mixed causation [6,7]. Further back, the third edition (revised) of DSM (DSM-III(R)) included the “male erectile disorder”, defined as a partial or complete, persistent and recurrent, incapacity to obtain and maintain the erection until the end of a sexual act, or recurrent/persistent lack of sexual excitation and pleasure during the sexual activity [8]. ED is included in the category of “male sexual dysfunctions”, together with delayed ejaculation, premature ejaculation, and male hypo-sexual desire disorder (Figure 3) [4-7].

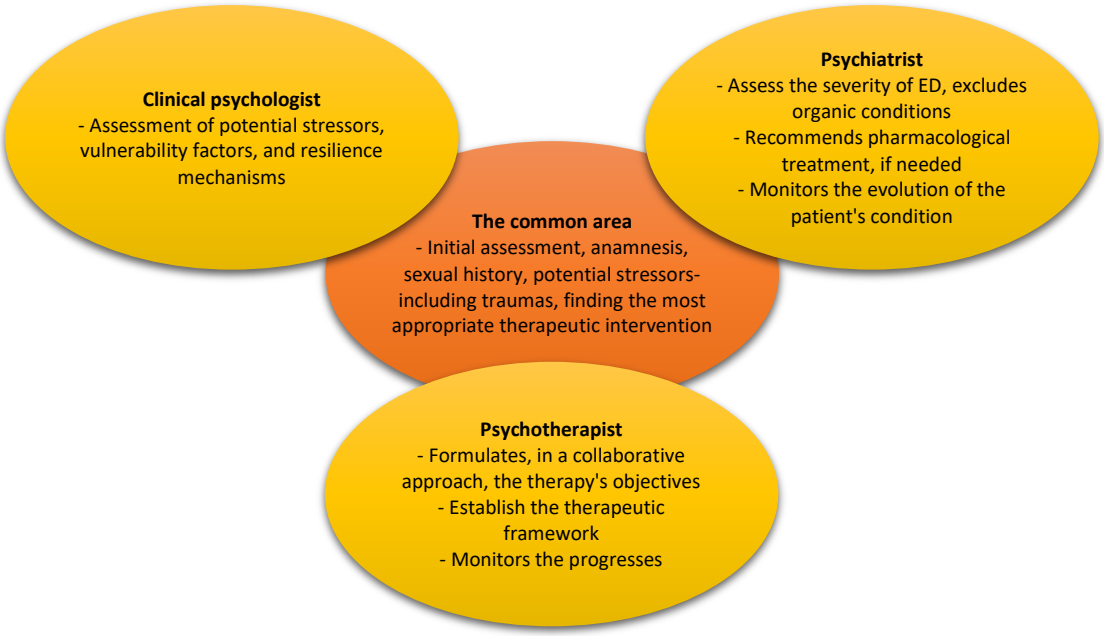

Figure 2: The roles of the clinical psychologist, psychiatrist, and psychotherapist in the assistance of patients with ED

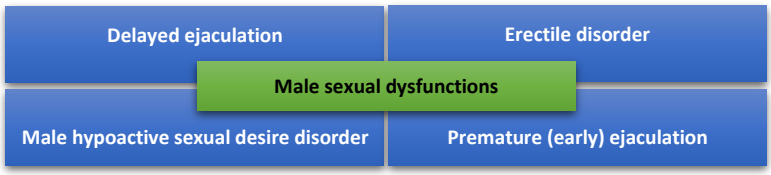

Figure 3: Male sexual dysfunctions [4-7]

The World Health Organisation's Classification of Diseases (ICD) mentioned in its latest edition (ICD-11), that ED is the "inability or marked reduction in the ability in men to attain or sustain a penile erection of sufficient duration or rigidity to allow for sexual activity" [9]. Also, ICD-11 specifies that ED appears although the libido and sexual stimulation exist, and its manifestations may be episodic or persistent (several months), and associated with significant distress at the clinical level [9]. In the previous edition, ICD-10 (the version for clinical research), ED had as its main clinical characteristics (1) the impossibility of participating in sexual activity according to the desire of the individual, (2) the dysfunction appears frequently, but may not be observed on every occasion, (3) the manifestations have a history of at least 6 months [10]. The ED may manifest itself as: a complete erection that may be obtained in the initial stage of sexual activity, but then disappears before ejaculation (if this appears); erection may be detected, but only in situations where the sexual activity is unprepared; partial erection, insufficient for sexual activity; or complete absence of erection [10].

The psychiatric assessment of patients with ED is recommended to be carried out from both the clinician's perspective (focusing on signs and symptoms) and the patient's (focusing on the consequences of dysfunction). There may be significant differences between the two perspectives, depending on the criteria for defining ED, for example, the duration of the manifestations, the frequency of their occurrence, and the functional impact. Sometimes the patient can admit the presence of erectile dysfunction and the associated discomfort, but at other times he avoids mentioning any aspect related to sexual functioning, presenting only general symptoms - "weakness", "permanent fatigue", "lack of initiative", etc.

Sexual dysfunction can be an isolated symptom or it can be integrated into a complex context, as it may appear together with multiple psychiatric symptoms (such as anxiety, insomnia, depression, psychotic manifestations, and substance addiction) or organic manifestations (like urinary hesitancy, high blood pressure, pelvic pain, etc.).

Among the main differential diagnoses in ED, major depressive disorder (MDD) is important to be considered, because a decreased libido has been detected in these patients during the acute episodes, and some of the adverse events of antidepressants involve sexual functioning [11-17]. According

to a systematic review and meta-analysis (n=49 reports), there is a high comorbidity rate of ED in patients with MDD (OR=1.39) and vice versa (OR=2.92), but a causal relationship can not be determined [11]. The incidence of sexual dysfunctions in patients undergoing treatment for MDD is difficult to be determined, but decreased sexual desire, decreased sexual excitement, reduced ability to reach orgasm, delayed orgasm, ED, delayed ejaculation, priapism, painful ejaculation, penile anesthesia were all reported in this population [12]. Therefore, sexual dysfunction may be a common MDD symptom, but also a frequent adverse effect of antidepressant treatment [14]. Due to this lastly-mentioned phenomenon, premature drug discontinuation in patients with MDD is not an uncommon event [14,18]. Nefazodone and bupropion have been associated with a lower incidence of sexual adverse events, due to their pharmacodynamic properties, unlike the selective serotonin reuptake inhibitors (SSRIs), which are among the most frequent drugs associated with such negative events [18]. At any rate, monitoring sexual adverse events during antidepressant treatment, as well as monitoring sexual functioning in these patients as part of the MDD clinical picture, are very important for preserving therapeutic adherence and quality of life [18-20].

Psychosocial stressors can trigger both major depression and erectile dysfunction [11]. Depression itself can reduce sexual functioning through multiple mechanisms (decreased initiative, fatigue, decreased self-esteem, ideas of uselessness and inability, etc.) [11-13]. Persistent erectile dysfunction can lead to reduced self-esteem, rumination about personal incapacity, and depression. In conclusion, patients with ED should be evaluated for signs of major depression and vice versa [11].

Psychotic disorders involve the presence of delusional ideas (some of which may have a sexual theme), hallucinations (some may be proprio- or interoceptive), thought and language disorganization, behavioral disorganization, negative symptoms (avolition, apathy, flat affect, anhedonia, alogia, etc.). Patients with psychotic disorders frequently associate sexual dysfunctions as a result of negative symptoms, antipsychotic medication, associated depression, etc [21-29]. In patients with psychotic disorders who had never received psychotropic treatment, 17% still accused the presence of sexual dysfunction [22]. Therefore, it is clear that not all sexual dysfunctions reported in this population are secondary to pharmacologic aspects, although a false causal relationship may be construed by patients, relatives, or even treating physicians. It must be mentioned that most mental health professionals do not ask their patients with psychosis about sexual dysfunctions, in clinical settings [23]. The sexual functioning in outpatients with schizophrenia spectrum disorders (SSD) has been associated with the level of subjective quality of life [24]. Also, a correlation between sexual dysfunctions in patients with SSD and their therapeutic adherence has been demonstrated, even when the dose, number, and duration of antipsychotics were not associated with the presence of sexual dysfunctions [25].

In an interesting study, patients with schizophrenia who were drug-naïve (N=20) were compared with patients who received long-acting first-generation antipsychotics (N=51) and with healthy controls (N=51), from the perspective of their sexual functioning [28]. Untreated patients with schizophrenia reported decreased sexual desire, while those who were receiving antipsychotics reported a restoration of the libido, although presented ED, orgasmic and sexual satisfaction problems [28]. Therefore, the structured assessment of sexual function is recommended to be conducted by mental health specialists in all patients diagnosed with SSD.

Anxiety disorders are included in the differential diagnosis of ED, but they may also be comorbid conditions with, or predisposing factors to sexual dysfunctions. While fear is the response to real or perceived imminent danger, anxiety is conceptualized as anticipating an unknown threat or an internal stressor [30]. Both anxiety and fear are associated with an activation of the sympathetic nervous system when anticipating an imminent danger, which prepares the individual for a fight or flight response [30-32].

One of the topics of interest for the current review is the presence of some specific phobias, like genophobia or erotophobia (fear of intimacy and sex), heterophobia (fear of the opposite sex), nosophobia (fear of microbes), coitophobia (fear of sexual intercourse), and haptophobia (fear of being touched by another person) [33-34]. These disorders may negatively affect the quality of sexual intercourse and even lead to avoidant behaviors.

Social anxiety disorder is characterized by fear of being evaluated or criticized by others when an individual is facing social situations, with severe negative consequences over all areas of functioning [35]. Unlike shyness, social anxiety disorder (or social phobia) has a severe impact on the quality of life in affected individuals [35]. According to a study (N=40 participants), men with social phobia report more frequent sexual dysfunctions and are more inclined to pay for sex [36]. The same study found moderate impairment in arousal, orgasm, sexual enjoyment, and subjective satisfaction with sexual activity in men with social phobia [36].

Panic disorder is defined by recurrent and spontaneous panic attacks, with significant negative functional impact [4-10]. Patients with panic disorder have a higher tendency to develop sexual dysfunctions vs. the general population [37]. According

to a review (n=6 reports), panic disorder was associated with a high prevalence of sexual aversion and sexual infrequency, while untreated patients with panic disorder may have a higher risk of ED vs. controls [37]. Patients with panic disorder can perceive the similarity between the neurovegetative activation during sexual intercourse and the one with onset during a panic attack [38]. Therefore, this symptomatic overlap maintains a negative conditioning that leads to the continuation of ED [38].

Agoraphobia is characterized by the fear of enclosed spaces, although the focus of this disorder is not limited to such triggers (i.e., newer classifications also include the fear of open spaces, being alone outside the home or being in a crowd, and using the public transportation) [4-10]. The exposure to feared situations can trigger discomfort, anxiety, or panic attacks [4-10]. The exploration of this disorder during the psychiatric evaluation of patients with ED is important because of the significant impact this type of phobia has on the overall functioning and quality of social relationships.

Generalized anxiety disorder has as its core criterion excessive anxiety or worries related to various daily activities, occurring for at least 6 months, and this disorder has been frequently associated with sexual dysfunctions in men [4-10,39]. In a study that evaluated 64 men with ED or premature ejaculation, 23.4% presented with anxiety disorders, and generalized anxiety disorder was the most frequently detected [39]. Men with preexisting anxiety disorders had a higher probability of presenting performance anxiety related to sex and of being diagnosed with premature ejaculation [39].

The prevalence of anxiety disorders in patients with ED ranges from 2.5% to 37%, followed closely by depressive disorders (18-35%) [40,41]. This association was most frequently reported in patients aged between 45 and 54 years [42]. Anxiety, as a non-specific symptom, can impair both sexual functioning and interpersonal behaviors. But the relationship between ED and anxiety is bidirectional because sexual dysfunction can induce anxiety, and consequently lead to a decrease in self-esteem, performance anxiety, and social avoidance.

Obsessive-compulsive disorders (OCDs) are currently conceived as a spectrum, with core symptoms represented by obsessions, compulsions, or both, that occur over a significant period of time or cause discomfort or significant dysfunctions to the patient [4-10]. Individuals with OCDs often present problems with sexual functioning, and 6% of them report the presence of ED, while 39% are dissatisfied with their sexual functioning [43,44]. The specific treatment for OCDs may also play an important role in the onset of ED, starting from tricyclics to SSRIs, especially because this disorder may require higher doses of drugs than MDD or anxiety disorders [44,45].

Body dysmorphic disorder (BDD) is considered the result of a preoccupation with self-perceived defects in physical appearance, which are not observable by others, or are considered minor when evaluated objectively [4-10]. ED was more commonly reported in men with BDD vs. the control group, although libido, frequency of intercourse, and masturbation were similar in the two groups [46]. Patients with BDD had more concerns about their penis size and tried to use different methods to increase it [46].

Post-traumatic stress disorder (PTSD) occurs following exposure (direct or indirect) to life-threatening or severe physical trauma or sexual violence, which translates into intrusion symptoms, avoidance of trauma-related stimuli, negative alterations in cognitions and mood, and alterations in arousal and affectivity [4-10]. The risk of erectile dysfunction in PTSD patients was significantly higher than in the general population [47]. PTSD was associated with overall sexual functioning, sexual desire and satisfaction, and sexual distress [47]. Avoidance and negative alterations in cognition/mood were associated with a higher risk for sexual dysfunctions in this population [47]. Psychotropic medication for patients with PTSD was not significantly associated with the risk of erectile dysfunction [48].

The presence of a defined sexual dysfunction does not exclude the existence of comorbid disorders from the same category. This observation is supported by the results of a study in which 86% of the respondents to an online questionnaire who had a sexual dysfunction also reported the presence of another sexual condition [49]. Men with one severe sexual dysfunction were significantly more likely to have such comorbidities [49]. ED and premature ejaculation were comorbid in 23-29% of cases explored in this study [49].

Substance use disorders may frequently cause the onset of sexual dysfunctions. Alcohol, opioid derivates, cannabis, and ecstasy were associated with higher scores on tests of sexual dysfunction vs. controls in a clinical study (N=101 vs. 43 participants) [50]. The association was not significant for alcohol and cannabis ($p>0.05$), but opioid and ecstasy users had significantly higher scores on the International Index of Erectile Function (IIEF) vs. controls [50]. Patients with alcohol dependence have a 72% rate of being diagnosed with one or more sexual dysfunctions, the most common being ED, reduced libido, and premature ejaculation, according to another study that enrolled 100 male subjects admitted to a detox center [51]. Additionally, illicit substances are sometimes used as aphrodisiacs to enhance sexual performance or pleasure. However, existing data show that most drugs have negative effects on erection, libido, and

ejaculation latency [50,51]. The data on cannabis use and its relationship with sexual functioning is controversial, because both men and women perceived this compound may increase their sexual functioning and satisfaction, especially desire and orgasm intensity [52]. Cannabinoids also have been associated with positive effects in a large variety of neurological disorders that may alter sexual functioning [53].

Pornography addiction or excessive use of online pornographic material represents a type of behavioral addiction with a potential negative impact on sexual functioning. There are several studies that have shown a high association between erectile dysfunction and excessive use of pornography, while other studies do not support this association [54-56]. Almost 60% of the patients with sexual dysfunction manifested when having intercourse with their partner, did not report this problem when using pornography [54]. However, a causal relationship between pornography and sexual dysfunctions does not exist, due to the lack of controlled studies and difficulties in designing such studies: sample biases, lack of clear diagnostic criteria, lack of validated instruments, the possibility that pornography abuse may be only a dimension of the more complex entity of sex addiction [54].

Cybersex is an excessive and uncontrolled use of online sexual activities leading to negative functional consequences [57]. Difficulties in forming intimate relationships have been associated with cybersex and pornography craving [58]. Men had higher scores for frequency of pornography online use than women and higher scores for craving the same materials [58].

Chronic organic conditions, such as diabetes mellitus (DM), blood hypertension, dyslipidemias, renal or hepatic failure, and endocrine pathologies (e.g., hypogonadism, hyperprolactinemia, hypothyroidism/hyperthyroidism) may all have an impact on sexual functioning [59]. Also, lifestyle factors such as smoking, chronic alcohol consumption, and excessive use of caffeine may be considered potential nocive stimuli on sexual functioning [59]. Neurological causes of ED that should be considered in the differential diagnosis are multiple sclerosis, spinal cord injuries, Parkinson's diseases, cerebrovascular accidents, etc., and the most common urological causes of ED are priapism, Peyronie's disease, and anatomical abnormalities [59]. The use of drugs like thiazides, spironolactone, clonidine, alpha-blockers, beta-blockers, antipsychotics, antidepressants, digoxin, ketoconazole, phenobarbital, etc. should be included among the potential triggers of ED [59]. Pelvic surgery and radiation therapy in the same area are other possible ED causes [59].

The pathogenetic factors of ED are traditionally split between psychological and organic, but this separation is artificial and, usually, there is mixed pathogenesis of this disorder in clinical practice (Figure 4). According to the results of a study focused on this distinction, in men ≤40 years old, approximately 85% had psychological causes for their ED and only 15% organic factors, while in men >40 years old, the rapport was 60/40 in favor of the organic causes [60]. Anxiety, depression, and relational conflicts were both causes and results of ED [60].

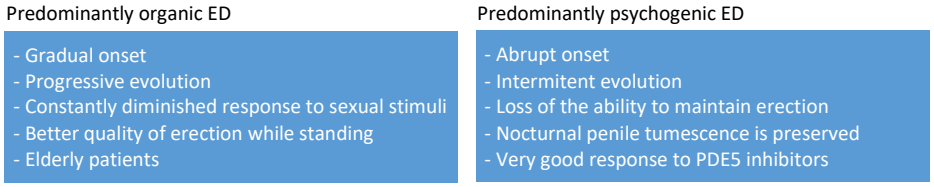

Figure 4: Indicators of psychogenic nature vs. organic causes of erectile dysfunction

Still, the dichotomous model of organic vs. psychological causes in ED is outdated, and the currently suggested multidimensional model, which includes vulnerability factors and precipitating factors, is preferred [61]. This model involves the participation of (1) immediate factors, such as performance anxiety, (2) personal history variables, like recent traumatic events/stressors, and (3) vulnerability factors in childhood and adolescence [61]. Additionally, there are some important elements that should be integrated into the initial evaluation of patients with ED, related to vulnerability: rigid pattern of education in childhood, dysfunctional intrafamilial relationships, uncertainty about gender roles during early phases of development, lack of sexual education, personality disorders [62-64]. Between the most commonly reported factors that contribute to the onset of ED, in combination with vulnerability factors, are a large variety of interpersonal stressors (e.g., dysfunctional marital relationships, extra-marital relationships), psychological reactions to organic diseases, recent physical or mental traumas, occasional failure of erection [61-63]. There are also a variety of elements that maintain sexual dysfunctions, such as performance anxiety, guilt, habituation, fear of intimacy, an untreated sexual dysfunction in the partner, low self-esteem, etc. [61-63].

Sensitivity to sociocultural aspects is needed when patients are evaluated for any sexual dysfunction. Perception of sexuality varies from one population to another, and the impact of social or professional stressors may vary across

different cultures [64,65]. Also, attitudes toward sex and the role of sexual activity are variables dependent on cultural context [65]. Gender roles are influenced by social and cultural factors, and they must be taken into account by practitioners when assessing patients with ED who are coming from another culture [64,65].

Studies exploring differences in sexual function and rates of sexual dysfunctions in homosexual and heterosexual populations are extremely few and their results are inconclusive [66]. ED in homosexual men was reported 1.5 times more frequently than in heterosexual individuals, according to a meta-analysis (n=4 studies) [66]. Homosexual men presented, in another study, a 45% higher risk of difficulties in obtaining an erection vs. heterosexual men [67]. Of these, 71% had little difficulty, 22% mild-moderate, 6% moderate, and 2% severe (according to IIEF-5 scores) [67]. Independent predictors for the presence of erectile dysfunction were age, a stable relationship, frequency of sexual activity with the partner, versatile sexual role, libido problems, ejaculation problems, and anodyspareunia [67]. Administration of phosphodiesterase-5 (PDE5) inhibitors was reported in 10% of homosexual men with ED, and 83% of them were satisfied with the effects of the medication [67]. Also, 43% used "legal drugs" to improve erectile dysfunction [67].

To conclude, the psychiatric evaluation of patients with ED involves the following steps: (1) formulating the diagnosis according to DSM-5 or ICD-11 criteria, based on an accurate collection of data on current mental and physical health status, personal medical history, and an assessment of vulnerability factors, triggers, and factors that maintain the sexual dysfunction; (2) verifying if there are concomitant sexual dysfunctions, besides ED; (3) exclusion of organic diseases, by recommending the adequate interdisciplinary and laboratory assessments; (4) formulating a therapeutic strategy, depending on the specific clinical aspects of the ED: psychotherapy, pharmacotherapy, or combined therapy. If non-psychogenic causes of ED or organic comorbidities have been identified, a multidisciplinary approach is recommended.

The psychological perspective

When evaluating a patient with possible ED, it is important to invite his partner to the initial interview, when possible, and to observe the dynamics of the couple's relationship [68]. The following aspects should be investigated during initial visits: fear of rejection, lack of satisfaction in the relationship, inappropriate sexual techniques, and sexual dysfunctions in the partner [68]. If one or more of the problems listed above are detected, the partner is invited to participate in psychotherapy [68].

However, there are also advantages to interviewing the patient alone: this way, it is possible to obtain a higher level of confidentiality, thus self-disclosure would be favored; various fantasies associated with guilt regarding his partner may be verbalized; aggressive sexual drives or traumatic personal experiences can be more easily revealed in the absence of the partner [68].

An example of structure for the first interview is the following: the couple can be interviewed for 10 minutes, to collect relevant historical data and establish the objectives of the assessment sessions; then each partner is interviewed separately, for 20 minutes; then the couple is brought together to formulate the conclusions [68]. Another assessment model considers the couple as the patient, and consequently, all assessments are made in the presence of both partners; the quality of the couple's relationship is a determining factor in the occurrence of erectile dysfunction and its resolution, therefore interviewing the couple is of paramount importance [68].

Forming an alliance with the patient is essential to a successful psychological interview, and to attain this result, the atmosphere must be safe, open, and non-judgmental; a strict agenda is not advisable, and flexibility is preferable [68]. The psychological assessment framework requires unconditional respect toward patients and an empathic, calm, and semantically adequate way to communicate with them [68,69]. The patient’s perspective on his own sexual dysfunction should be assessed and de-structured if based on myths or wrongfully understood scientific information [68,69]. The patient's personality needs to be assessed, as well as his core values, and attitudes towards life and others, his major existential goals, and the importance of relationships for the patient [68,69].

More specifically, the psychologist needs to assess the educational and cultural background of the patients, in order to formulate the questions and conclusions in an adequate manner; the main mechanisms of defense should be assessed, as well as his main coping strategies; gender identity disorders, sexual preferences, paraphilias, other sexual dysfunctions, childhood trauma (i.e., incest, rape, sexual or physical abuse) are essential elements to be evaluated; current level of functioning in all major areas is important to determine- actual relationships, professional performances, social support, relationships with nuclear family, hobbies; stressors in his current environment- bullying, mobbing, sexual harassment, discrimination, conflicts with peers; detailed analysis of the current sexual relationship; which are the main reasons for lack of satisfaction or failure in any functional domain?; dysfunctions in the main stages of the sexual cycle; factors that are associated with the onset and maintenance of the ED; how does it work a typical sexual intercourse for the evaluated

individual?; which are the possible factors that negatively interfere with the stages of sexual cycle?; personal sexual history and possible relationship with the current problems [69].

The structured assessment of ED symptoms and functional consequences is done using validated psychological instruments. Arizona Scale for Sexual Experiences (ASEX) was developed by the Department of Psychiatry and Psychology at the University of Arizona and the Department of Psychiatry and Behavioral Sciences at Stanford University [70]. This instrument is self-administered, but it can also be completed during an interview, by a mental health specialist [70]. It measures the following dimensions: sexual desire, sexual arousal, penile erection/vaginal lubrication, ability to achieve orgasm, and orgasmic satisfaction [70]. There are two versions, one for women and one for men [70]. Each item is scored on a six-point Likert scale, with higher scores indicating greater severity of the respective sexual dysfunctions [70].

The Brief Sexual Function Inventory (BSFI) is intended for the evaluation of sexual dysfunction for men only [71]. This inventory has 11 items scored on a Likert scale, which assesses 4 dimensions - sexual desire, the ability to achieve or maintain an erection, ejaculatory function, and satisfaction with one's own sexual activity [71]. Low scores on this scale indicate high severity of sexual dysfunction [71]. For erectile dysfunction there are 3 items, scored from 0 (no dysfunction) to 5 (severe/ persistent dysfunction): frequency of partial/complete erections in the presence of sexual stimulation in the last 30 days; the presence of erections consistent enough to allow sexual intercourse in the last month; difficulty getting an erection in the last 30 days [71]. The scale is self-administered, although it was originally created to be administered by the clinician [71-73].

The UKU scale (Udvalg for kliníske Undersogelser) is an instrument for the assessment of adverse effects of psychotropic medication, sexual adverse events included [74]. It has 48 items scored from 0 to 4 on a Likert scale included in its first section; the second section comprises the assessment of the functional impact of the reported adverse effects; the third part refers to the management of the respective adverse effects [74]. The items represent symptoms and clinical signs distributed by categories: mental (difficulties concentrating, drowsiness, depression, etc.), neurological (dystonia, stiffness, tremors, etc.), neurovegetative (diarrhea, constipation, palpitations, etc.), and other (pruritus, increased/weight loss, photosensitivity, etc.) [74].

The scale also includes 6 items dedicated to sexual functioning: increase/decrease in sexual desire, erectile dysfunction, ejaculatory dysfunction, orgasmic dysfunction, and lack of vaginal lubrication [74]. The scale enables global assessment of the functional impact these symptoms may have [74]. The causal relationship between the medication and the adverse effect is also evaluated as being improbable, possible, or probable [74]. Low scores on this scale mean reduced severity of the respective manifestation [74]. It is administered by a clinician, but there is also a simpler self-administered form (UKU-SERS-Pat) [74,75]. All items can be applied or only relevant ones can be selected; the full version is recommended for clinical trials [75].

International Index for Erectile Dysfunction (IIEF) is a multidimensional tool for assessing sexual dysfunction and comprises 5 factors - erectile function, orgasmic function, sexual desire, sexual satisfaction, and overall satisfaction [76]. These factors result from 15 items formulated as questions, referring to erection frequency, erection firmness, penetration capacity, erection maintenance frequency, sexual satisfaction, etc. [76]. Items are scored from 0-5 and refer to the manifestations detected in the last few weeks; the instrument is self-administered [76]. The scores were used as indicators of the possibility of initiating sildenafil administration (scores below 14 out of a maximum of 30 in the "erectile dysfunction" domain) or psychosexual counseling (scores between 14 and 25 in the same domain) [76]. IIEF version 1 is only administrable to heterosexual men, but IIEF version 2 is applicable to both heterosexual and homosexual men [76,77]. A shortened 5-item version was also in use (IIEF-5), with a total score between 0 and 30; a high severity of dysfunction is present at scores 0–10; scores below 26 indicate the presence of some degree of erectile dysfunction [78].

Erectile Firmness Scale (EHS) contains only one item and is a self-administered instrument [79]. It rates the firmness of the erection from (0) (absent) to 5 (complete) and it is used in clinical trials to determine response to medication [79].

Still, the most important tool for the psychological assessment of patients with sexual dysfunctions remains a detailed history focusing on the history of sexual development. Aspects related to the partner's perception of sex life are essential for building an accurate picture of sexual dysfunction. All the scales previously mentioned are in no way meant to be a substitute for an accurate collection of personal history and psychological examination.

However, these instruments help the psychologist in detecting ED symptoms when patients are embarrassed to talk about the subject, and they are also useful for the monitoring process (i.e., effectiveness or tolerability of a drug or the efficacy of psychotherapy). They may also be needed for screening purposes in vulnerable populations. The main limitations of the scales are the lack of cross-cultural validation and possible

differences in symptom reporting based on patient age [80].

The psychotherapeutic approach

According to the results of a review [60], the most frequently therapeutic intervention for ED is sexual therapy (50%), followed by cognitive-behavioral sex therapy (33%), while other types of psychosocial approaches have an equal representation (16%) – cognitive-behavioral therapy, group therapy, and couple therapy.

The psychotherapies dedicated to ED are derived from various interpretative models of this disorder. For example, the cognitive model of ED states that explicit/implicit requests for sexual performance lead to an expectation of performance (e.g., messages received from the partner create the expectation of an erection) [81]. In normal conditions, the attention of the individuals is focused on erotic stimuli, which increases the vegetative excitation, and favors the onset of erection (positive feedback); on the contrary, negative feedback involves catastrophic expectations, lack of perceived control over own body, focus of attention on partner's opinion about a potential failure or on other, non-sexual, stimuli, which increases the vegetative activation and focus on negative consequences, thus leading to ED [81].

The cognitive-emotional model of ED is based on a presumed occasional failure to obtain an adequate erection (no triggers are identified); following this random event, the individual activates cognitive schemata focused on personal incompetence and the misconceptions about the "macho" role; ruminations about future failures and an internal pressure to perform better next time is the following step; possibly as a defense mechanism that tries to protect the individual against a future failure, thoughts and images related to sexual activity disappear, therefore the stimulation decreases; the final stage is the "natural" consequence of the previous steps, and consists in ED's onset [82].

The main questions that help the therapist to formulate a treatment plan are: "What kind of phantasies and related emotions are associated with the current partner?", "Who has the initiative or how does the engagement in sexual activity appear?", "Is achieving sexual arousal within the current relationship and in general an easy process or a difficult one?", "How does the patient consider he can satisfy his partner and how does the partner perceive the satisfaction she receives?" [83]. Also, the therapist should explore the ability of an individual to achieve orgasm and under what conditions is it achieved; to verify what happens when one of the partners does not reach orgasm, and if this becomes a source of relational conflict; which is the definition of satisfactory sexual activity the same for both partners; to determine the level of satisfaction with sex life and with life in general in both partners [83].

According to other therapists specialized in sex-marital therapy, the specialist must keep detailed reports on the patient's status at each visit and of the suggested techniques; the therapist offers operationalized definitions of the problems he/she wants to elaborate on; the therapist evaluates the global functioning of the patient, not only of his sexual function; the therapist schedules monitoring visits at pre-determined intervals [84].

The PLISSIT model comprises "permission", i.e., the patients want to be considered "normal people", not "perverted", "deviant", "sick" or "abnormal"; often they are not disturbed by a certain behavior, but by the thought that they might do something bad or wrong; therefore, reassurance of normality is important for fantasies, thoughts, and dreams (e.g., those related to other people, or homosexual phantasies), for anxiety that can be associated with sexual stimulation (e.g., the appearance of spontaneous erections in inappropriate conditions); there are, of course, limits to validation, like fantasies with extreme sadism, pedophilic drives, etc. [85]. "Limited Information" is another core concept of this model that refers to the fact that clinicians provide limited information on relevant aspects of sexual concerns presented by the patient; the information is adjusted to the particularities of the patient, avoiding unnecessary scientific terminology and over-generalization; it is considered helpful in combating sexual myths [85]. "Specific Suggestions" refer to the fact that the therapist should formulate her/his suggestions according to the specific information obtained from the patient; these are direct attempts to change problematic behavior; these suggestions take place within the theoretical framework of short therapy [85]. "Intensive Therapy" includes specific techniques of sexual therapy [85]. During psychotherapy, the patient is encouraged to elaborate on the topic of his sex life [85]. The psychotherapist's attitude is one of empathetic listening and guiding the interview toward areas of interest in a discreet, non-confrontational manner [85]. The therapist is non-critical, normalizes, and helps validate the patient's emotions [85].

A frequently used technique in sex therapy is sensation focus [86]. These exercises progress from non-genital to genital tactile stimulation, with the instruction for the patient to refrain from vaginal penetration; thus, the patient's anxiety related to the sexual act will be reduced (desensitization), and the partner can be stimulated even in the absence of focusing on the genital area; after the patient has regained the confidence in himself, by managing to focus his attention on bodily sensations, penetration can be re-introduced into the therapy plan, gradually; it is recommended to formulate as objectives sensory focus sessions, 2 times a week, 10 minutes

each; by changing roles between partners, each of them being active and passive, the patient gains experience in giving and receiving sexual stimulation [86].

Cognitive therapy and sexual fantasy training involve masturbation using guided imagery to regain control over erection, coupled with cognitive restructuring; homework includes scoring the satisfaction obtained by the patient and his partner [86].

Psychoeducation targets the increase of patients' level of information on the phases of the sexual cycle and encourages open discussions about sexuality [87]. This psychoeducational approach may have as an objective the modification of sexual scripts, which largely depend on social myths and misperceptions about sexual functioning [87].

CONCLUSIONS

The etiology of ED is usually mixed and, as a result, a multidisciplinary approach to both its diagnosis and treatment is recommended. Therefore, this review supports the conclusions of currently available guidelines for sexual dysfunctions, that a multidisciplinary, pharmacological, and psychosocial treatment is superior to monotherapy [86-88]. It is important for mental health specialists to approach the subject of ED in an empathic, non-conflictual, and non-judgmental manner, in order to obtain the information needed not only for the diagnosis but also for the formulation of the therapeutic objectives.

The main factors to be approached during the initial interview are the circumstances of the ED onset, vulnerability factors and triggers, sexual orientation of the patients, gender-related problems, and personal history of trauma [86]. Questionnaires and structured interviews are useful both initially and during the therapeutic process, as a way to document the evolution of ED symptoms. These assessment methods are selected according to the specifics of the case, but a combination of unstructured, structured interviews and scoring techniques using a validated clinical tool is preferred.

There are multiple psychometric methods for evaluating the severity of erectile dysfunction, quality of life, general functionality, etc., and the mental health specialist is the one who selects the most appropriate tools for each individual patient based on objective (severity of symptoms, functional impact), but also on subjective factors (personal experience). The stages of evaluating a patient with ED are flexible but generally involve a stage of constructing the psychiatrist/ psychologist/psychotherapist-patient relationship, describing the objectives and methods used for the evaluation, then a stage of investigating the sexual history, and a phase summarizing the collected data, which paves the way to a therapeutic solution. The desired outcomes of the therapy should be framed around satisfaction, pleasure, and quality, rather than quantity [86]. Directive and non-directive techniques can be combined depending on the particularities of the case. For an experienced mental health specialist, cultural, social, or psychological "barriers" can not represent major obstacles in the relationship with the patient. These aspects are discussed and re-interpreted through the lens of the objectives established by mutual agreement with the patient. The clinician is attentive to cultural, social, and educational details and integrates the patient's values into his/ her discourse. Active listening is essential to obtain "sensitive" information and to create an appropriate therapeutic framework.

Conflicts of interest and sources of funding

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Authors' contribution

OV conceived the idea of the review. OV, AGM, BMP, CAC, CFP and DU designed the study. OV, CFP, AGM, CAC, CFP, DU, MD, DGV, CN and CAS screened the studies during the primary search. OV, AGM, BPM and CAS drafted the first version of the review. OV, CAC, CFP, DU, MD, DGV, CN and CAS revised the manuscript and elaborated the conclusions of the review. All authors had full access to all data contained in the review and decided to submit the manuscript for publication.

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ORIGINAL ARTICLE

Management of Urinary Dysfunction in Patients with Multiple Sclerosis

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Abstract: *Besides the motor and sensory dysfunction that characterizes multiple sclerosis, during the disease evolution and with the accumulation of new demyelinating lesions in specific areas of the central nervous system, autonomic dysfunction be-comes more prevalent among patients suffering from multiple sclerosis. Although not usually addressed, the complex spectrum of autonomic dysfunction manifestations has the potential of a negative impact on the daily activities of multiple sclerosis patients, needing specific investigations and therapeutic measures destined to alleviate symptoms and increase the quality of life. Since urinary dysfunction is evident from the early stages of multiple sclerosis on urodynamic studies even in patients without urinary complaints, it is indicated to thoroughly screen for urinary tract dysfunction as well as other autonomic dysfunctions in all the multiple sclerosis patients.*

Keywords: *autonomic dysfunction, urinary dysfunction, multiple sclerosis*

INTRODUCTION

Multiple sclerosis (MS) is one of the most common causes of disability in young patients. Clinically it does manifest uniquely for every patient, but depending on the site of the demyelinating lesions, some symptoms have a higher frequency in different combinations, particularly sensory symptoms, motor weakness, and bladder dysfunction. Most commonly, patients will present with numbness, tingling, diplopia, Lhermitte sign, and motor weakness, that last for at least 24 hours and may or may not resolve completely. Many patients may have residual symptoms, heat sensitivity with worsening of symptoms in case of increased body temperature, known as Uhthoff's symptom [1–3].

AUTONOMIC DYSFUNCTION IN MULTIPLE SCLEROSIS

As the disease follows its course over the years, inevitably patients will also experience damage to the autonomic nervous system with the involvement of both sympathetic and parasympathetic parts in different proportions. The promoters of the autonomic dysfunction appear to be lesions located in the nuclei of the periventricular regions of the fourth ventricle, medulla, but it was also seen in patients who presented cervical spinal cord atrophy with or without demyelinating lesions, suggesting that autonomic dysfunction may also result from axonal depletion [4].

Although severely overlooked, autonomic dysfunction has a great impact on the quality of life of patients suffering from multiple sclerosis, the most commonly addressed issues being bowel and bladder disturbances, which tend to be more bothersome in the case of longer disease, greater disability, or recent relapses [5].

Approximately 50% of patients experience orthostatic intolerance manifested by dizziness, loss of postural tonus, and tachycardia while transitioning from a supine to an upright position or in case of prolonged standing. The impaired sympathetic vasoconstrictor reflex responsible for maintaining

normal blood pressure leads to an increased frequency of orthostatic hypotension among those patients[4].

Sleep disturbances in patients with multiple sclerosis are not very well studied, the most common symptom being difficulty initiating and maintaining sleep. There are several factors like muscle spasms, trigeminal neuralgia, restless legs syndrome, anxiety, depression, and side effects of medication used to treat fatigue, that contribute to insomnia, which can be searched for and addressed [2,4].

Another common but less addressed pathological aspect of multiple sclerosis is the sexual dysfunction that can be a consequence of multiple lesions including spinal cord lesions of the motor and sensory pathways and can be aggravated by the urinary incontinence and the spasticity accompanying upper motor lesions. About 50% of MS patients tend to become sexually inactive during the disease, with consequences like decreased libido, impotence, and infertility [2,5,6].

Bowel dysfunction is present in up to 50% of MS patients, about 43 % of them will complain of constipation partially due to disease exacerbations, and partially to decreased ambulation and certain medications that may alter bowel movement. A great proportion of patients with bowel dysfunction will present fecal incontinence as a consequence of impaired external anal sphincter function [2,4,7].

UROLOGICAL DYSFUNCTIONS

The most frequent autonomic dysfunction present in patients suffering from multiple sclerosis is bladder dysfunction, the prevalence of neurologic lower urinary tract dysfunction varies between 65 to 92% [8]. Approximately 75% of patients report bladder dysfunction secondary to a storage phase dysfunction leading to incontinence, or the voiding phase dysfunction leading to urinary retention and incomplete bladder emptying [2,4]. Bladder control requires complex coordinated functions of the cerebral cortex, pontine and sacral micturition centers, and the integrity of the peripheral nervous system [6]. The demyelinating lesions localized at the spinal cord level disrupt the fiber connections from the pontine micturition center to the parasympathetic sacral micturition center causing the silent C fibers, usually insensitive to bladder filling in normal conditions, to be hypersensitive leading to detrusor hyperactivity [9,10]. Spinal cord centers activate storage reflexes in the filling phase of the bladder, while the brain centers of micturition are responsible for the act of urine output [4,11]. Detrusor overactivity, which translates in clinical terms to symptoms like urgency to void that occurs at a lower bladder volume than normal, frequency or urge and incontinence are the leading complaints. On the opposite side lies detrusor sphincter dyssynergia described as detrusor contraction without urethral sphincter relaxation, which is responsible for the impossibility of bladder emptying causing symptoms like hesitancy, or interrupted stream. While the former urologic abnormality is caused by demyelinating lesions of the frontal cortex, the second one is a consequence of lesions of the pontine centers of micturition or lesions above the sacral parasympathetic centers [2,6,12]. Unlike spinal cord lesions due to multiple sclerosis, spinal cord injuries, even in patients with relatively preserved neurological function, are characterized by universal bladder dysfunction, meaning that there is an impairment of both sensation of bladder fullness as well as the motor control of bladder and sphincter [6,11].

For many patients, urological symptoms arise only in the later stages of disease progression with a proportion of over 50% of patients with severe MS presenting severe bladder symptoms, whereas only 5-10% of MS patients have mild bladder abnormalities at the beginning. Nevertheless, a retrospective study conducted in Ankara, Turkey demonstrated that 52% of their multiple sclerosis patients without urological complaints had altered urodynamic results which could be explained by a subclinical impairment [13,14].

Patients with overactive bladder syndrome usually complain of urinary urgency, increased daytime frequency or nocturia, and sometimes incontinence in the absence of urinary tract infections or other associated pathology that could explain the symptoms. Obstructive symptoms are also an important point of complaint especially when leading to urinary tract infections. These manifestations can be associated and can have a huge impact on patients' quality of life in terms of decreased quality of sleep, decreased work productivity, and depressive symptoms [15,16].

Urine retention is a consequence either of neurogenic underactive bladder or of detrusor sphincter dyssynergia causing bladder obstruction of urine flow, leading to frequent lower urinary tract infections that can be another cause of disease relapse through the autoimmune process which it sets off [9,17,18]. Regarding the complications of the upper urinary tract there have also been mentioned vesica-urethral reflux, hydronephrosis, and end-stage kidney disease have as consequences of the morphological changes in the bladder wall caused by high intravesical pressures [16,17,19–21].

EVALUATION OF LOWER URINARY TRACT SYMPTOMS

Urological evaluation should be specifically made for every patient considering their disease type and duration, the level of disability on the EDSS scale, comorbidities, and current medications especially opiates, antipsychotics, and

antidepressants, starting with a complete history taking. The clinical dialogue should be directed towards describing urinary symptoms like frequency, urgency, hesitancy, retention, incontinence, and nocturia and the impact these symptoms have on the quality of the patient's life [16,22]. A bladder diary can provide additional information about the patient's habits of fluid ingestion and the exact frequency of bladder emptying [9].

Regarding physical examination, besides local inspection and examination of pelvic and genital organs including sacral cord mediated reflexes (bulbocavernosus, anal reflex), the neurologist should look for pontine, cerebellar as well as pyramidal signs which are indicative of lower urinary tract symptom severity [16,22]. For an initial evaluation of patients with multiple sclerosis, a bladder and renal ultrasound and a questionnaire for assessing overactive bladder and voiding dysfunction are in order, while for patients with red flags (recurring urinary tract infections, immunosuppressive treatment, EDSS >6, male patients, age >55) should be referred to a neuro-urology clinic or specialist [23].

Furthermore, some investigations can help us better understand the nature of the urological dysfunction. Firstly, all patients complaining of urinary symptoms should undergo a urinalysis and urine culture to assess the presence of a possible urinary tract infection as well as serum creatinine. Ultrasonography can allow us to measure the post-void residual volume, and it can be done at different times a day for better quantification of the bladder emptying pattern. Patients with a known high risk of upper urinary tract disease should benefit from periodical ultrasonography examinations for early detection of upper urinary tract dilation. Since a high post-void residual volume is not enough to distinguish between detrusor contractility problems and obstruction, urodynamic studies are required. Urodynamic studies represent a combination of cystometry and pressure flow study, useful in evaluating the outlet function and compliance of the detrusor and bladder. It can be used as a prognostic tool as well as for guiding the best treatment approach. In the case of hematuria, a urethrocystoscopy evaluation is required to rule out bladder tumors, bladder stones, and urethral stricture [16,20].

TREATMENT APPROACHES IN MULTIPLE SCLEROSIS-ASSOCIATED URINARY DYSFUNCTION

Physical therapy

One of the areas worth addressing is physical therapy, which in this case refers to physical exercises like pelvic floor exercises alone or combined with intravaginal electrical stimulation. Cognitive behavioral therapies may be helpful in some patients in trying to develop a bladder voiding schedule according to each patient's natural voiding pattern which combined with restricted water intake before bedtime can improve sleep quality and reduce complaints like fatigue [16].

Medical treatment

Regarding the drugs efficient for urinary dysfunction, there are several options depending on the main cause of dysfunction, that can be assessed by urodynamic studies. The first treatment line for neurogenic bladder is antimuscarinic drugs (oxybutin, tolterodine). Anticholinergics can alleviate complaints of neurogenic bladder as well as phosphodiesterase inhibitors and desmopressin which can be used as an alternative to antimuscarinics or as an adjuvant [6,20]. In an observational prospective study on the bladder and urodynamic alterations in patients with multiple sclerosis, the degree of urinary symptom severity decreased following symptomatic urological medication by lowering the bladder pressure, improving muscle spasms, and reducing pain caused by urine storage [24].

In case of bladder outlet resistance, autonomic dysreflexia, and postvoid residual volume, alpha-blockers (prazosin, terazosin) can help sphincter relaxation by lowering bladder pressure during contraction, but administered with caution because they can aggravate hypotension [6,20,25].

In patients with hypotonic bladders, cholinergic medications (bethanechol) may help empty the bladder, while in patients with pathologic relaxation of the vesical sphincter, alpha-adrenergic medication may increase bladder storage [6,17].

Minimally invasive treatment

Catheterization, self-catheterization, or third-party catheterization is the preferred method for patients who cannot otherwise empty their bladder. Patient medical education in managing a sterile intermittent catheterization can help reduce complications like urinary tract infections or bacteriuria. The habit of keeping a bladder diary can also help choose the frequency of catheterization so that the volume of the bladder at catheterization does not exceed 400-500 ml. As short-term methods, patients can dispose of condom catheters and adult diapers.

Botulinum toxin injections in the detrusor cause reversible chemical denervation with effects lasting for approximately 9 months, but also with known side effects like urinary tract infections, elevated postvoid residual volume, and increasing need for intermittent catheterization. In the case of bladder neck and urethral obstruction, to reduce the outlet resistance and to protect the upper urinary tract, patients may benefit from chemical denervation of the detrusor sphincter using botulinum toxin [6,17,20].

Surgical treatment

Sphincterotomy for reducing the bladder outlet resistance without completely losing the closure of the urethra comports the risk of secondary narrowing of the bladder neck and needs to be repeated at regular intervals. Bladder augmentation is indicated in patients with overactive or low compliance bladder when conservative methods have not been beneficial [20]. Urethral stents, sphincterotomy, and electrical implantation devices can be taken into account in selected cases, but with limited evidence of efficacy [6,26].

CONCLUSIONS

Multiple sclerosis is a central nervous system inflammatory demyelinating disease with multiple clinical manifestations, including autonomic dysfunctions. The most common and the most addressed is bladder dysfunction, but it is only a small part of the many bothersome effects of multiple sclerosis lesions, hence the need to evaluate and treat accordingly to ensure a higher quality of life for patients.

Conflicts of interest and sources of funding

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Authors' contribution

Conceptualization, C.G.L., C.A.S. and D.M.; methodology, V.I.V., R.A.A., F.A.A. and A.M.R..; resources, D.M., C.A.S., and F.A.A..; data curation, C.G.L., V.I.V., and A.M.R.; writing—original draft preparation, C.G.L., C.A.S., F.A.A., and A.M.R..; writing—review and editing, V.I.V., and R.A.A. All authors have read and agreed to the published version of the manuscript.

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ORIGINAL ARTICLE

Chronic Pulmonary Sequels on CT Scans of Patients with COVID-19

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Abstract: *Background: Lung tissue changes following COVID-19 may persist for a long time after discharge and recovery, but not enough information is available about these chronic pulmonary sequels. We addressed to evaluation of chronic pulmonary sequels on CT scans of patients with COVID-19. Methods: This cross-sectional study was conducted on 94 patients with COVID-19 who were evaluated by CT scan at the time of discharge and also underwent continuous evaluation by CT scan of the lungs for at least three months. Through reviewing the hospital archive files, patients' backgrounds and three-month follow-up information were collected. Results: Chronic pulmonary involvement during the follow-up of patients was found in 83%. Peripheral and diffuse involvements were revealed in 79.8% and 3.2% respectively. Also, single-lobar and multi-lobar involvements were found in 69.1%% and 13.8% respectively. In terms of abnormal lung findings on CT scan, ground-glass opacity was found in 20.2%, Crazy-paving pattern in 2.1%, reticulation in 3.2%, parenchymal band in 12.8%, consolidation in 1.1% and bronchiectasis in 3.2%. The mean CT score was also 4.21 ± 3.45. The appearance of findings in CT patients was strongly influenced by the patients 'age, body mass index, underlying comorbidities, and also, the severity of initial COVID-19. Conclusion: Within at least three months of disease recovery in hospitalized patients with COVID-19, evidence of pulmonary involvement in 83% in the form of chronic pulmonary sequels can be expected. The most common findings related to pulmonary involvement included GOO in 20.2% and parenchymal band in 12.8% of cases.*

Keywords: *Chronic pulmonary sequels, CT scan, Covid-19*

INTRODUCTION

Although several effective vaccines with high immunity have been developed to prevent the spread of COVID-19, preventing its transmission is now believed to be the only way to combat the disease. In recent months, much effort has been published to explain the epidemiology, clinical manifestations, and clinical features and imaging of COVID-19 [1-4]. In this regard, the occurrence of fever and dry cough are the first and most common symptoms of the disease [5]. However, these symptoms are similar to those of other infections, such as the flu A and B, making it difficult to diagnose. Also, laboratory evaluation in patients is often time-consuming and its diagnostic kit may not be available for homogeneity. On the other hand, laboratory tests are sometimes associated with high levels of false negatives. In this regard, imaging plays a key role in the diagnosis and management of COVID-19-induced pneumonia. CT scan is the first diagnostic modality in patients with suspected disease and also a desirable protocol in following the effect and treatment process in these patients [6]. Especially in patients with a negative RT-PCR result, CT scan evaluation can be very useful [7,8]. Various studies have evaluated and described radiographic changes in the acute phase of COVID-19 disease and have correlated these changes with the severity of clinical manifestations [9-11]. Recent studies have also shown that about 94% of hospitalized patients still experience pulmonary parenchymal changes at discharge [12, 13]. In addition, some have shown that pulmonary opacities persisted in 53% of patients with mild COVID-19 without developing negative and complicating scaffolds for up to three weeks after discharge and clinical recovery [14]. Therefore, it seems that lung tissue changes, even the occurrence of pulmonary fibrosis, may persist for a long time after discharge and recovery of patients [15-17]. But not enough information is available about these chronic pulmonary sequels. What we addressed in the present study was the evaluation of chronic pulmonary sequels on CT scan of patients with Covid-19.

MATERIALS AND METHODS

This cross-sectional study was conducted on patients with COVID-19 who were admitted to our medical center during 2020 and 2021 and were evaluated by CT scan at the time of discharge and also underwent continuous evaluation by CT scan of the lungs for at least three months after treatment and recovery. Initially, through reviewing the hospital archive files, patients' background information including sex, age, history of underlying diseases, body mass index (BMI), and clinical characteristics of patients were collected. Also, in the initial CT scan of patients (during hospitalization) and also at the time of follow-up of patients (at least three months after discharge) and by an experienced radiologist, a complete interpretation of pulmonary involvement patterns was provided in terms of form and extent of involvement and CT score and classified according to the underlying characteristics of the patients. The severity of COVID-19 was stratified into four types mild, moderate, severe, and critical on the basis of patients' symptoms, laboratory results, and imaging findings at admission as previously described [18]. Pulmonary involvement was also assessed based on the type of lesion (peripheral or diffuse), the pattern of lobar involvement (single-lobar or multi-lobar), and the type of pulmonary lesions. To determine the CT score, each of the five lung lobes was visually scored on a scale of 0 to 5, with 0 indicating no involvement and 5 indicating more than 75% involvement. The total CT score was determined as the sum of lung involvement, ranging from 0 (no involvement) to 25 (maximum involvement) [19].

For statistical analysis, results were presented as mean ± standard deviation (SD) for quantitative variables and were summarized by frequency (percentage) for categorical variables. Continuous variables were compared using the t-test or Mann-Whitney test whenever the data did not appear to have normal distribution or when the assumption of equal variances was violated across the study groups. The categorical variables were compared using the Chi-Square test or Fisher's exact test if required. P values of ≤ 0.05 were considered statistically significant. For the statistical analysis, the statistical software SPSS version 23.0 for Windows (IBM, Armonk, New York) was used. Interventional studies involving animals or humans, and other studies that require ethical approval, must list the authority that provided approval and the corresponding ethical approval code.

RESULTS

A total of 94 patients were evaluated and followed up for at least three months after discharge. The average age of patients was 53.02±4.86 years ranged 40 to 62 years and 59.6% were male. The mean BMI was also 25.41±1.91 kg/m^2 and 5.3% were obese. Regarding comorbidity, 22.3% were hypertensive, 13.8% were diabetics, and 6.4% had a history of ischemic heart disease. With respect to disease severity, 4.3% were classified as mild, 86.2% as moderate, 6.4% as severe, and 3.2% as critical.

First, there was no evidence of pulmonary involvement in CT scans in 17.0% and therefore the frequency of chronic pulmonary involvement during the follow-up of patients was 83%. In terms of how the lesions were spread, 79.8% of the involvement was peripheral, and only 3.2% was diffuse. Also, single-lobar and multi-lobar involvements were revealed in 69.1%% and 13.8% respectively. In terms of abnormal lung findings on CT scan, GOO was found in 20.2%, Crazy-paving pattern in 2.1%, reticulation in 3.2%, parenchymal band in 12.8%, consolidation in 1.1% and bronchiectasis in 3.2%. The mean CT score was also 4.21 ± 3.45.

As shown in Table 1, the patterns of chronic pulmonary sequels in patients suffering from COVID-19 were independent of patients' gender. However, the pattern and distribution of pulmonary involvements were different in the younger and the elderly, and the diffuse and multi-lobar patterns were more found in older adults. Also, abnormal lung lesions were more prevalent in older patients. Totally, the mean CT score was significantly higher in older adults than in the younger. Similar patterns were revealed in obese as compared to non-obese patients and thus obesity was found to be another factor predicting more severity of chronic sequels on CT. As shown in Table 1, the presence of comorbidities was in line with more severe pulmonary expansion of abnormal lesions. Also, as indicated in Table 2, we showed diffuse and multi-lobar lesions as well as a higher rate of pulmonary lesions in those with more severe initial COVID-19 disease. There was also a significant association between CT score and disease severity (Figure 1).

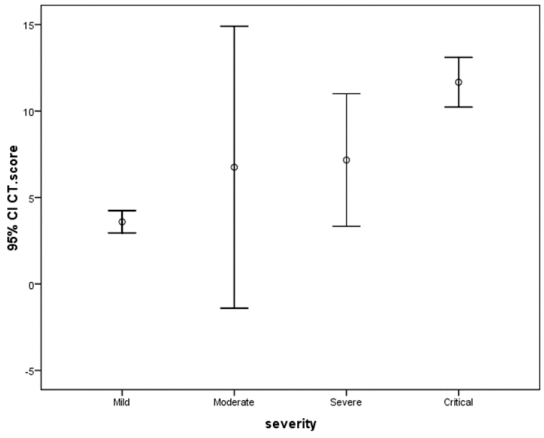

Figure 1: The correlation between CT score and severity of primary Covid-19

Table 1: Status and pattern of pulmonary involvement according to underlying factors

Item	Gender		Age		Obesity		Morbidity	
	Male	Female	≤50	>50	Obese	Non-obese	Present	Absent
Distribution								
Peripheral	76.8	84.2	63.4	92.5*	50.0	81.1*	78.3	80.3*
Diffuse	3.6	2.6	0.0	5.7	50.0	1.1	13.0	0.0
Pattern								
Single-lobar	64.3	16.1	53.7	81.1*	50.0	70.0*	52.2	74.6*
Multi-lobar	76.3	10.5	9.8	17.0	50.0	12.2	39.1	5.6
GOO	17.9	23.7	17.1	22.6	50.0	18.9*	43.5	12.7*
Crazy-paving	3.6	0.0	0.0	3.8	25.0	1.1*	8.7	0.0*
Reticulation	3.6	2.6	0.0	5.7	50.0	1.1*	13.0	0.0*
Parenchymal band	14.3	10.5	9.8	15.1	0.0	13.3	26.1	8.5*
Consolidation	1.8	0.0	0.0	1.9	0.0	1.1	4.3	0.0
Bronchiectasis	3.6	2.6	0.0	5.7	0.0	3.3	13.0	0.0*
Mean CT score	4.62±3.49	3.61±3.33	3.37±3.47	4.78±3.32*	7.25±4.92	4.08±3.34*	5.83±3.97	3.69±3.11*

Table 2: Status and pattern of pulmonary involvement according to disease severity

Item	Mild	Moderate	Severe	Critical	P value
Distribution					0.001
Peripheral	100	80.2	100	0	
Diffuse	0	0	0	100	
Pattern					0.001
Single-lobar	77.8	25.0	16.7	0	0.001
Multi-lobar	2.5	75.0	83.3	100	0.001
GOO	9.9	75.0	83.3	100	0.001
Crazy-paving	0	0	0	66.7	0.001
Reticulation	0	0	0	100	0.001
Parenchymal band	50.0	7.4	50.0	33.3	0.201
Consolidation	0	0	0	33.3	0.001
Bronchiectasis	0	1.2	16.7	33.3	0.004
Mean CT score	3.59±2.92	6.75±5.12	7.17±3.65	11.67±0.57	0.001

DISCUSSION

In the acute phase of Covid-19, different patterns of pulmonary involvement are examined in depth and the severity and extent of the disease are classified accordingly. But the question remains, how long will these involvements last after recovery from the disease, and in other words, will there still be evidence of lung involvement due to the disease months after the symptoms have improved? Obviously, the presence of these abnormal findings in the form of chronic scoliosis in CT scans of patients, weeks and months after discharge from the hospital can indicate the continuation of clinical manifestation of the disease and sometimes even exacerbation of these symptoms. What we did in the present study was the evaluation of chronic pulmonary edema on CT scans of patients with COVID-19 and related underlying factors. First, the study showed that for at least three months after treatment and recovery from COVID-19 disease, evidence of pulmonary involvement would still be expected in 83% of patients even after improvement in clinical symptoms. The most common findings of CT as chronic disease sequels included GOO patterns in 20.2% and parenchymal band in 12.8%. Other findings included reticulation in 3.2%, bronchiectasis in 3.2%, Crazy-paving pattern in 2.1%, and consolidation in 1.1% of patients. Therefore, even after about three months of the disease and the relative recovery, the occurrence of some evidence in CT patients with COVID-19, especially GOO, is still predictable. Additionally, in terms of the pattern of involvements, the lesions are mainly in the form of peripheral, and in most cases, they will be single-lobar involvements rather than multi-lobar or diffuse ones. As a second finding, we found that the appearance of these

findings in CT patients during the months following recovery from COVID-19 was strongly influenced by the patients 'age, BMI, underlying comorbidities (such as hypertension, diabetes, and history of ischemic heart disease) and also, the severity of initial Covid-19. In other words, the incidence of chronic pulmonary sequels in CT is more predictable in the months following recovery from COVID-19 at older ages, in obese individuals with underlying comorbidities, and in individuals with severe underlying COVID-19.

Various studies have reported the persistence of chronic pulmonary lesions in the weeks and months after discharge from the hospital, although due to differences in patients' underlying characteristics, different follow-up periods, and different intensities of underlying COVID-19 disease, CT scans were quite different during patient follow-up. In a study by Caruso et al and at 6-month follow-up, 72% of patients showed fibrosis-like changes and 42% of patients showed evidence of GOO [20]. In a study by Han et al., fibrotic-like changes were observed in 35% of cases, while 65% of cases either had completely clean lungs or had grand glass opacity or increased interstitial thickness. Age over 50, heart rate above 100 at admission, length of hospital stay over 17 days, respiratory distress syndrome, non-invasive mechanical ventilation, and CT scan score above 18 as factors associated with fibrotic-like changes over six months after discharge [21]. In another study by Dehan Liu et al during the first to third weeks, the GOO opacity and pulmonary fibrosis remained constant [22]. In a study by Safont et al., during a 180-day follow-up with a CT scan, 66% had chronic pulmonary lesions. Among this evidence, 68.3% had GOO and 38.4% had parenchymal bands [23]. In the study by Blanco et al., 19 patients with COVID-19 were evaluated by CT scan during a six-month follow-up period, in which only 48% had a normal CT scan [24]. Also, in the study of Hossein et al., 42.3% had pulmonary sequels in CT scan follow-up. The most common residual patterns in CT scans included GOO at 54.5% followed by grand glass mix at 31.8% and parenchymal bands at 13.7% [25]. Therefore, a wide range of findings related to COVID-19 can be seen in the weeks and months after recovery, sometimes without symptoms and sometimes with signs and symptoms associated with COVID-19. It seems that follow-up of these patients and simultaneous and continuous evaluation of lung CT scan findings along with clinical manifestations in patients will be necessary even after partial recovery and hospital discharge.

CONCLUSIONS

As a final conclusion, within at least three months of disease recovery in hospitalized patients with COVID-19, evidence of pulmonary involvement in 83% in the form of chronic pulmonary sequels can be expected. The most common findings related to pulmonary involvement included GOO in 20.2% and parenchymal band in 12.8% of cases. The presence of chronic pulmonary lesions in COVID-19 patients is strongly influenced by old age, obesity, the presence of underlying comorbidities as well and the severity of early COVID-19 disease.

Conflicts of interest and sources of funding

The authors declare no conflict of interest.

Acknowledgments

None.

Authors' contribution

Design and concepts: TFL, SK, MH, KO; Data gathering and analysis: TFL, SK, MH, KO; Preparing the manuscript: TFL, SK, MH, KO.

Ethics approval and consent to participate

The ethical committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran confirmed the proposal of this study with IR.SBMU.MSP.REC.1400.253 code.

Patient consent for publication

Informed consent was obtained from all subjects involved in the study.

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ORIGINAL ARTICLE

Investigation of Mutations in H19, IGF2, CDKN1C, KCNQ1, and NSD1 Genes in Iranian Children Suspected of Silver-Russell Syndrome (SRS) and Beckwith-Wiedemann Syndrome (BWS) with MS-MLPA

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Abstract: *Background: Silver-Russell Syndrome (SRS) and Beckwith-Wiedemann Syndrome (BWS) are two syndromes that are poorly diagnosed in many affected people due to mild and subtle symptoms, genetic complexity, and lack of familiarity with the hallmarks. Objective: The present study was conducted with the aim of determining mutations in H19, IGF2, CDKN1C, KCNQ1, and NSD1 genes in Iranian children suspected of SRS and BWS by the MS-MLPA method. Methods: In this case series study, which was conducted in 2022 in Pars Genome Laboratory, Karaj, Iran, 10 children suspected of SRS or BWS syndrome were included. These 10 Iranian children were referred by pediatric endocrinologists. 5 ml of peripheral blood was taken per patient for DNA extraction. MS-MLPA method was undertaken for possible mutations (methylation and deletion) in H19, IGF2, CDKN1C, KCNQ1, and NSD1 genes. Results: The interpretation of MS-MLPA results showed that out of 10 children (4 boys and 6 girls) suspected of having SRS or BWS syndrome (based on the pediatric endocrinologist's diagnosis), only 3 children were definitively diagnosed with SRS or BWS syndrome. Based on this, methylation changes in the promoter of ICR1 and ICR2, which are related to the genes H19, IGF2, CDKN1C, KCNQ1, and NSD1, lead to the development of SRS or BWS syndrome. Conclusion: The present findings showed that methylation changes in H19, IGF2, CDKN1C, KCNQ1, and NSD1 genes are associated with the occurrence of SRS or BWS syndrome. In this study, we show that MS-MLPA can serve as a rapid, inexpensive, and reliable method for the molecular diagnosis of these syndromes.*

Keywords: *Silver-Russell Syndrome (SRS), Beckwith-Wiedemann Syndrome (BWS), Methylation, MS-MLPA*

INTRODUCTION

Silver-Russell Syndrome (SRS) is a rare disorder characterized by intrauterine growth restriction, poor postnatal growth, macrocephaly, a triangular facial appearance, prominent forehead, body asymmetry, and significant feeding difficulties [1]. Patients can have a wide range of clinical findings. The majority of people with SRS have normal intelligence but have motor or speech delays. SRS is genetically heterogeneous, which means that different genetic abnormalities cause this disorder [2]. In around 60% of people with SRS, the disease is related to mutations in chromosome 7 or 11. Genetic defects that may be responsible for this syndrome include uniparental disomy of maternal chromosome 11P15.5, deletion of chromosome 7, or duplication of chromosome 7 or mutation of the KCNQ1, H19 (located on 11p15) and NSD1 (located on 5p35). However, in about 40% of patients with SRS, the cause is unknown. In general, the diagnosis of SRS is based on clinical findings. Since many symptoms are subtle and nonspecific, SRS remains difficult to diagnose [3]. Consensus guidelines for the evaluation and diagnosis of SRS have recently been published based on the Netchine-Harbison clinical scoring system for SRS [4]. Testing for known genetic causes of SRS (chromosomes 7 and 11) can confirm the clinical diagnosis in up to 60% of individuals. Knowing the main genetic cause can also help to select the appropriate treatment approach [5]. Treatment of this syndrome focuses on managing the symptoms [6,7]. SRS is present at birth and many symptoms improve as the patient grows [8].

Beckwith-Wiedemann Syndrome (BWS) is an overgrowth syndrome that affects certain chromosomes of the fetus. People with this syndrome may experience mild to severe signs and symptoms. One out of every 11,000 infants is affected by BWS, which is equally common in both boys and girls. This syndrome causes excessive growth of some body parts. BWS affects many areas of the body and makes the child appear significantly larger than their peers. Patients show different signs and symptoms depending on the affected organs [9,10], including macrosomia, hemihyperplasia, macroglossia, hypoglycemia in infancy, and sometimes long-term hypoglycemia (due to high insulin). The management of this syndrome focuses on its common symptoms and complications.

Physicians monitor and check affected children for low blood sugar and increase the frequency of feeding to obtain optimal blood glucose levels. There are several genetic causes of BWS, usually resulting in changes in one or more genes in a region of chromosome 11 known as 11p15 [11,12]. In most cases, there is genetic mosaicism. Other genetic changes in this syndrome include methylation defects in the region of 11p15, uniparental disomy (UPD) in 11p15, or mutation of the CDKN1C and IGF2. The diagnosis of BWS is clinical and it is often confirmed by genetic testing. To assay these abnormalities, a blood sample or other tissue samples should be taken [13,14].

In addition to organ growth defects, BWS patients are susceptible to other diseases, including cancer (such as Wilms' kidney tumor, hepatoblastoma, and neuroblastoma), heart disease (pericarditis, cardiomyopathy, etc.), hypoglycemia, hemihyperplasia, and digestive disorders [15]. Conducting investigations of H19, IGF2, CDKN1C, KCNQ1 (located 11p15), and NSD1 (located 5p35) genes in these syndromes and increasing information in this field can lead to a more accurate and precise diagnosis; preventing secondary diseases including cancer; and also be effective in preventing the transfer of these genes to the fetus before and during pregnancy [3,13,14]. Considering that no study in this field has been done in Iran, the present research aims to investigate mutations in H19, IGF2, CDKN1C, KCNQ1, and NSD1 genes in children suspected of SRS and BWS with Methylation-Specific Multiplex Ligation-Dependent Probe Amplification (MS-MLPA) method.

MATERIALS AND METHODS

Study procedures

In this case series study that was conducted in 2022 at Pars Genome Laboratory in Karaj, Iran, 10 children suspected of SRS or BWS were included. These 10 Iranian children were referred to us by a pediatric endocrinologist for final confirmation of the diagnosis. 5 ml of peripheral blood was taken for DNA extraction from these patients. MS-MLPA method was undertaken to identify mutations (methylation and deletion) in H19, IGF2, CDKN1C, KCNQ1, and NSD1 genes [16].

Inclusion criteria

The pediatric endocrinologists referred children under 12 years of age and clinically suspicious for the diagnosis of SRS or BWS. Written informed consent from parents and guardians of the children was documented.

Exclusion criteria

Children with multiple comorbidities

DNA extraction and purification

5 ml of peripheral blood was taken from each patient. DNA extraction from peripheral blood leukocytes was performed for all children using a DNA extraction kit (DENA Zist Asia, Cot.No:S-1031-1, made in Iran). Then, the quantity of extracted DNA was determined and confirmed with the nanodrop device, and the quality of the DNA samples was confirmed by using 1% agarose gel.

MS-MLPA

MS-MLPA probe mixture (ME030BWS/SRS) was purchased from the manufacturer (MRC-Holland, Amsterdam/NL).

This mix contains 42 probes, 26 of which are specific for the BWS/SRS region in 11p15. Ten of the 11p15 probes are methylation-specific since they contain a HhaI recognition site. Two probes for NSD1 and 13 reference probes (without HhaI recognition site) and one digestion control probe.

We used a total of 200 ng of genomic DNA extracted from peripheral blood by the salting-out procedure. 10 Mmol/l Tris-EDTA (pH 8.0) was added to DNA if dilution was necessary. After 16 h of hybridization at 60C, samples were equally split into two aliquots. The first aliquot underwent ligation only, whereas the second one underwent ligation followed by enzymatic digestion with HhaI [a restriction enzyme recognizing only unmethylated DNA] and PCR amplification using universal primers.

In the latter case, amplification products were obtained and detected by capillary electrophoresis only if the CpG included in the HhaI site was methylated. Ligation, enzymatic digestion, and PCR amplification were performed according to the manufacturer's instructions.

All samples were run in duplicate and at least two normal samples were analyzed in each MS-MLPA experiment, as controls. PCR products (1 ml) were mixed with 0.4 ml of internal size standard (500LIZ Genescan; Applied Biosystems)

and 9 ml of deionized formamide and injected into an ABI-3130 genetic analyzer (Applied Biosystems) equipped with a 36 cm, four capillaries array using POP7 as polymer. The samples were injected for 20 s at 1.5 kV and run for a total time of 1200 s at 15 kV. Genemarker v1.95 software is used for analysis, which performs all steps of data normalization and sloping data correction.

Ethical considerations

This study was approved by the ethics committee of Azad University of Tehran Medical Sciences (code: IR.IAU.PS.REC.1401.062). Throughout the study, researchers adhered to the Declaration of Helsinki. Written informed consent was obtained from the patients in this research. All information related to children was kept confidential. This study did not interfere with the process of providing medical services and care by physicians in the clinic. This research did not impose any additional costs on the patient.

RESULTS

The interpretation of MS-MLPA findings demonstrated that out of 10 children (4 boys and 6 girls) suspected of SRS or BWS (based on the diagnosis of a pediatric endocrinologist), only 3 were definitively diagnosed with these syndromes. None of the children were the result of a consanguine marriage. No deletion or addition changes were observed in any of the children, especially in the NSD1 gene.

The first patient

A newborn baby girl was diagnosed by an endocrinologist with suspicion of BWS. DNA was extracted from the blood sample and molecular analysis was done with MS MLPA genetic test. Copy number analysis showed that no deletions or duplications occurred in the chromosomal segments and therefore deemed normal (Figure 1A). Methylation analysis showed that hypomethylation of IC2 occurred in the 11p15 section [Figure 1B]. Therefore, this infant indeed had BWS.

	Probe Name	Bin Size	1734-030-2_B1
1	CDKN1C-exon1	347.1	1.536
2	CDKN1C_exon1	196.6	1.137
3	CDKN1C_exon3	445.1	1.121
4	Ctrl-2p25	460.6	0.881
5	Ctrl_02q24	309.5	0.995
6	Ctrl_07q31	154.5	0.823
7	Ctrl_10q21	382.6	1.023
8	Ctrl_10q25	127.3	0.904
9	Ctrl_12q13	208.4	1.144
10	Ctrl_14q24	148.2	1.057
11	Ctrl_16q22	337.3	0.941
12	Ctrl_17p12	258.8	1.107
13	Ctrl_18q21	425.5	0.969
14	Ctrl_22q11	202.4	0.978
15	Ctrl_3q29	292.4	1.009
16	Ctrl_9q21	177.2	0.839
17	H19-upstream	214.3	0.874
18	H19-upstream	451.7	0.940
19	H19DMR/IC1	184.1	0.934
20	H19DMR/IC1	300.1	1.008
21	H19DMR/IC1	133.7	0.957
22	H19DMR/IC1	239.9	0.968
23	H19_exon5	228.5	1.108
24	H19_upstream	190.5	1.032
25	H19_upstream	160.0	0.977
26	IGF2_EXON4	171.3	1.025
27	IGF2_exon7	283.8	1.024
28	KCNQ1_ex13	267.4	0.979
29	KCNQ1_ex15	373.4	1.144
30	KCNQ1_ex17	409.9	1.156
31	KCNQ1_exon2	434.7	1.029
32	KCNQ1_exon3	221.9	0.975
33	KCNQ1_exon7	328.1	1.292
34	KCNQ1_exon8	363.0	1.115
35	KCNQ1_exon9	399.4	0.921
36	KVDMR/IC2	139.4	1.078
37	KvDMR/IC2	274.4	1.278
38	KvDMR/IC2	165.2	1.003
39	KvDMR/IC2	391.7	1.191
40	NSD1-EXON23	317.8	1.020
41	NSD1-exon21	417.4	0.900
42	Y-fragment	106.0	0.000
43	digestion cont	354.8	1.135
44	x-fragment	101.5	0.990

A. Copy number analysis

	Probe Name	Bin Size	1734D-030-5_E
1	CDKN1C-exon1	347.1	0.878
2	CDKN1C_exon1	196.6	1.204
3	CDKN1C_exon3	445.1	0.975
4	Ctrl-2p25	460.6	1.029
5	Ctrl_02q24	309.5	1.078
6	Ctrl_07q31	154.5	0.824
7	Ctrl_10q21	382.6	0.929
8	Ctrl_10q25	127.3	0.923
9	Ctrl_12q13	208.4	1.244
10	Ctrl_14q24	148.2	1.027
11	Ctrl_16q22	337.3	1.033
12	Ctrl_17p12	258.8	1.293
13	Ctrl_18q21	425.5	0.968
14	Ctrl_22q11	202.4	1.039
15	Ctrl_3q29	292.4	1.072
16	Ctrl_9q21	177.2	0.902
17	H19-upstream	214.3	0.893
18	H19-upstream	451.7	0.932
19	H19DMR/IC1	184.1	0.956
20	H19DMR/IC1	300.1	1.100
21	H19DMR/IC1	133.7	0.951
22	H19DMR/IC1	239.9	1.011
23	H19_exon5	228.5	1.099
24	H19_upstream	190.5	1.067
25	H19_upstream	160.0	0.855
26	IGF2_EXON4	171.3	0.000
27	IGF2_exon7	283.8	1.084
28	KCNQ1_ex13	267.4	1.027
29	KCNQ1_ex15	373.4	1.243
30	KCNQ1_ex17	409.9	1.116
31	KCNQ1_exon2	434.7	1.014
32	KCNQ1_exon3	221.9	1.074
33	KCNQ1_exon7	328.1	1.446
34	KCNQ1_exon8	363.0	1.050
35	KCNQ1_exon9	399.4	0.948
36	KVDMR/IC2	139.4	0.315
37	KvDMR/IC2	274.4	0.404
38	KvDMR/IC2	165.2	0.290
39	KvDMR/IC2	391.7	0.301
40	NSD1-EXON23	317.8	1.028
41	NSD1-exon21	417.4	0.960
42	Y-fragment	106.0	0.000
43	digestion cont	354.8	-1
44	x-fragment	101.5	1.072

B. Methylation analysis

Figure 1: MLPA genetic test findings related to the first patient

The second patient

A 21-month-old girl, with the examination of an endocrinologist, was diagnosed with suspected SRS. DNA was extracted from the blood sample and molecular analysis was done with MS-MLPA genetic test. Copy number analysis showed that no deletions or duplications occurred in the chromosomal segments, hence a normal finding (Figure 2A). Methylation analysis showed that hypomethylation of IC1

occurred in the 11p15 segment. This hypomethylation occurred in probes 19 to 22 (Figure 2B). Therefore, this baby definitely had SRS.

	Probe Name	Bin Size	D07_4-769-2-03
1	CDKN1C-exon1	347.1	0.729
2	CDKN1C_exon1	196.1	0.944
3	CDKN1C_exon3	442.4	0.907
4	Ctrl-2p25	459.7	1.155
5	Ctrl_02q24	311.0	0.907
6	Ctrl_07q31	154.0	1.047
7	Ctrl_10q21	381.6	1.010
8	Ctrl_10q26	127.2	0.893
9	Ctrl_12q13	207.5	1.003
10	Ctrl_14q24	147.6	0.958
11	Ctrl_16q22	338.9	1.041
12	Ctrl_17p12	259.8	0.912
13	Ctrl_18q21	423.6	1.013
14	Ctrl_22q11	202.1	0.988
15	Ctrl_3q29	293.7	1.033
16	Ctrl_9q21	177.3	1.023
17	H19-upstream	215.0	1.002
18	H19-upstream	450.4	1.046
19	H19DMR/IC1	183.7	1.010
20	H19DMR/IC1	301.5	1.068
21	H19DMR/IC1	133.0	0.987
22	H19DMR/IC1	241.1	1.026
23	H19_exon5	229.5	1.045
24	H19_upstream	190.4	1.004
25	H19_upstream	160.2	0.985
26	IGF2_EXON4	170.2	0.975
27	IGF2_exon7	285.4	0.975
28	KCNQ1_ex13	269.4	1.130
29	KCNQ1_ex15	372.4	0.847
30	KCNQ1_ex17	407.8	0.982
31	KCNQ1_exon2	433.3	1.019
32	KCNQ1_exon3	223.0	1.007
33	KCNQ1_exon7	329.3	0.909
34	KCNQ1_exon8	361.0	1.017
35	KCNQ1_exon9	398.8	1.133
36	KVDMR/IC2	138.6	0.961
37	KvDMR/IC2	276.0	1.002
38	KvDMR/IC2	165.1	1.024
39	KvDMR/IC2	390.9	0.950
40	NSD1-EXON23	319.9	1.060
41	NSD1-exon21	417.1	0.993
42	Y-fragment	105.9	0.000
43	digestion cont	353.5	0.975
44	x-fragment	101.4	0.976

A. Copy number analysis

	Probe Name	Bin Size	F07_6-769B-03
1	CDKN1C-exon1	347.1	1.188
2	CDKN1C_exon1	196.1	0.945
3	CDKN1C_exon3	442.4	0.960
4	Ctrl-2p25	459.7	1.089
5	Ctrl_02q24	311.0	0.893
6	Ctrl_07q31	154.0	1.106
7	Ctrl_10q21	381.6	0.975
8	Ctrl_10q26	127.2	1.014
9	Ctrl_12q13	207.5	1.033
10	Ctrl_14q24	147.6	0.986
11	Ctrl_16q22	338.9	1.058
12	Ctrl_17p12	259.8	0.911
13	Ctrl_18q21	423.6	1.039
14	Ctrl_22q11	202.1	1.012
15	Ctrl_3q29	293.7	0.993
16	Ctrl_9q21	177.3	1.018
17	H19-upstream	215.0	1.106
18	H19-upstream	450.4	1.030
19	H19DMR/IC1	183.7	0.433
20	H19DMR/IC1	301.5	0.383
21	H19DMR/IC1	133.0	0.594
22	H19DMR/IC1	241.1	0.500
23	H19_exon5	229.5	1.024
24	H19_upstream	190.4	0.996
25	H19_upstream	160.2	1.077
26	IGF2_EXON4	170.2	0.000
27	IGF2_exon7	285.4	0.973
28	KCNQ1_ex13	269.4	1.097
29	KCNQ1_ex15	372.4	0.854
30	KCNQ1_ex17	407.8	1.087
31	KCNQ1_exon2	433.3	0.912
32	KCNQ1_exon3	223.0	0.957
33	KCNQ1_exon7	329.3	0.928
34	KCNQ1_exon8	361.0	0.957
35	KCNQ1_exon9	398.8	0.946
36	KVDMR/IC2	138.6	1.089
37	KvDMR/IC2	276.0	1.000
38	KvDMR/IC2	165.1	1.078
39	KvDMR/IC2	390.9	0.975
40	NSD1-EXON23	319.9	0.966
41	NSD1-exon21	417.1	0.964
42	Y-fragment	105.9	0.000
43	digestion cont	353.5	x1
44	x-fragment	101.4	1.095

B. Methylation analysis

Figure 2: MLPA genetic test findings related to the second patient

The third patient

A 2-year-old boy was suspected of having BWS according to the examination of an endocrinologist. DNA was extracted from the blood sample and molecular analysis was done with MS-MLPA genetic test. Copy number analysis showed that no deletions or duplications occurred in the chromosomal segments and therefore, it was deemed normal [Figure 3A]. Methylation analysis showed that hypermethylation of IC1 occurred in the 11p15 segment. This hypomethylation occurred in probes 36, 38, and 39. IC2 hypomethylation was detected in the 11p15 segment. This hypomethylation occurred in probes 19 to 22 (Figure 3B). Therefore, this boy definitely had BWS. Uniparental disomy occurred in this patient and he inherited both chromosomes from his father.

The fourth patient

A 9-year-old boy was suspected of SRS according to the examination of an endocrinologist. DNA was extracted from the blood sample and molecular analysis was done with MS-MLPA genetic test. Copy number analysis showed that no deletions or duplications occurred in the chromosomal segments and therefore, it was deemed normal (Figure 4A). Methylation analysis showed a normal pattern in the IC1 and IC2 (Figure 4B). Therefore, this child did not suffer from either SRS or BWS.

Fifth to tenth patients

The fifth to seventh patients were a 5-year-old girl, an 8-year-old girl, and a 12-year-old girl, respectively, suspected of SRS. The eighth patient was a 4-year-old boy, the ninth patient was a 6-year-old girl, and the tenth patient was a 7-year-old boy who was suspected of having BWS; according to MS-MLPA, it was found that none had either SRS or BWS.

DISCUSSION

The main reason for investigating mutations and methylation changes in H19, IGF2, CDKN1C, and KCNQ1 (located on 11p15) and NSD1 (located on 5q35.3) genes lies in their function. Alterations in these genes affect function and can give rise to the phenotypes associated with SRS or BWS. The gene CDKN1C (Cyclin Dependent Kinase Inhibitor 1C) is a tumor suppressor and a strong inhibitor and a negative regulator of cell

proliferation.

	Probe Name	Bin Size	756-030-3_C08
1	CDKN1C-exon1	347.2	0.933
2	CDKN1C_exon1	196.6	1.037
3	CDKN1C_exon3	444.4	1.000
4	Ctrl-3p26	460.2	1.021
5	Ctrl_82q24	309.9	0.887
6	Ctrl_87q31	154.6	1.043
7	Ctrl_18q21	382.3	0.968
8	Ctrl_18q26	127.3	1.031
9	Ctrl_12q13	208.5	0.900
10	Ctrl_14q24	148.2	1.024
11	Ctrl_16q22	337.9	0.927
12	Ctrl_17p12	259.4	0.999
13	Ctrl_18q21	424.9	1.032
14	Ctrl_22q11	202.7	0.995
15	Ctrl_3q29	292.7	0.940
16	Ctrl_9q21	177.5	0.957
17	H19-upstream	214.8	1.027
18	H19-upstream	451.4	1.061
19	H19DMR/IC1	184.2	1.004
20	H19DMR/IC1	350.4	1.128
21	H19DMR/IC1	133.6	1.064
22	H19DMR/IC1	240.6	1.097
23	H19_exon5	229.0	0.992
24	H19_upstream	190.8	0.926
25	H19_upstream	160.3	0.977
26	IGF2_EXON4	171.3	1.037
27	IGF2_exon7	284.3	1.007
28	KCNQ1_ex13	268.1	1.013
29	KCNQ1_ex15	373.2	1.027
30	KCNQ1_ex17	409.3	0.973
31	KCNQ1_exon2	434.2	1.029
32	KCNQ1_exon3	222.6	0.990
33	KCNQ1_exon7	328.5	1.001
34	KCNQ1_exon8	382.4	1.006
35	KCNQ1_exon9	399.1	0.991
36	KVDMR/IC2	139.3	0.962
37	KVDMR/IC2	274.9	1.021
38	KvDMR/IC2	165.4	1.049
39	KvDMR/IC2	391.4	0.955
40	NSD1-EXON23	318.4	0.958
41	NSD1-exon21	417.3	0.969
42	Y-fragment	106.0	1.019
43	digestion cont	354.4	1.015
44	x-fragment	101.6	0.959

A. Copy number analysis

	Probe Name	Bin Size	756D-030-7_G08
1	CDKN1C-exon1	347.3	0.959
2	CDKN1C_exon1	196.6	1.041
3	CDKN1C_exon3	444.4	1.025
4	Ctrl-3p26	460.3	1.068
5	Ctrl_82q24	309.9	0.976
6	Ctrl_87q31	154.6	1.017
7	Ctrl_18q21	382.3	1.116
8	Ctrl_18q26	127.3	1.032
9	Ctrl_12q13	208.4	0.946
10	Ctrl_14q24	148.3	0.977
11	Ctrl_16q22	337.9	1.007
12	Ctrl_17p12	259.4	0.991
13	Ctrl_18q21	424.9	1.020
14	Ctrl_22q11	202.6	1.012
15	Ctrl_3q29	292.7	0.931
16	Ctrl_9q21	177.4	1.021
17	H19-upstream	214.7	1.084
18	H19-upstream	451.4	1.013
19	H19DMR/IC1	184.1	1.614
20	H19DMR/IC1	350.4	1.824
21	H19DMR/IC1	133.5	1.715
22	H19DMR/IC1	240.6	1.792
23	H19_exon5	229.0	1.029
24	H19_upstream	190.7	0.963
25	H19_upstream	160.2	1.068
26	IGF2_EXON4	171.3	0.888
27	IGF2_exon7	284.3	1.028
28	KCNQ1_ex13	268.1	1.026
29	KCNQ1_ex15	373.2	0.975
30	KCNQ1_ex17	409.3	1.095
31	KCNQ1_exon2	434.2	1.081
32	KCNQ1_exon3	222.6	1.017
33	KCNQ1_exon7	328.5	0.931
34	KCNQ1_exon8	382.4	1.057
35	KCNQ1_exon9	399.1	0.999
36	KVDMR/IC2	139.2	0.487
37	KvDMR/IC2	274.9	0.725
38	KvDMR/IC2	165.4	0.393
39	KvDMR/IC2	391.4	0.478
40	NSD1-EXON23	318.4	1.044
41	NSD1-exon21	417.3	1.042
42	Y-fragment	106.0	1.096
43	digestion cont	354.4	-1
44	x-fragment	101.6	0.564

B. Methylation analysis

Figure 3: MLPA genetic test findings related to the third patient

Mutation in this gene can cause abnormal height and abnormal bladder structure and function. It also provides instructions for making proteins that help growth regulation [17]. NSD1 (Nuclear Receptor Binding SET Domain Protein 1) acts as a basic transcription factor and a regulator in transcription. Mutation in this gene can cause childhood acute myeloid leukemia and abnormal body height [18]. KCNQ1 [potassium voltage-gated channel subfamily Q member 1] gene belongs to a large family of genes responsible for making potassium channels [19]. The IGF2 gene (insulin-like growth factor 2) plays an essential role in the growth and development of the fetus before birth, and studies also showed that IGF2 causes the growth and division (multiplication) of cells in many different tissues [20]. The H19 gene is located near the IGF2 gene and provides instructions for making a non-coding RNA. Despite many genes, the H19 gene does not contain instructions for making a protein. The function of this non-coding RNA produced from this gene is unknown, but researchers believe it may act as a tumor suppressor. The H19 gene is highly active before birth in various tissues and appears to play an important role in early development [21].

The molecular defects of BWS and SRS are very heterogeneous. Many of these abnormalities involve epigenetic or copy number changes within the 11p15.5 gene cluster. Identification of these defects usually requires the use of multiple assays. Molecular diagnosis of SRS and BWS is currently possible only in one-third of patients [22] The aim of this study was to evaluate the methylation status and copy number of genes H19, IGF2, CDKN1C, KCNQ1, and NSD1 in blood samples of 10 Iranian children suspected of SRS or BWS.

Analysis of methylation, deletion, and addition in the mentioned genes in 10 children suspected of SRS or BWS, led to the diagnosis of SRS in one child and BWS in 2 children. In line with the present study, Gaston et al. showed that analysis of KvDMR1 methylation in leukocyte DNA alone identified BWS in 60% of all patients (64% of patients with severe form and 53% of patients with incomplete form of BWS). The analysis of the H19 gene methylation identified 13 other BWS patients with H19 hypermethylation, which confirms the effect of methylation in the occurrence of BWS in the two mentioned genes in line with our study [23].

Therefore, KCNQ1OT and H19 gene analysis is useful in routine

evaluation and leads to the diagnosis of BWS or SRS in children suspected of these syndromes [24].

	Probe Name	Bin Size	731-030-2_B08_
1	CDKN1C-exon1	347.2	1.373
2	CDKN1C_exon1	196.6	0.997
3	CDKN1C_exon3	444.4	1.085
4	Ctrl-2p25	460.2	0.934
5	Ctrl_02q24	309.9	0.900
6	Ctrl_07q31	154.6	0.778
7	Ctrl_10q21	382.3	0.992
8	Ctrl_10q25	127.3	0.847
9	Ctrl_12q13	208.5	1.031
10	Ctrl_14q24	148.2	0.961
11	Ctrl_16q22	337.9	0.950
12	Ctrl_17p12	259.4	1.028
13	Ctrl_18q21	424.9	1.100
14	Ctrl_22q11	202.7	0.998
15	Ctrl_3q29	292.7	1.054
16	Ctrl_9q21	177.5	0.820
17	H19-upstream	214.8	0.884
18	H19-upstream	451.4	1.123
19	H19DMR/IC1	184.2	0.846
20	H19DMR/IC1	300.4	1.011
21	H19DMR/IC1	133.6	0.850
22	H19DMR/IC1	240.6	0.902
23	H19_exon5	229.0	1.099
24	H19_upstream	190.8	0.989
25	H19_upstream	160.3	0.769
26	IGF2_EXON4	171.3	0.928
27	IGF2_exon7	284.3	1.009
28	KCNQ1_ex13	268.1	0.908
29	KCNQ1_ex15	373.2	1.150
30	KCNQ1_ex17	489.3	1.081
31	KCNQ1_exon2	434.2	1.023
32	KCNQ1_exon3	222.5	0.919
33	KCNQ1_exon7	328.5	1.190
34	KCNQ1_exon8	382.4	1.131
35	KCNQ1_exon9	399.1	0.978
36	KvDMR/IC2	139.3	0.851
37	KvDMR/IC2	274.9	1.109
38	KvDMR/IC2	165.4	0.917
39	KvDMR/IC2	391.4	1.116
40	NSD1-EXON23	318.4	1.033
41	NSD1-exon21	417.3	1.014
42	Y-fragment	186.0	0.943
43	digestion contr	354.4	0.972
44	x-fragment	101.6	0.525

	Probe Name	Bin Size	731D-030-6_F08
1	CDKN1C-exon1	347.3	1.050
2	CDKN1C_exon1	196.6	1.067
3	CDKN1C_exon3	444.4	0.928
4	Ctrl-2p25	460.3	0.851
5	Ctrl_02q24	309.9	1.033
6	Ctrl_07q31	154.6	0.805
7	Ctrl_10q21	382.3	0.961
8	Ctrl_10q25	127.3	0.934
9	Ctrl_12q13	208.4	1.157
10	Ctrl_14q24	148.3	1.104
11	Ctrl_16q22	337.9	0.976
12	Ctrl_17p12	259.4	1.069
13	Ctrl_18q21	424.9	0.984
14	Ctrl_22q11	202.6	1.036
15	Ctrl_3q29	292.7	0.939
16	Ctrl_9q21	177.4	0.943
17	H19-upstream	214.7	0.937
18	H19-upstream	451.4	0.871
19	H19DMR/IC1	184.1	0.984
20	H19DMR/IC1	300.4	0.960
21	H19DMR/IC1	133.5	0.958
22	H19DMR/IC1	240.6	0.935
23	H19_exon5	229.0	1.101
24	H19_upstream	190.7	1.049
25	H19_upstream	160.2	0.846
26	IGF2_EXON4	171.3	0.863
27	IGF2_exon7	284.3	0.992
28	KCNQ1_ex13	268.1	0.940
29	KCNQ1_ex15	373.2	1.065
30	KCNQ1_ex17	489.3	1.019
31	KCNQ1_exon2	434.2	0.931
32	KCNQ1_exon3	222.6	0.957
33	KCNQ1_exon7	328.5	1.134
34	KCNQ1_exon8	382.4	1.029
35	KCNQ1_exon9	399.1	0.837
36	KvDMR/IC2	139.2	0.981
37	KvDMR/IC2	274.9	0.976
38	KvDMR/IC2	165.4	0.888
39	KvDMR/IC2	391.4	0.867
40	NSD1-EXON23	318.4	0.996
41	NSD1-exon21	417.3	0.914
42	Y-fragment	186.8	1.099
43	digestion contr	354.4	-1
44	x-fragment	101.6	0.564

A. Copy number analysis B. Methylation analysis

Figure 4: MLPA genetic test findings related to the fourth patient

Gaston et al. showed complete or nearly complete demethylation (KvDMR1 MI >90%) in 70% of BWS patients, but about one-third showed partial demethylation, suggesting that this abnormality can occur with mosaicism. These findings were supported by the analysis of other pathological tissues that show different demethylation patterns from blood cells. For example, KvDMR1 is incompletely demethylated in the tongue tissue samples of patients while it is fully demethylated in blood cells, indicating that the analysis of blood samples was sufficient to diagnose BWS [23]. Gaston et al., observed that demethylation of the KCNQ1OT gene was never associated with abnormal methylation of the H19 gene [23] except in patients with UPD 11p15, indicating at least two imprinting centers at 11p15.5. These findings were consistent with a recent study by Engel et al [12].

In the present study, the analysis of H19, IGF2, CDKN1C, KCNQ1, and NSD1 genes was normal in 7 out of 10 children. In line with these findings, Gaston et al reported that the analysis of KCNQ1OT and H19 genes was normal for 26 patients (26.8%). CDKN1C gene sequencing was performed in 10 of these patients [5 sporadic forms and 5 patients from families]. No abnormalities were found in sporadic cases. CDKN1C mutations were found in the families of 2 patients, and both patients had exomphalos [23]. Other unknown 11p15 abnormalities may account for other cases. Various mechanisms may cause over-growth syndrome by inducing the activity of growth factors such as IGF2. Several transgenic mouse models have provided evidence for this by showing increased levels of bioactive IGF2 induced by inhibition of the IGF type 2 receptor [IGF2R] or glypican 3, two agents known to bind and downregulate IGF2 expression, making it less accessible to the IGF1R [25]. Further studies on 11p15 genes and IGF2-related regulators in overgrowth syndromes should be conducted to determine the association between genotype and phenotype and to elucidate functional mechanisms.

In a study by Jurkiewicz et al., the MS-MLPA screening test identified the triplication of the IGF2/H19 domain for all probes of these genes in the ME030 kit, although this result was contrary to the results of our study because we did not find any duplication or deletion in individuals. They also

showed that the H19 (ICR1) region had increased methylation and also showed normal methylation patterns for the IGF2R KCNQ1OT1 genes, which are in line with the results of our study [26].

In the present study, MS-MLPA was used for the molecular diagnosis of BWS and SRS. The results showed that MS-MLPA is a sensitive and reliable tool for further identification of molecular defects of BWS and SRS. These findings are in line with the study of Priolo et al., which was conducted in Italy [27]. Most epigenetic defects of BWS and SRS occur sporadically and exist in the mosaic form in individuals [28,29]. Therefore, it is important to use a sensitive diagnostic method to identify all mosaic cases. MS-MLPA method for the detection of 11p15.5 defects is very accurate and sensitive [27].

The great advantage of MS-MLPA is that this method simultaneously determines the copy number and methylation level of the loci. This is clearly shown in the third patient who had both hypomethylation and hypermethylation. While the ligation-only reaction revealed an increase in copy number, the ligation-digestion reaction provided information about the parental origin of the amplified region. Another study also confirmed that MS-MLPA can be considered a robust assay for the analysis of chromosome 11p5.5 defects [30]. In conclusion, our experience with these 10 suspected children showed that MS-MLPA is an easy, low-cost, and reliable method for the molecular diagnosis of BWS/SRS cases with im-printed and repetitive defects on chromosome 11p15. However, due to the small sample size as one limitation in this study, these results need to be confirmed in a larger series of patients. Also, for 7 children who were not confirmed to be suffering from BWS and RSS syndromes in this study, we suggest performing a WES test for them because the doctor may have made a mistake in diagnosing their symptoms and they may be suffering from another disease.

CONCLUSIONS

This was the first study to document these two syndromes in Iran. The present findings showed that methylation changes in the control regions of ICR1 and ICR2, which are related to H19, IGF2, CDKN1C, KCNQ1, and NSD1 genes, lead to the development of SRS or BWS. A diagnosis of the two syndromes is not accurate solely based solely on clinical symptoms and without the use of cellular and molecular genetic testing. MS-MLPA is suggested as a low-cost, reliable, and rapid method for the molecular diagnosis of BWS/SRS with imprinting and duplication defects on chromosome 11P15.

Conflicts of interest and sources of funding

The authors declare no conflict of interest.

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Authors' contribution

MAE, ME, OM, NS, and SS were responsible for the study concept and design. MAE, ME, OM, NS, and SS led data collection. MAE, ME, OM, NS, and SS were responsible for the analysis and interpretation of data. MAE, ME, OM, NS, and SS wrote the draft.

Ethics approval and consent to participate

This study was approved by the ethics committee of Azaz University of Medical Sciences [code: IR.IAU.PS.REC.1401.062]. Throughout the study, researchers adhered to the Declaration of Helsinki. Written informed consent was obtained from the children's parents in this research.

Patient consent for publication

Informed consent was obtained from all subjects involved in the study.

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ORIGINAL ARTICLE

Body Composition Differences in Army Paratroopers: Effect of Speciality, Age, and Gender

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Abstract: *Body composition has been associated with better performance in military populations. However, there is no consensus about the most suitable body composition values for army paratroopers. To analyze body composition differences among the type of specialty, age group, and gender of army paratroopers, for a better improvement of their operability. 104 paratroopers of the Spanish Army were analyzed. Anthropometric and morphological data were analysed throw an In-Body720. There was a significant effect of age on intracellular water. Older soldiers had lower values of intracellular water, protein, and skeletal muscle mass than middle-aged soldiers. No significant differences (P ≤ 0.05) for the effect of the kind of work and age together or taken separately. Men had significantly higher values than women for height, weight, extracellular water, protein, fat-free mass, and skeletal muscle mass, and significantly lower values for the percentage of body fat, while no differences were found for body fat mass and body mass index. We conclude that paratroopers' body composition depends on age in skeletal muscle mass, intracellular water, and protein. For better unit readiness, soldiers must be monitored to analyze the effect of training and nutrition habits on their body composition.*

Keywords: *military; body fat mass; lean mass; body water*

INTRODUCTION

Physical training is a key factor for soldiers, who are expected to perform efficiently in physical tasks and maneuvers, i.e. lifting and carrying weights, deployments, long displacements, or fighting [26]. Traditionally, Body Composition (BC) has been associated with better performance in military populations [13], like in load-carriage jobs, in which lean mass (together with aerobic power) has been identified as more important than speed of movement [21]. Despite these facts, there is still a tendency towards considering perceived assumptions, like appearance, rather than empirical data to evaluate the capabilities of each soldier, and weight plus Body Max Index (BMI) by themselves are not enough to assess military training since many other factors, like lean mass gain, are positive aspects of training that need to be measured [22,24].

Although some standards have been used and considered in the Army to encourage soldiers to keep fit and with good nutritional habits, there is no consensus about the most suitable BC for all the healthy ranges of weight, soft lean mass, or body fat percentage. This attempt becomes more complicated if we consider the wide range of duties required by the different units of the Army and the different soldiers´ ages [14,28]. The incorporation of women in the Army further complicates this issue, because there is a need to define gender-appropriate standards to maximize female soldiers' health and performance [14]. Taking sports, as a reference, different specializations would require different standards of body composition [2,37], like different jobs would require different BC in the Army, as it was reported for the Army [16,17,38] and Air Forces [4]. Moreover, BC is a variable that could be easily managed by any motivated soldier both with good fitness and nutrition habits, with the aim, not to predict the performance, but to obtain health benefits, resistance to disease or injuries [18,27], and better general capabilities, both mental and physical due to a balanced lifestyle [14,25].

Paratroopers are considered an elite unit of the Army since their deployments are highly operational and involve a parachute jump, which has been reported to increase heart rate and decrease fine motor skills [10,34]. Apart from the physical readiness required for a quick setup, prior to entering

the battlefield, they face additional hazards related to high altitudes, like hypoxia, disorientation, or night flights [3,5,6]. The principal tactical jumps are automatic para-chute jumps, in which the soldier has no control of the parachute opening, and manual parachute jumps, in which the subject has control of the parachute in all the maneuvers. Previous research found that both jumps produced a large stress response increasing cortisol, blood lactate, and sympathetic activation [8,10,11]. The health and performance of these kinds of units, which are under extreme psychological and physiological environments [7], can be undermined and there is a need to monitor their body composition [36], especially during their deployment in crisis management operations [29].

MATERIALS AND METHODS

Experimental approach to the problem

To implement the body composition assessment, we followed previous procedures [4,9,30] in which the participants stood upright on foot electrodes on the instrument platform, with arms not touching the torso. They were barefooted and without excess clothing. Four-foot electrodes were used, two of which were oval-shaped and two heel-shaped, and prior to testing the skin was cleaned and dried. Participants were asked to grip the palm and thumb electrodes. The system was calibrated before the testing session and the contacting surface of the electrodes was cleaned with alcohol before each measurement.

Participants abstained from alcohol consumption and vigorous exercise for 24h before the measurement and were measured in the morning after overnight fasting to control hydration status, as in previous research with bioelectrical impedance.

Subjects

A cross-sectional experimental design was conducted to analyze body composition differences of paratroopers, depending on their specialty, group age, and gender. 7 female paratroopers (specialty: automatic parachute; age: M = 32.1, SD = 8.79), and 97 male paratroopers were analyzed and classified into groups of specialty (43 automatic, age: M = 33.3, SD = 9.75; 54 manual, age: M = 34.8, SD = 7.58) and age (21 younger, under 29 years; 48 middle-aged, between 29 and 38 years; and 28 older, above 38 years) of the Spanish Army with a qualification of "fit" according to the periodic medical examination as recorded in the ministerial order 23/2011. Besides, during the research, they were carrying out the periodic aeromedical training included in the ministerial order 23/2011 and the STANAG 3114 "Aeromedical Training of Flight Personnel" (NATO regulations). Before starting the research, the experimental procedures were explained to all the participants, who gave their voluntary written informed consent under the Declaration of Helsinki. The procedures conducted in the present research were designed and approved by the Medical Service of the Aerospace Medicine Instruction Centre of Spanish Air Forces and the University Ethic Committee (CIPI/18/093).

Instruments

A bioimpedance analyzer (InBody 720, Biospace Co. Ltd., Seoul, South Korea) was used for body mass measurement (to the nearest 0.1 kg) and body height (to the nearest 0.1 cm). InBody 720 is a multifrequency impedance body composition analyzer, which uses an eight-point tactile electrode method to take readings from the body. It measures the resistance at five specific frequencies (1 kHz, 50 kHz, 250 kHz, 500 kHz, and 1 MHz) and reactance at three specific frequencies (5 kHz, 50 kHz, and 250 kHz) on each of five segments (right arm, left arm, trunk, right leg, and left leg). Bioelectrical-impedance analysis (BIA) is one of the methods available for measuring body composition in healthy populations. Its simplicity, portability, cost, and subject acceptance make it a very desirable technique. The reliability of BIA compared to the gold standard body composition measurement (DXA) has been successfully demonstrated for a wide range of normal and overweight populations [15,35] and military samples [1].

Data were electronically imported to Excel using Lookin'Body 3.0 software. The following parameters were analyzed: i. Body mass (kg), ii. Height (cm), iii. Body Mass Index (BMI) (kg/m^2), iv. Total Body Water (TBW) (kg) (including left and right arms, trunk, and left and right legs), v. Intracellular Water (ICW) (l) (including left and right arms, trunk, and left and right legs), vi. Extracellular Water (ECW) (l) (including left and right arms, trunk, and left and right legs), vii. Proteins (kg), viii. Minerals (kg), ix. Body Fat Mass (BFM) (kg) (including left and right arms, trunk, and left and right legs, in kg and %), x. Percentage of Body Fat (PBF) (%), xi. Soft Lean Mass (SLM) (kg), xii. Fat-Free Mass (FFM) (kg) (including left and right arms, trunk, and left and right legs, in kg and %), xiii. Skeletal Muscle Mass (kg).

Statistical analysis

The SPSS statistical package (version 21.0; SPSS, Inc. Chicago, Illinois) was used to analyze the data. Normality assumptions were checked with a Shapiro-Wilk test for all the subgroups and a Kolmogorov-Smirnov test for the whole group. Gender differences were analyzed for the same specialty (automatic parachute) with a T-Student test for parametric samples and a Mann-Whitney test for no parametric samples. Homoscedasticity was tested with the Levene test. A factorial ANOVA test was used to compare the type of work, the group of age, and the interaction between the type of work and the group of age on each parameter together with a Bonferroni post hoc test to analyze pairwise comparisons. The level of

significance for all the comparisons was set at P ≤ 0.05.

RESULTS

The results are reported with their mean and standard deviation. Table 1 shows the results of body composition. No significant differences were found for the effect of type of work and age together, but there was a significant effect of age [F(2, 91)=3.172, p = 0.047, η2 = 0.06], in ICW. Older soldiers (M = 28.3, SD = 2.8) had lower values (p = 0.041) of ICW than middle-aged (M = 30.3, SD = 3.7). There was a significant effect of age [F(2, 91)=3.108, p = 0.049, η2 = 0.06], in protein. Older soldiers (M = 12.2, SD = 1.2) had lower values (p = 0.043) of protein than middle-aged (M = 13.0, SD = 1.6). There was a significant effect of age [F(2, 91)=3.154, p = 0.047, η2 = 0.07], in SMM. Older soldiers (M = 34.9, SD = 3.6) had lower values (p = 0.041) of SMM than middle-aged (M = 37.5, SD = 4.9).

Table 1: Results of body composition variables by specialty and age groups

	Age < 28				28 < Age < 38				Age > 38				Age and specialty effect		
	Automatic		Manual		Automatic		Manual		Automatic		Manual				
	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	F	p	η^2
Height (cm)	178.8	7.5	177.5	7.0	177.3	6.2	175.2	5.9	174.3	5.8	175.0	5.7	0.47	0.66	0.01
Body Mass (kg)	75.3	7.1	79.4	14.1	83.4	15.4	78.9	10.6	73.6	9.4	76.5	9.2	1.40	0.25	0.03
BMI (kg/m^2)	23.6	2.6	25.1	3.6	26.3	3.5	25.6	2.8	24.2	3.0	24.9	2.6	1.03	0.35	0.02
TBW (l)	46.4	3.1	47.6	5.5	49.0	7.8	47.5	4.8	45.5	4.5	45.3	4.4	0.47	0.62	0.01
ICW (l)	29.1	1.9	29.9	3.6	30.7	4.9	30.0	3.0	28.4	2.9	28.3	2.8	0.36	0.69	0.01
ECW (l)	17.2	1.2	17.7	2.0	18.3	2.9	17.5	1.8	17.0	1.5	17.0	1.6	0.72	0.48	0.02
Proteins (kg)	12.6	0.8	12.9	1.5	13.2	2.1	12.9	1.3	12.2	1.2	12.2	1.2	0.33	0.71	0.00
Minerals (kg)	4.2	0.4	4.5	0.7	4.7	0.8	4.4	0.5	4.3	0.4	4.2	0.5	1.17	0.31	0.03
BFM (kg)	11.9	4.1	14.3	10.1	16.3	7.4	13.9	6.4	11.5	6.5	14.6	5.1	1.86	0.16	0.04
PBF (%)	15.6	4.4	16.9	9.1	19.0	6.3	17.1	5.7	15.1	6.8	18.8	5.3	1.83	0.17	0.04
SLM (kg)	59.8	4.0	61.3	7.2	63.2	10.0	61.3	6.2	58.5	5.8	58.3	5.6	0.45	0.64	0.01
FFM (kg)	63.3	4.3	65.1	7.7	67.0	10.8	64.9	6.6	62.1	6.1	61.8	6.0	0.48	0.61	0.01
SMM (kg)	36.0	2.5	37.1	4.7	38.1	6.4	37.1	3.9	35.0	3.8	34.9	3.6	0.38	0.68	0.01

Note. M = Mean; SD = Standard Deviation; F = Fisher-Snedecor test; η2 = Partial eta squared; BMI = Body Mass Index; TBW = Total Body Water; ICW = Intracellular Water; ECW = Extracellular Water; BFM = Body Fat Mass; PBF = Percentage of Body Fat; SLM = Soft Lean Mass; FFM = Fat Free Mass; SMM = Skeletal Muscle Mass

Table 2 shows the results of FFM and BFM by body segments. No significant differences were found for the effect of kind of work and age together or taken separately.

Table 2: Results of Fat-Free Mass and Body Fat Mass by body segments

	Age < 28				28 < Age < 38				Age > 38				Age and specialty effect		
	Automatic		Manual		Automatic		Manual		Automatic		Manual				
	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	F	p	η2
FFM Right Arm (kg)	3.65	0.31	3.76	0.52	3.93	0.76	3.79	0.49	3.56	0.55	3.58	0.47	0.39	0.67	0.01
FFM Left Arm (kg)	3.61	0.30	3.72	0.54	3.89	0.78	3.78	0.51	3.50	0.59	3.78	0.51	0.34	0.71	0.01
FFM Trunk (kg)	28.1	1.79	28.7	3.20	29.6	4.44	28.9	2.93	27.2	3.43	27.6	2.88	0.39	0.67	0.01
FFM Right Leg (kg)	10.0	1.01	9.94	1.09	10.1	1.49	9.71	1.02	9.32	0.91	9.37	1.00	0.42	0.65	0.01
FFM Left Leg (kg)	9.92	1.02	9.89	1.13	10.0	1.52	9.64	1.02	9.32	0.80	9.32	0.97	0.37	0.69	0.01
BFM Right Arm (kg)	0.52	0.26	0.75	0.86	0.86	0.56	0.68	0.56	0.55	0.51	0.75	0.35	1.67	0.19	0.04
BFM Left Arm (kg)	0.55	0.28	0.78	0.90	0.90	0.58	0.67	0.56	0.55	0.49	0.76	0.36	1.97	0.14	0.04
BFM Trunk (kg)	6.16	2.59	7.45	5.70	8.80	4.34	7.36	3.74	5.83	3.81	7.78	3.16	1.93	0.15	0.04
BFM Right Leg (kg)	1.82	0.47	2.07	1.22	2.30	0.90	1.98	0.77	1.74	0.82	2.10	0.57	1.87	0.16	0.04
BFM Left Leg (kg)	1.79	0.46	2.02	1.25	2.27	0.89	1.98	0.76	1.74	0.82	2.08	0.59	1.60	0.20	0.03

Note. M = Mean; SD = Standard Deviation; F = Fisher-Snedecor test; η2 = Partial eta squared; FFM = Fat-Free Mass; BFM = Body Fat Mass

Table 3 shows the results of body water (total, intracellular, and extracellular) by body segments. No significant differences were found for the effect of type of work and age together, but there was a significant effect of age [F(2, 91)=3.314, p = 0.041, η2 = 0.06], in the ICW of the trunk. Older soldiers (M = 13.3, SD = 1.51) had lower values (p = 0.035) in the ICW of the trunk than middle-aged (M = 14.2, SD = 1.71). Men had significant higher values than women for height (p=0.000; M = 176.9, SD = 6.6 vs M = 164.1, SD = 5.9), weight (p=0.000; M = 78.0, SD = 12.2 vs M = 60.0, SD = 6.8), TBW (M = 47.2, SD = 5.8 vs M = 34.0, SD = 2.1), ECW (p=0.000; M = 17.6, SD = 2.1 vs M = 12.8, SD = 0.73), ICW (p=0.000; M = 29.5, SD = 3.6 vs M = 21.2, SD = 1.3), Protein (p=0.000; M = 12.7, SD = 1.5 vs M = 9.2, SD = 0.6), Minerals (p=0.000; M = 4.4, SD = 0.62 vs M = 3.27, SD = 0.27), FFM (p=0.000; M = 64.4, SD = 8.0 vs M = 46.5, SD = 2.96), SLM (p=0.000; M = 60.7, SD = 7.5 vs M = 43.8, SD = 2.76), SMM (p=0.000; M = 36.5, SD = 4.8 vs M = 25.7, SD = 1.83), and significant lower values for PBF (p=0.034; M = 16.8, SD = 6.0 vs M = 22.0, SD = 4.3). No significant differences were found for the effect of gender between men and women in BFM (p=0.955; M = 13.5, SD = 6.5 vs M = 13.4, SD = 4.3) and BMI (p=0.065; M = 24.8, SD = 3.29 vs M = 22.34, SD = 2.9).

Table 3: Results of Body Water by Body Segments

	Age < 28				28 < Age < 38				Age > 38				Age and specialty effect		
	Automatic		Manual		Automatic		Manual		Automatic		Manual				
	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	F	p	η^2
TBW Right Arm (l)	2.84	0.23	2.92	0.40	3.05	0.59	2.94	0.38	2.77	0.42	2.78	0.37	0.39	0.67	0.01
TBW Left Arm (l)	2.80	0.23	2.89	0.42	3.02	0.61	2.93	0.40	2.72	0.46	2.77	0.38	0.37	0.69	0.01
TBW Trunk (l)	21.8	1.42	22.3	2.46	23.0	3.45	22.4	2.28	21.1	2.66	21.4	2.20	0.44	0.64	0.01
TBW Right Leg (l)	7.78	0.80	7.71	0.85	7.86	1.15	7.52	0.79	7.23	0.70	7.28	0.77	0.47	0.62	0.01
TBW Left Leg (l)	7.71	0.80	7.68	0.89	7.81	1.18	7.48	0.80	7.24	0.62	7.25	0.76	0.39	0.67	0.01
ICW Right Arm (l)	1.77	0.14	1.83	0.25	1.91	0.36	1.84	0.23	1.73	0.27	1.73	0.23	0.37	0.68	0.01
ICW Left Arm (l)	1.75	0.14	1.81	0.26	1.89	0.37	1.84	0.24	1.70	0.29	1.73	0.24	0.31	0.72	0.01
ICW Trunk (l)	13.7	0.85	14.0	1.60	14.4	2.17	14.1	1.43	13.2	1.71	13.3	1.40	0.32	0.72	0.01
ICW Right Leg (l)	4.90	0.47	4.88	0.53	4.95	0.73	4.77	0.49	4.55	0.46	4.55	0.49	0.29	0.74	0.01
ICW Left Leg (l)	4.83	0.48	4.82	0.54	4.90	0.74	4.72	0.49	4.53	0.39	4.52	0.47	0.27	0.76	0.01
ECW Right Arm (l)	1.06	0.09	1.08	0.14	1.14	0.22	1.09	1.14	1.04	0.15	1.04	0.14	0.43	0.64	0.01
ECW Left Arm (l)	1.05	0.09	1.08	0.15	1.13	0.23	1.09	1.15	1.02	0.16	1.04	0.14	0.47	0.62	0.01
ECW Trunk (l)	8.15	0.59	8.28	0.88	8.61	1.29	8.27	0.85	7.95	0.95	8.07	0.81	0.68	0.50	0.02
ECW Right Leg (l)	2.88	0.32	2.83	0.33	2.91	0.43	2.74	0.30	2.68	0.24	2.72	0.28	0.86	0.42	0.02
ECW Left Leg (l)	2.87	0.33	2.85	0.35	2.91	0.45	2.75	0.31	2.17	0.22	2.72	0.29	0.63	0.53	0.01

Note. M = Mean; SD = Standard Deviation; F = Fisher-Snedecor test; η2 = Partial eta squared; TBW = Total Body Water; ICW = Intracellular Water; ECW = Extracellular Water

DISCUSSION

This study aimed to analyze body composition differences between the type of specialty, age group, and gender of paratroopers.

The first hypothesis was fulfilled since there were no differences in body composition between specialties. The second hypothesis was partially fulfilled since there were differences in intracellular water, protein, and skeletal muscle mass between middle-aged and older soldiers. The third hypothesis was not fulfilled since there were no significant differences in body composition between male and female paratroopers.

The main parachute jumps conducted by paratroopers' units are automatic or manual. The characteristics of both jumps are different since in automatic parachute jumps there is a lack of parachute control during the flight and especially during the landing (with high impacts that directly affect the muscle and bone structures of jumpers).

On the contrary, in manual jumps the flight and landing phases allow for greater control by the jumpers, leading to a less traumatic landing. But independently of these differences, we did not find differences between jump specialties, a fact probably due to the similar training, instruction, and nutritional regimen of both groups. The only difference was the tactical maneuvers after both jumps, but the rest of the preparation and military context were similar in both groups.

Differences between ages were found in intracellular water, protein, and skeletal muscle mass. The middle-aged group had a better mean than the older group in all those variables. Since

the skeletal muscle is mainly built upon protein and water, these results show a tendency towards skeletal muscle loss with age.

The differences found could be related to the fact that middle-aged soldiers have complete growth, they are in their most operational phase in the unit, where they perform a higher number of jumps and the training is more intense, facts that could explain the higher intracellular water, protein, and skeletal muscle mass. This fact would join, with the investigations that support that resistance training promotes an increase in body water, principally by intracellular content [32]. On the other hand, older and younger participants perform a lower number of jumps: older paratroopers begin with the natural muscle mass loss process associated with age [33] and the younger paratroopers have not yet reached their growth zenith and their training in the unit is not yet maximum.

It is important to note that body fat mass, percentage of body fat, and soft lean mass, which have been usually reported as crucial for the performance of the units [13,21] were not different between groups, supporting the idea of the adequate habits of these troops during their service. Also, data analysis for body segments, reinforces the idea of the correct BC maintenance of these units, throughout their military service, despite their age, since no significant differences were found between groups of work and age.

Besides, if we compare the values of body mass index (24.12 kg/m^2 vs 25.88 kg/m^2 for age <28, 25.89 kg/m^2 vs 26.94 kg/m^2 for age between 28 and 38, and 24.64 kg/m^2 vs 26.86 kg/m^2 for age > 38); and body fat percentage (16.0% vs 16.6% for age < 28, 17.8% vs 18.8% for age between 28 and 38, and 17.2% vs 19.7% for age > 38) with some US Army standards, they are better for all the age groups [20]. Nevertheless, we should bear in mind that these groups are considered “elite” inside the Spanish army, and we are comparing them with a combination of soldiers from the combat support, combat arms, and combat service support units. However, results of body mass index and body fat percentage were higher than those reported for soldiers after 6 months of deployment in a crisis management operation (25.89 kg/m^2 vs 24.50 kg/m^2 and 17.8% vs 13.7%), a fact that highlights the need to monitor body composition during field activities, and especially on those with higher length, intensity or more stressful type [36]. Not only fat mass but fat-free mass and protein could be undermined during operational training or deployment [23,24].

If we compare these results with other Army units, body mass index and percentage of body fat are quite similar to airplane and helicopter pilots, but lower to other aircrew support members (25.49 kg/m^2 and 23.79% respectively) [4]. If we take body segments, there is a tendency towards having more fat-free mass in the right arm, which is consistent with previous research in the Air Forces [4], light forces [12], and even in females [39], which could be due to the prominence of right-handedness.

Regarding our third hypothesis about differences in BC according to sex, most of the BC male parameters in absolute terms were higher, while BFM and BMI did not show significant differences. Further investigation is recommended on this issue since the female sample size does not allow the values to be extrapolated and generalized.

Finally, regarding body water composition, there are no significant differences between age groups and manual or automatic parachute jumps in any of the variables analyzed, total, intracellular, and extracellular water. These values suggest an optimal balance of hydration among the healthy population and are correlated with a lower and healthier BMI [19], consequent with the normal-weight distribution among age and type of jump. Indeed, similar values of intracellular and extracellular values have been found in professional athletes depending on their composition whether it is an athlete or not [31].

The main limitation of this study was the small sample size of women. Secondly, we did not have permission to gather other demographic data (like nutrition, economic, education, and physical activity levels in leisure time) that could have helped us to better understand the differences between groups. These background variables could be studied further to design longitudinal research to understand the mechanisms of BC changes.

CONCLUSIONS

Paratroopers' Body Composition is not affected by their type of parachute specialty (automatic or manual) but depends on age for skeletal muscle mass, intracellular water, and protein. Older soldiers had lower values of ICW than middle-aged. Older soldiers had lower values of protein than middle-aged. Older soldiers had lower values of SMM than middle-aged. This data suggests good nutrition and training habits among the paratroopers. When comparing these data with previous research, it suggests that the body fat percentage of paratroopers is similar to airplane pilots, lower than aircrew personnel and combat and service soldiers, but higher than soldiers deployed. Body composition should be monitored to analyze the effect of training and nutrition habits during service, especially during stressful deployments. These facts must be considered to better prepare each unit.

Conflicts of interest and sources of funding

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Authors' contribution

Conceptualization, V.J.C.S. and A.B.S.; methodology, V.J.C.S. and A.B.S.; formal analysis, V.J.C.S. and A.B.S.; investigation, V.J.C.S. and A.B.S..; resources, V.J.C.S. and A.B.S.; data curation, A.B.S.; writing—original draft preparation, A.B.S.; writing—review and editing, V.J.C.S. and A.B.S..; supervision, V.J.C.S.; project administration, V.J.C.S.. All authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of UNIVERSIDAD EUROPEA DE MADRID (protocol code CIPI/18/093).

Patient consent for publication

Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

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ORIGINAL ARTICLE

Functional and Dysfunctional Coping Strategies in Patients Diagnosed with Cancer – From Initial Assessment to Therapeutic Interventions

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Abstract: *The assessment of coping mechanisms in patients diagnosed with oncological diseases is essential for mental health specialists, who have to design the most appropriate case management strategy for comorbid mood disorders, anxiety disorders, adjustment disorders, and other psychiatric conditions that may be detected in this vulnerable population. The adequate treatment of these disorders is important for the preservation of mental health status, quality of life, and overall functionality in patients diagnosed with cancer. Coping mechanisms modulate the vulnerability toward psychiatric disorders, but they also have an impact on treatment adherence, which is an important factor correlating with prognosis. Appraisal-focused, problem-focused, emotion-focused, and occupation-focused coping represent the most well-defined strategies patients use when confronted with a stressful life situation, like a diagnosis with potentially vital consequences. Maladaptive coping strategies may also be identified in these patients, e.g., withdrawal from reality, including complete or partial denial of the disease, substance abuse, behavioral addictions, refusal of the recommended treatment, etc. The psychotherapeutic approach in patients with oncological diseases should include an initial evaluation of the coping strategies used either currently or in the past stressful conditions, an assessment of all the psychosocial resources the patients have (e.,g., support group, professional insertion, hobbies), and screening for mood and anxiety disorders that may have been triggered by the cancer diagnosis. Consequently, within the psychotherapeutic framework, a trained specialist can enhance the role of adaptive coping strategies and highlight the disadvantages of dysfunctional ones. This process may possess a favorable impact on treatment adherence, mental health status, and quality of life in patients with cancer.*

Keywords: *coping mechanisms, treatment adherence, quality of life, cognitive behavioral therapy, executive functions*

INTRODUCTION

Coping mechanisms are defined as thoughts and behaviors activated when finding a solution to either internal or external stressful situations is required [1]. These triggers may vary from different feared situations, a perceived lack of control over own impulses, or difficult life decisions (when internal events are taken into consideration), to physically traumatic stimuli, imposed changes in lifestyle, or interpersonal stressors (when external events are the focus of attention). Combined, internal and external triggers, may exist, as in severe physical conditions, such as oncological diseases, when the diagnosis, the treatment, the stigma, and the overall functionality (personal, familial, social, and professional) are all affected.

A traditional difference exists between the „coping mechanisms", which are conscious and voluntary, and the „defense mechanisms", which are psychodynamically-based constructs, and by their definition unconscious. Coping mechanisms represent sets of structured, stable responses that have a well-defined adaptive function [1]. Defense mechanisms, on the other hand, have as their purpose to disguise internal impulses, in order to protect the Ego; therefore they operate outside the sphere of the conscious mind [2-5]. However, not all authors agree with the

assumption that all defense mechanisms are unconscious, because of examples like suppression or humor, which involves, at least partially, a participation of the Ego [6-8]. At any rate, it is considered that defense mechanisms act as „conscious suppression", and this may be observed in interpersonal relationships [2].

There are many classifications of coping mechanisms because these psychological processes are numerous and quite diverse, therefore different authors group them according to more or less arbitrary criteria [9-15]. One example of such a classification has as its core criterion the targeted stressor(s), and includes (1) reactive coping – the individual responds to a certain stress factor; people who activate this type of coping have presumably better adaptive skills in an unstable environment with many hard-to-predict stressors; (2) proactive coping – the individual activates behaviors/thoughts aimed at neutralizing the effects of anticipated stressors; people who activate this coping perform better in a stable environment with routine tasks [1,16].

Another classification of coping mechanisms includes (a) problem-focused strategies – address the origin of the discomfort, and these may be manifested as active coping, planning, restrictive coping (not acting prematurely, holding back until a good opportunity to act appears), and inhibition of concurrent activities; (b) emotion-focused strategies – targeting the decrease of negative emotions correlated with the ongoing stressor, e.g., positive reframing, acceptance, recourse to religion, and humor; (c) meaning-based strategies, i.e., extraction and management of the meaning the stressful situation has for an individual in a certain moment; (d) social or support-based strategies, which refer to the ability of stress reduction by seeking emotional or behavioral support from the group to which that person belongs [1,17].

The coping mechanisms mentioned above are „adaptive" processes, but there is also a category of „maladaptive" coping strategies [18]. It is supposed that problematic coping may develop in the early stages of conscious life when individuals have to face overwhelming stressors like maltreatment, abuse, or neglect [18]. For example, the excessive activation of primitive coping strategies, lack of modeling for healthy coping alternatives, and repeated exposure to intense stressors in the immediate environment are potential factors that may increase the individual's vulnerability to the development of maladaptive coping strategies [18]. These coping mechanisms are associated with a negative evolution of mental health problems and more severe levels of psychopathological manifestations [1]. Examples of maladaptive or dysfunctional coping are represented by disengagement (defined by efforts to move away from the stress triggers), emotional suppression (i.e., inhibition of emotional reactions), cognitive avoidance (manifested by distraction, worry, and thought suppression), and denial (i.e., the unwillingness to accept reality) [19-21].

The role of maladaptive coping mechanisms in psychopathology has been explored intensively, and conditions like posttraumatic stress disorder, anxiety disorders, depressive disorders, psychosomatic disorders, and somatoform disorders have all been correlated with avoidance-based coping or other maladaptive strategies [1]. The situation of treatment or diagnostic refusal in patients with psychiatric disorders, but also in those presenting with different organic illnesses, including cancer, has been associated with maladaptive coping (i.e., denial) [21].

The rationale for exploring coping mechanisms in patients with oncological and other severe diseases is the need to understand better the psychological strategies activated by patients in stressful situations and to work with these strategies during psychotherapy. One aspect of this process is to allow for the formation of a more solid therapeutic relationship, by exploring the adaptive strategies. Other objectives that may involve coping mechanisms are the training of functional coping skills and helping patients to deconstruct the dysfunctional ones.

There is an additional aspect for exploring these mechanisms in patients with cancer, and it refers to the risk of psychiatric comorbidities. As can be anticipated, this diagnosis, as well as the associated treatment and prognosis, may lead to the onset of adjustment disorders, depressive disorders, stress-related pathology, etc. Many authors are highlighting the need for identifying and treating depression in this specific population because this would have a favorable impact on the quality of life and mortality [22-24]. Cases of major depressive disorders (MDD) have frequently been detected in patients with cancer, reaching prevalences of 2-43.5% [22-24]. The importance of coping mechanisms derives from their impact on the evolution of clinical manifestations and therapeutic responses in patients with MDD [25]. A study exploring this impact concluded that maladaptive mechanisms, mainly ruminating, catastrophizing, and self-blaming were the most frequently used by patients with depression [25]. Accepting the current situation, positive refocusing and re-evaluation are functional coping mechanisms, with a protective role in these patients, and these strategies can be used during the psychotherapeutic process [25].

In another study, the importance of coping strategies for resilience was evaluated in patients with panic disorder [26]. The severity of clinical manifestations and maladaptive coping skills decreased during cognitive-behavioral therapy (CBT) administered in a group format [26]. CBT also had beneficial effects on increasing resilience and quality of life in patients

with panic disorder, thus highlighting the role of psychotherapy in modulating the influence of coping mechanisms on daily functioning, besides its positive clinical impact [26].

Adjustment disorders illustrate the failure of coping mechanisms' functionality in preserving a safe distance toward stressful stimuli' negative impact [27]. Also, acute stress reactions and posttraumatic stress disorder are consequences of coping strategies failure, but in these cases, the stressors represent vital threats [27]. Psychotherapy in these cases targets enhancing coping abilities with the specific stressor and increasing the adaptive responses [28]. This is usually done by identifying negative thoughts and replacing them with more adaptive thoughts (i.e., cognitive restructuring), monitoring the experiences aimed at increasing self-efficacy, and behavioral activation [28-30].

FROM CONCEPTUAL ANALYSIS TO CLINICAL SITUATIONS – AN ANALYSIS OF HOW THE COPING MECHANISMS FUNCTION IN SPECIFIC SITUATIONS

As mentioned in the introductory chapter, coping mechanisms are usually activated in stressful situations, and configuring the individual profile of these mechanisms allows the therapist to represent the specific vulnerability features and the resilience factors to stressful situations, like a vital threat raised by a cancer diagnosis. The presentation of several theoretical situations is considered helpful for a better understanding of how the coping mechanisms may work in clinical settings. For example, when a patient finds out that she has been diagnosed with early-stage breast cancer, she may engage in seeking information about this disease and/or making an appointment for further investigations (which implies active, problem-focused coping); also, she may decide to join a self-help group for patients with the same diagnosis (i.e., a coping based on social support), or she may look for spiritual support from a priest (defining a coping based on recourse to religion). However, continuously looking for information that may invalidate the oncological diagnosis, by exploring different online resources, or by repeated medical consultations in the hope of finding a doctor willing to offer another diagnosis, is a maladaptive coping strategy based on denial. In the case of a patient with prostate cancer who has already been investigated by collecting all the necessary laboratory analyses (prostate biopsy included) and by undergoing all imagistic investigations required, the delay of treatment initiation is caused by his lack of trust in the results of these investigations. Due to this maladaptive coping strategy, the treatment initiation is delayed, and the prognosis may worsen.

Another example is represented by a woman with breast cancer who has undergone a total mastectomy and developed a depressive disorder because she refuses to accept her new body image. As a manifestation of her depression, she does not want to consult a mental health specialist, thus activating a coping strategy based on denial and avoidance.

Yet another typical situation is when a person who goes through a divorce caused by health and financial problems ends up consuming alcohol excessively, to suppress his/her negative emotions. This way of facing the specific life situation is characterized by disengagement and avoiding confrontation with own emotions, i.e., two types of maladaptive coping.

On the other end of the coping mechanisms spectrum, a person who has to retire prematurely due to an oncological disease (now in remission, but with several negative functional consequences) may go initially through an adjustment disorder caused by the recent change of the daily activity regime, but then she/he resignifies the situation and considers that she/he has more free time to spend with own family. This is an example of active coping, planning, and coping based on significance because the individual perceives her/himself as a survivor of the disease and focuses on future prospects.

Of particular importance for understanding how coping mechanisms work, is the exploration of the strategies activated and the solutions each patient has found when confronted previously with difficult situations, such as interpersonal conflicts, social isolation, work conflicts, economic difficulties, and severe illnesses [31].

NEUROBIOLOGICAL FOUNDATION OF COPING MECHANISMS AND THE STRUCTURED APPROACH TO THEIR CLINICAL ASSESSMENT

The prefrontal cortex, responsible for executive functioning (i.e., planning, analysis and synthesis, abstracting, problem-solving, verbal fluency, etc.), and the nucleus accumbens, which is part of the reward system or the mesocorticolimbic circuit, have been associated with the functioning of the coping mechanisms [32,33]. These central nervous system (CNS) structures are sensitive to chronic stress, and disruptions in the functioning of the prefrontal cortex have negative consequences on the ability to perform complex cognitive tasks [32,33]. Executive functions, including cognitive flexibility, planning, inhibitory control, error detection, working memory, and verbal fluency, are largely dependent on the morphological integrity and functionality of the prefrontal cortex [34-39]. These functions are essential for constructing coping mechanisms, their preservation or inhibition, and mediating the relationship between coping strategies and different psychiatric disorders, such as MDD [34,35]. It was even demonstrated that interventions targeting the enhancement of executive functions and/or coping

mechanisms are useful in decreasing the risk of MDD recurrence [34]. Executive functions have a role in increasing the risk of suicidality by mediating the relationship between high levels of stress and avoidant coping, therefore enhancing executive functioning through computerized training and cognitive rehabilitation may reduce the impact of stressors on individuals [35]. Also, executive dysfunction and mnestic impairments were associated with lower use of active coping mechanisms in patients with schizophrenia, highlighting the important correlations existing between these variables [39].

The activity of the hypothalamic-pituitary-adrenocortical axis (HPAA) and the plasma level of cortisol have no confirmed direct effect on coping mechanisms, but they may influence intermediate variables with impact on, or associated with, these mechanisms [33,40,41]. Such mediators are depressive mood, anxiety, insomnia, psychomotor restlessness, and attentional and memory deficits [33,40,41]. For example, in a study (N=30 participants), the presence of MDD was correlated with a higher level of morning serum cortisol vs. healthy individuals, and an inverse correlation between these levels and the frequency of activated prosocial coping mechanisms was observed [42].

Serotonergic and dopaminergic pathways in the CNS are responsible for modulating mood, strengthening/decreasing certain behaviors, and influencing memory and attention [43-48]. The prefrontal cortex, hippocampus, and corpus striatum, which are involved in cognition, are densely populated by dopaminergic and serotonergic neurons originating within the raphe complex, mesocortical, and nigrostriatal systems [43,49]. Processes like learning and memory depend on the modulation of serotonergic and dopaminergic activity and imply short- and long-lasting synaptic modifications [43]. Modulation of serotonergic and dopaminergic pathways using pharmacological agents, as is the case with MDD and schizophrenia spectrum disorders, can influence not only the core clinical symptoms of these disorders but, indirectly, may modulate the coping mechanisms [50-53].

The impact of vasopressin and oxytocin on psychological mechanisms that are important for the modulation of coping strategies was also explored [54,55]. These pituitary neuropeptides are responsible for shaping social behavior, aggression, and social memory, according to preclinical studies [54,55].

Data about the importance of vasopressin and oxytocin in humans also emerged recently, and functional neuroimaging studies showed that impaired operation of these neuropeptides appears in mental disorders that imply an early attachment disruption or difficulties in forming social relationships, such as autism spectrum disorders (ASD), social anxiety disorder, borderline personality disorder, and schizophrenia spectrum disorders (SSD) [55].

In conclusion, the biological foundation of cognition is extremely complex, and the adequate functioning of the cognitive processes (as a prerequisite for the operational coping mechanisms) depends heavily on the integrity of multiple neurotransmission and neurohormonal pathways.

The assessment of coping mechanisms is conducted by mental health specialists trained in using validated instruments. Besides the general evaluation, based on a psychological interview targeting current mental status and personal history, the specialists may use various scales, inventories, and questionnaires constructed and validated for this purpose.

Brief-COPE is a 28-item, self-administered instrument that quantifies functional and dysfunctional coping methods in stressful situations [56]. It is often used in clinical settings to assess how patients cope with stressors such as cancer diagnosis, heart failure, trauma, and mental and behavioral disorders [56]. Based on interpreting this questionnaire's results, psychological counseling strategies may be formulated [56]. Brief-COPE evaluates three types of coping: (1) problem-focused – includes the following dimensions: active coping, planning, suppression of competing activities, restraint coping, and seeking instrumental social support; a high score indicates coping strategies aimed at changing stressful situations, psychological stability, a pragmatic approach and is a positive prognostic factor; (2) focused on emotions – includes seeking social support, positive reinterpretation, acceptance, denial, and appeal to religion; high scores indicate strategies aimed at regulating stress-related emotions; (3) avoidant – includees emotional venting, behavioral disengagement, mental disengagement; a high score suggests physical or cognitive disengagement efforts, low scores suggest adaptive coping [56-61]. The original COPE inventory included 15 dimensions, each with 4 items [57,62]. However, this form was criticized because the number of factors differed across studies, varying from 3 to 5, it was considered too long, and the distinction between its dimensions was not supported by further analyses [57].

The most frequently used tools for evaluating coping mechanisms, besides the COPE and Brief-COPE, according to a meta-analysis, are the Ways of Coping Questionnaire, Coping Strategies Questionnaire, Coping Response Inventory, Coping Strategies Inventory, Adolescent Coping Scale, Pain Coping Questionnaire, Religious COPE, and Utrecht Coping List [63]. The same source mentioned that coping scales are often used for the structured assessment of patients with various health problems, such as illnesses, pain, and medical diagnoses [63].

COPING MECHANISMS IN PATIENTS WITH ONCOLOGIC DISEASES

Coping mechanisms influence the decision-making process regarding participation in treatment and implicitly shape the evolution and prognosis of the condition. The same mechanisms impact the patient's desire to be informed about their own illness, their perception of self-efficacy, their relationship with others during the illness, etc. The main coping mechanisms identified in a study (N=603 patients with cancer) were (1) seeking or using social support, (2) focusing on the positive aspects of life, (3) distancing, (4) cognitive avoidance/cognitive abandonment, and (5) behavioral avoidance/behavioral abandonment [64]. This study concluded that the type of oncological disease, the time elapsed since the diagnosis of the disease, and the presence of treatment correlated non-significantly with the type of coping [64]. The stress perception associated with the oncological condition correlated significantly with the activation of coping based on social support, but also with cognitive and behavioral disengagement [64]. Coping based on social support, focusing on the positive, and distancing were all associated with less emotional distress, while cognitive/behavioral withdrawal was associated with more emotional distress [64].

The relationship between coping strategies, quality of life, and mood in patients with incurable oncological conditions was investigated in a randomized trial of palliative care, that enrolled patients with advanced stages of lung or gastrointestinal cancer (N=350), who were in the first 8 weeks after the diagnosis was confirmed [65]. All main outcomes were assessed in a structured way, and the most frequently detected coping mechanism was emotional support (77%), followed by acceptance (49%), self-blame (38%), and denial (28%) [65]. Coping based on emotional support was significantly associated with better quality of life and less severe depressed mood [65]. In the same study, acceptance correlated significantly with better quality of life and inversely with depression and anxiety, denial and self-blame correlated significantly inversely with quality of life and non-significantly with depression and anxiety [65]. The study's authors concluded that interventions aimed at improving the quality of life and mood of these patients should include increasing the use of adaptive coping strategies [65]. It also may be stated that patients with incurable forms employ different coping mechanisms than those with oncological conditions presenting a favorable prognosis [65]. The explanation is that the emotional burden and severity of physical symptoms create a different setting for activating coping mechanisms in the two population subgroups [65].

Types of coping activated by patients with breast cancer predict long-term psychological adjustment, according to the results of a study that explored the strategies for facing difficulties related to this diagnosis in a group of 55 patients [66]. Coping mechanisms, decisional control, expression of frustration, and psychological adaptation in the first 6 months after finding out the diagnosis were assessed initially and after 3 years [66]. Patients who were initially depressed and who responded to finding out the diagnosis with cognitive avoidance (acceptance/resignation) showed significantly less chance of psychological adjustment 3 years later [66]. The poor psychological adjustment was significantly associated with cognitive avoidance and minimal activation of proximity/ contact-based coping [66].

The relationship between quality of life and coping mechanisms was evaluated in women extensively treated for advanced-stage genital cancer (N=64) [67]. A control group included women with genital cancer in the early stages, who consequently received less aggressive treatment [67]. The main stressors in the study group were the adverse effects of chemotherapy, poor physical condition, and poor prognosis [67]. Using the COPE inventory to assess coping mechanisms, it was observed that avoidance and engagement were the dominant strategies in patients with severe forms of cancer [67]. Also, these patients presented more frequently both types of coping (commitment/avoidance) vs. patients in the control group [67]. Avoidant coping strategies correlated with more negative mood and poorer overall well-being, while avoidance and seeking instrumental support were associated with worse prognosis in patients with severe forms of cancer, but not in those with early forms [67]. Thus, coping mechanisms have a stronger impact on prognosis in patients with severe forms of genital cancer [67]. Avoidant coping strategies in particular may adversely affect the quality of life and mood in this population [67].

The predictive value of coping mechanisms in patients who were diagnosed with stage I or II breast cancer no more than 12 months ago was the objective of a longitudinal study that included 70 patients [68]. This study explored the abilities of these patients to activate situational-specific coping mechanisms and hope (considered a more stable factor), as predictors of adaptation after finding out their diagnosis [68]. Active acceptance-based coping at the moment of diagnosis predicted better adjustment over time in this population, while avoidance-based coping correlated with greater fear of cancer recurrence, independent of the patient's initial medical condition and age [68]. Religion-based coping proved more effective for women who had a lower initial level of hope [68]. The conclusion of the study was that protective and risk factors for adaptation in first-year breast cancer survivors can be identified even before surgery [68]. Mood characteristics, such as hope, as well as situation-specific coping strategies, must be

considered by the case manager [68].

Optimism was explored as a variable mediated by coping strategies in patients with breast cancer in the early stages, in a study with 59 participants [69]. The level of optimism was determined one day before surgery, 10 days after it, and 3-, 6-, and 12 months post-surgery [69]. Acceptance-based coping, positive reframing, and religion were the most frequently activated strategies, and optimism correlated inversely with the level of distress at each of the study visits [69]. Denial and behavioral disengagement were the least frequently activated coping strategies in this population [69]. In conclusion, optimism and pessimism are mediated by the activation of different coping mechanisms in the adaptive process and they have an important role in favoring or hindering this process [69].

The correlation between coping strategies and psychological distress was investigated in patients with advanced stages of cancer [70]. In such a study, that enrolled 132 patients diagnosed with breast, ovarian, lung, colorectal, and other types of cancer, a correlation between distancing, cognitive avoidance, and behavioral abandonment, on the one hand, and psychological distress, on the other, was demonstrated [70]. Distancing coping correlated negatively with psychological distress, while cognitive and behavioral avoidance correlated positively with the same distress [70]. Overall, coping methods explained 36% of the variation in the level of psychological distress [70]. The most important coping mechanism contributing to psychological distress was behavioral avoidance, followed by cognitive avoidance [70].

Emotional expressive coping has predictive value for physical and mental adjustment to breast cancer diagnosis, according to a prospective, 3-month study (N=92 participants) [71]. Active processing of emotions is a coping mechanism based on promoting emotional contact, that favors adaptation and health status [71]. Women who reported emotional expression-type of coping had fewer visits to the doctor for cancer-related symptoms, had better physical health and energy levels, and had lower levels of distress over the next 3 months compared to patients who had less emotional expression [71]. Quality of life was also better in patients with emotionally expressive coping [71].

MDD, personality disorders, and coping strategies were studied as moderators of the quality of life in patients with non-metastatic breast cancer [72]. A cross-sectional study included 120 patients diagnosed with breast cancer 7 months ago, and the main outcomes were quality of life, coping strategies, biological and physiological variables, presence of MDD and personality disorders, and pain severity [72]. Poorer physical quality of life was associated with MDD, younger age, more severe initial tumor stage, and behavioral disengagement coping [72]. A lower emotional quality of life was associated with MDD, the presence of a personality disorder, more severe pain, a high level of self-blame, and a lower use of acceptance-based coping [72]. Also, lower global quality of life was associated with MDD, the presence of a personality disorder, more intense pain, high levels of self-blame, less use of reframing coping strategies, and the absence of hormone therapy [72]. In conclusion, lower quality of life was associated with premorbid patient characteristics (personality disorder) and coping variables (depression, personality, and specific strategies), less so with cancer-related variables (type of treatment, disease severity) [72].

TRAINING OF THE COPING MECHANISMS IN ONCOLOGICAL PATIENTS

Psychotherapy and psychological counseling in patients diagnosed with severe organic illnesses have, between their objectives, the enhancement of functional coping mechanisms and the reduction of maladaptive ones, in order to enhance resilience to stressful situations (e.g., pain, invalidity, changes in lifestyle, adverse events of drugs). Coping skills training programs have been associated with improving some biological and psychosocial parameters in patients with chronic somatic conditions [73]. For example, a decrease in HbA1c levels and an increase in self-efficacy have been observed in patients with diabetes after applying a coping skills training program [73]. The same programs led to increased self-care capacity in diabetic patients, decreased the severity of depressive symptoms, improved the prognosis of chronic obstructive pulmonary disease (COPD), and ameliorated the quality of life in individuals with various somatic conditions [73]. The training of these skills involves lectures, Q&A sessions, group discussions, role-plays, etc., under the direction of a clinical psychologist/psychotherapist; nurses trained for this purpose can also lead such support groups [73]. The topics addressed are the principles of effective self-care, stress management, communication skills training, cognitive-behavioral restructuring techniques, and problem-solving techniques [73].

A brief coping skills training program for oncology patients was designed to increase the patient's feelings of self-efficacy, personal control, and resilience to stress [74]. Applied in the perioperative framework, this program was created for nurses, and they were trained on how to enhance the patient's trust in their own abilities to manage dysfunctional cognitions and behaviors [74]. This training was used to prepare the patients for solving situations or events that may occur before or after surgery [74]. Providing information, education, emotional support, and specific interventions – adapted to the

objectives of each individual case, were part of this brief training [74]. Relaxation based on breathing, cognitive restructuring, mindfulness, goal setting, and motivational interviewing may be part of more comprehensive training [74].

Encouraging caregivers of oncology patients to participate in the training of coping mechanisms is considered an important aspect of case management. A study that included 233 patients with lung cancer and their relatives compared two interventions – 14 telephone sessions of coping skills training assisted by a relative vs. education/support with the help of a relative [75]. Patients in both groups showed improvements in anxiety and self-efficacy during the 4-month follow-up [75]. However, family-assisted coping skills training has been associated with better outcomes in patients with stage II or III cancer [75]. Caregiver-assisted education/psychological support was more effective in patients with stage I lung cancer [75].

The effects of coping skills training on caregivers of oncological patients were also explored. Such a study enrolled 70 participants and looked at the effects of coping skills training on the quality of life in daughters of patients with breast cancer [76]. The intervention consisted of an educational program, with workshop-type lectures, followed by group discussions [76]. Relatives' quality of life was considerably improved by this intervention, according to Pediatric Quality of Life Inventory scores [76]. Physical, and emotional functioning and participation in school activities were improved vs. the control group [76].

Particular aspects of adaptive strategies training depend on the specific elements of oncological conditions. The approach in a psychotherapeutic setting to patients with cancer must include the training of coping skills, depending on the particularities of each case. It is essential for the therapist to work with the patient in identifying the stress factors that activate coping mechanisms: self-image changing (e.g. mastectomy, limb amputation), reduced mobility, adverse effects of chemotherapy and radiotherapy (e.g. alopecia, persistent vomiting, swallowing disorders, sexual dysfunctions), lifestyle changes (eg, colostomy, indwelling urinary catheter), physical pain, psychiatric symptoms/ disorders – depression, anxiety, restlessness, insomnia, apathy, anhedonia, etc.

Therapeutic interventions may be focused on the activation of functional coping mechanisms, based on action and social support, e.g. looking for a support group for patients who have undergone similar surgical interventions; planning activities based on the spared skills; analyzing the pros and cons of correcting deficits (prosthetics, plastic surgery); re-signification of the situation, e.g. post-operative scars are an indicator of the battle with the disease and of the fact that the person has overcome it, assuming the state of "survivor" or "victor"; creating an optimal framework for expressing emotions, concerns, fears related to the new situation in which the person finds him/herself, dysfunctions of the social image, self-image, and relationships with those close to the patient, decreased professional functioning, etc.; encouraging acceptance of the present situation and redirecting resources towards functional, healthy aspects of own life; acceptance does not mean resignation; adding non-pharmacological methods to drug pain therapy – mindfulness, progressive muscle relaxation, diaphragmatic breathing, guided imagery, planning hobby activities; specific techniques for augmenting the effect of medication in depressive, anxiety, adaptation disorders, insomnia, etc., e.g., cognitive restructuring in depression and anxiety, aversive therapy or relaxation techniques in insomnia, guided imagery and activity planning for isolated affective/anxiety symptoms, etc.

CONCLUSIONS

Coping mechanisms in patients diagnosed with oncological diseases have an important role in preserving mental health, quality of life, and general functionality. Coping mechanisms modulate the vulnerability towards mood and anxiety disorders in patients diagnosed with cancer, but these mechanisms also have an impact on therapeutic adherence, thus representing important factors that correlate with prognosis. The best-defined functional coping strategies are those centered on realistic assessment, problem-solving, emotion-expression, and occupational activation, therefore it is recommended that the therapist train these healthy mechanisms in patients with severe organic diseases.

Coping resources activated in stressful life circumstances include the participation of family or friends, recourse to religious rituals, at least partial maintenance of professional roles whenever possible, and self-help therapy (online sessions, organized groups, etc.).

In oncology patients, maladaptive coping strategies can also be identified, for example, withdrawal from reality, including complete or partial denial of the disease, substance abuse, behavioral addictions, treatment refusal, requesting the administration of insufficiently validated treatments not recommended by a specialist, etc.

The psychotherapeutic approach to patients with cancer should include an initial assessment of coping strategies used both now and in the past under stressful conditions, an evaluation of the psychosocial resources that patients have previously activated in life-threatening situations, and a screening for mood, stress-related, and anxiety disorders that

may have been triggered since the onset of the current illness (Figure 1).

Figure 1: The algorithm for assessing and training coping mechanisms in patients with cancer

After this stage, a psychiatric consultation may be recommended, if necessary (acute psychiatric pathology, the need for pharmacological treatment). The initiation of psychological counseling, psychotherapy, and, in specific cases, psychopharmacological treatment may be prescribed, depending on the results of the initial evaluation. Monitoring the mental health status by using standardized tools, and planning follow-up visits to update patients' knowledge and coping skills learned is necessary. The psychotherapeutic intervention should be focused on increasing adaptive coping through systematic training and highlighting the disadvantages of dysfunctional coping mechanisms. This therapeutic process can have a significant favorable impact on treatment adherence, mental health status, and quality of life in patients with cancer

Further research on this topic should investigate the impact of structured coping skills training programs on more homogenous populations, such as patients with a certain type of oncological disease and in a specified stage of the disease's evolution [77-79]. Of major interest would be the exploration of combined therapeutic interventions in this population, and also the assessment of the impact different types of surgical interventions may have on mental health and quality of life [77-83]. As a limitation of this review, it should be mentioned that it was not designed as a systematic approach to this topic, its immediate purpose is to enhance the awareness of mental health specialists about the importance of assessing and addressing during therapy the important aspect of coping strategies.

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Authors' contribution

OV conceived the idea of the review. OV, AGM, BMP, CAC, CFP and REBB designed the study. OV, CFP, REBB, AFF, MCP, CAS and CN screened the studies and abstracted the primary sources. OV, AGM, BPM and CAS drafted the first version of the review. OV, CAC, CFP, AFA, MCP and CAS revised the manuscript and elaborated the conclusions of the review. All authors had full access to all data contained in the review and decided to submit the manuscript for publication.

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ORIGINAL ARTICLE

Confidentiality in the Pharmaceutical Managed Entry Agreements in Romania

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Abstract: *Background and Aim: Health technology assessment for drugs is a necessary step in developing health policies that are focused on patients and getting the best value for scarce resources. One important feature of health technology assessment is transparency. In many countries, health technology assessment is followed by negotiations between pharmaceutical companies and health authorities to determine whether a drug will be publicly funded. These negotiations often result in Managed Entry Agreements, which typically include confidentiality clauses covering the final price of the drug. Methods: We reviewed Romanian legislation starting in 2014 to assess the level of confidentiality and transparency in drug pricing and reimbursement. Results: We found that for drugs with Managed Entry Agreements, the level of discounts is confidential, the public does not know how much the government is paying for each of these drugs, the volumes (units) of drugs are not transparently published by the payer, the outcomes, in terms of patients treated by therapeutic area, therapeutic success, and resource utilization, are confidential. Conclusions: We consider that too much confidentiality can prevent the public from knowing if the government is getting good value for money, but too little confidentiality can lead to higher drug prices or a lack of drugs from the market.*

Keywords: *managed entry agreements, health technology, confidentiality, pricing, reimbursement*

INTRODUCTION

Every year, the pharmaceutical industry introduces new drugs on the market, constantly developing new solutions for known illnesses. The licensing mechanisms of new drugs are already fully regulated, and the beneficial effects and potential risks have been proven through numerous preclinical and clinical studies [1].

One of the main concerns of each country in establishing its policy on medicines is the permanent increase in the price of new medicines, which generates a growing pressure on the budgets allocated to health [2]. Once they enter the market, the big challenge for a new drug is to be accepted for financing from public funds (or through the health care mechanisms specific to each country). For this, in most of the EU countries, each drug is passed through the very fine sieve of the Health Technology Assessment (HTA) process, the ultimate goal of which is to provide health decision-makers with sufficient relevant information based on which to establish priorities in the field of care, such as the reasonable cost that they afford, and the level of financing, their efficiency, respect for legal and ethical values, and equity.

To develop patient-focused health policies that seek to achieve the best value for scarce resources, one characteristic of the HTA is the transparency of the process [3]. Together or after the HTA process, in numerous countries, there is a practice of negotiations between the Marketing Authorization Holder (MAH) and health authorities to get access to a drug to public financing using Managed Entry Agreements (MEA). In 1993, first-time conventions, lately called MEAs, were introduced between producers and third-party payers, targeting discounts and rebates for innovative drugs [4,5].

The need for these contracts was born from the producers' desire to make price reductions only in the conditions in which they obtain guarantees regarding the avoidance of parallel import and the downward spiral of the price as a result of the fact that the visible price decrease in one country attracts a decrease in all other countries and finally even in the respective country as a result of the use of reference prices. From this perspective, MEAs have a common denominator,

respectively, the confidentiality covering the final price of the drug generally as a means to avoid price decreases in other countries.

On the other hand, the third-party payer is interested in keeping costs within a predictable and bearable margin, obtaining superior outcomes for patients, and spending the available resources as efficiently as possible [4]. MEAs can be divided into financial agreements that directly affect the budget (confidential discount or rebate, price-volume agreements, volume or expenditure cap, free initial treatment, etc.) and performance-based agreements that evaluate the treatment's performance (coverage with evidence development, payment by result, conditional treatment continuation, etc.) [4,6,7].

In Romania, a scorecard model called HTA was introduced in 2014. This is based primarily on the results of the assessments or reimbursement status for the respective drugs in EU countries. The health authorities decided in 2015 to increase the financial sustainability of the new drugs through the transfer of financial risk to MAHs via Managed Entry Agreements (known locally as Cost-Volume contracts, which are financial-based agreements, and Cost-Volume-Results contracts, which are performance-based agreements) [8]. The introduction of MEAs allowed a thawing of the introduction of innovative products on the reimbursement list for medicine (the List), after many years of impasse.

To conclude a functional MEA for a drug, there are several mandatory steps in Romania, some of which are confidential and some of which are transparent. These steps are (1) publishing of the List prices in the Official Gazette; (2) submitting the HTA dossier, obtaining the HTA body's approval and publishing the HTA Report; (3) negotiating the MEA, if applicable; and (4) adding the drug on the List.

METHODS

We performed a critical review of the Romanian legislation and rules, from the last 10 years, looking for the confidentiality and transparency aspects covering the pricing and reimbursement of drugs with MEA.

In our research, we worked to identify the elements of the lack of transparency in the price negotiation process for medicines or related to it that could lead to a discrepancy between the benefits offered to patients and the actual price paid.

RESULTS

The Romanian regulations do not stipulate the confidentiality of the final purchase prices. The joint order from the Minister of Health and the President of the National Health Insurance House (NHIH) requires that any confidential information obtained during the execution of the MEA contract (Cost-Volume contract) be used only to fulfill the MEA's purpose and not be disclosed or utilized for other reasons, including the final price of the medication in question [9].

Our analysis revealed that drug reimbursement in Romania through MEAs involves a blend of public and confidential data.

In Romania, there are two List Price catalogs: (1) one used for public reimbursement (Canamed) [10] and (2) one used only by pharmacies or health care providers not having a public reimbursement (Public Catalogue) [11]. Both catalogs are publicly available in the Official Gazette and on the Ministry of Health website [12], while Canamed can be seen also with the reimbursement price on the local payer website [13] (last update on July 1st, 2021). For the International Reference Pricing process done by other countries, the Ministry of Health recommends the use of the Public Catalogue (with higher List prices than Canamed).

The submission of the HTA dossier is a highly confidential process, with most of the steps not transparent: the content of the HTA dossier, the members of the evaluation team from the HTA body, the deadline for the evaluation, and the involvement or not of the Specialty Commissions from the Ministry of Health. Every month, the HTA body (National Agency for Drugs and Medical Devices – ANMDM) publishes a list of the drugs received for HTA evaluation. Next, the HTA report for each drug and the decision and the meeting minutes of the Appeal Committee (only for the drugs where the MAH was not satisfied with the results of the HTA report) are also published.

The HTA methodology allows the MAH to offer (part of the HTA dossier) a "confidential discount" to the payer (NHIH), one key aspect of confidentiality, resulting in a lower price compared to its competitor. This "confidential discount" should be part of a Protocol between MAH and the payer to include the obligation of the MAH to lower the net price (not visible) once the HTA report is positive and then the drug is included in the Reimbursement List 2020 [14].

The negotiation of a MEA is mainly a confidential process. The decision to apply for an MEA relies on the MAH level and also the proposal to be addressed to the Negotiation Committee. The negotiation is contingent upon the number of patients under contract and the eligible population. Ministry of Health (MoH) Specialty Commissions establish the eligible population and publish it on the payer website. Often, these Commissions are not transparent about the data they use to define and establish the eligible population. The number of contracted patients is strictly confidential because the ratio of contracted patients to eligible patients determines the discounts to be

paid by MAH as part of MEA (between 25-75%). The table with these discounts is publicly available and published in the Official Gazette (Table 1) [9,15]. This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

Table 1: Discounts for MEA depending on the market share covered

Percent (%) applied to quarterly consumption (single drug)	Percent (%) applied to quarterly consumption (multiple winners for substitutable drugs)	Percent (%) no. of contracted patients from the no. of total eligible patients
25%	30%	<5%
30%	35%	5-15%
35%	40%	15-25%
40%	45%	25-35%
45%	50%	35-45%
50%	55%	45-55%
55%	60%	55-65%
60%	65%	65-75%
70%	75%	>=75%

The term for these MEAs is one year and is publicly available, their starting point is the first month after the inclusion of the drug in the List.

The renegotiation process involves a simpler approach, with a publication of the moment for negotiation and a clear request for the Specialty Commissions to provide arguments regarding the change in the eligible population.

Other optional aspects of the negotiation, like the coverage by the MAH of some testing or other services, are usually available to the public on the websites of the local affiliates of the payer, due to the need to inform the providers of these aspects [9].

After the conclusion and the execution of the MEA, the MAH will pay the final discount to the payer according to the effective patient share, irrespective of the contracted patient share and discount. The respective data is provided by the payer only to the MAH and nobody else could access this information.

The Parliament approves the payer budget for MEA negotiation and contracting through the State Budget Law. The process of determining this MEA budget is not transparent to the public [16]. Meanwhile, the payer discloses the remaining budget after every round of MEA negotiations. The payer typically reserves a certain amount of its drug budget for drugs that require a new yearly MEA contract to ensure the continued operation of existing MEAs. If the contracting budget is over, no new MEA is signed and for the existing MEAs, there is no renegotiation; only the included patients can continue their treatment and there are no new inclusions of patients.

The budget for drugs with MEA is not available to the public on a monthly or quarterly basis, but at the end of each year, the authorities issue a note detailing the total budget used. MEA's budget information is also included in the Payer Yearly Report, published in the second half of the following year.

The decision to include the drug on the List is highly political, as it requires budget allocation for its introduction. The process for the calculation of budget needs for new drugs is not transparent. The information is only publicly available once the draft of the List is published for public comment.

DISCUSSION

Some authors have pointed out that the classically invoked reasons, namely that the prices of new drugs are very high because most of the income is reinvested in research, are no longer in line with the reality of the time. Drug manufacturers often carry out a series of additional purely financial operations, such as mergers and acquisitions and stock transactions, and play an active role in the capital market, etc. [9,17]. In recent years, concern has grown regarding the confidentiality of the final purchase prices of medicines; in 2019 WHO recommended to member states that they take measures in this regard [18].

The MEA process's opacity creates advantages and disadvantages for the MAH and NHIH regarding the confidentiality of final prices paid by the public payers. The opaque process of setting new budgets extends also for the case when MEAs and the random addition of expenses every time a new product is conditionally approved creates confusion among drug beneficiaries and competing MAHs. Other beneficiaries of the health-care system who require additional

funds (except for new drugs) frequently raise concerns.

The legislation for the MEA budget grants access to new drugs on a first-come, first-served basis. Priority criteria are also considered and include the unmet need, the drugs with EU emergency approval, and the public health reasons [15].

The payer does not transparently publish the volume (units) of drugs, including those with MEAs. The only publicly available data on the payer's website is at the ATC3 level and the INN level for the top 10 drugs in terms of value reimbursed in open pharmacies for each category (A, B, C, D, and E) of the reimbursement List [19]. Contract monitoring is challenging because currently there is no way to access treatment outcome data or resource utilization for patients receiving MEA treatments; the payer does not conduct cost-effectiveness analyses or publish data; and manufacturers do not have access to confidential patient outcomes.

The confidentiality stipulated in the MEA prevents not only price comparisons but also the comparisons of the treatment outcomes, making it impossible for any researcher to develop any pharmacoeconomic analysis or to monitor the physician's performance.

There are no transparent rules on the term to publish the updated List (and include the newly evaluated drugs), as the legislation only specifies an update once a year. The practice has shown that shorter terms are needed.

The payer agrees to keep the final prices confidential to avoid the risk of the producer choosing not to sell in Romania than to affect the prices in other countries and decrease the access to new technologies for the population, which would be discriminatory.

The confidentiality of the final price could also be viewed as means to respond to limited resources available in the healthcare sector and is linked with delayed access to innovative technology which could address also the quality of care and patient safety, but even with more resources these aspects will be removed only through a better understanding from the health care professionals [20].

Before 2017, payers and manufacturers would sign collaboration protocols, which included confidential discounts. Protocols aimed to decrease prices in Romania's public healthcare system without affecting the visible drug price or international reference price. Due to unclear legislation, these protocols were abolished and replaced with a new type of protocol where the MAH could support some of the financing burden of the payer [21].

The Payer determines the reimbursement prices (reference price) based on a specific methodology, but its application is not transparent [22]. Anyone can find prices on NHIH's website, and pharmacies will charge co-payments if drug prices are higher than the reimbursement price.

As other authors have already pointed out, the various confidentiality elements of the MAH process prevent other possible cost-containment policies from being used. Meanwhile, in Romania, some of these policies are also used (biosimilars/generic stimulation for prescription, reference pricing, protocols and guidelines for prescriptions, some open tenders for exclusivity) while others are waiting to be implemented (price ceilings, value-based pricing, pay for performance, etc.) [23]

Looking at the future development of MEA in Romania, there are plans to expand from simple cost-volume contracts to other types of MEA, including the payment for performance mechanisms. For this aspect, there will be a need for merging patient-level data with drug-patient data, yet not generally available in Romania at the health system level, but only in some of the hospitals, and the definition and the confidentiality of these patient outcomes will be a new issue during the implementation [24,25].

The future of MEA in Romania will be linked with the new EU Directive on HTA, which will become effective in 2025, and some local projects funded by the MOH in regarding pricing, reimbursement, and HTA. The utilization of a classic HTA model with cost-effectiveness in place could replace the actual scorecard HTA model. This will create the case for the confidentiality of prices used in the cost-effectiveness analysis but will benefit from the public availability of outcomes measures like the local values for Quality Adjusted Life Years (QALY) and will catalyze the efforts to determine the cost of QALY in Romania [26,27,28].

CONCLUSIONS

The negotiation process for the MEA in Romania results in a confidential final price, making it difficult to compare with other EU countries.

Confidential price rises question the legitimacy of some decisions and, in particular, the impossibility of checking over time the cost-effectiveness of drugs in the absence of the knowledge of the cost effectively paid. The solution is finding proper ways to provide more transparency in the process, especially regarding budget allocation. This can be obtained using guidelines as a result of cooperation between all interested actors.

From this perspective, the entire process of delivering drugs to the public domain is a new paradigm that requires new means of increasing the equity of financial allocations within the

healthcare system while ensuring profitability for the MAH and accessibility for the patients. The key solution is better collaboration and communication between stakeholders keeping in mind to meet patients' needs and expectations.

Conflicts of interest and sources of funding

The authors declare no conflict of interest. This research received no external funding.

Authors' contribution

Conceptualization, M.S.P. and C.P.R. methodology, M.S.P. and C.P.R., validation, M.S.P. and C.P.R., formal analysis, M.S.P., C.P.R., and B.C.P. resources, M.S.P., C.P.R., B.C.P., data curation, M.S.P., writing—original draft preparation, M.S.P., writing—review and editing, C.P.R., and B.C.P. visualization, B.C.P.; supervision, M.S.P. All authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

Not applicable

Patient consent for publication

Not applicable.

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ORIGINAL ARTICLE

Sentinel Node Biopsy Positivity: Is Axillary Dissection Always Necessary? – A Retrospective Analysis

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Abstract: *Breast cancer remains a significant health concern, with predictions indicating a rise in global incidence. While the primary focus is on oncological radicality, the aesthetic and psychological impacts of surgical interventions, such as radical mastectomy, cannot be ignored. This study, conducted over 8 years, aimed to identify factors that can prevent unnecessary lymphadenectomy. The research analyzed various parameters, including age, BMI, tumor size, and immunohistochemistry, to determine their correlation with axillary lymph node invasion. The results highlighted the importance of tumor size and estrogen receptor status in predicting axillary lymph node metastasis. The study underscores the value of sentinel lymph node biopsy in early breast cancer treatment, emphasizing its benefits over complete axillary lymph node dissection.*

Keywords: *breast cancer, lymphadenectomy, sentinel node biopsy*

INTRODUCTION

According to the American Cancer Society, one out of eight women in the United States will develop breast cancer during their lifetime [1]. Predictions regarding the global incidence of breast cancer in women outline the idea that the incidence will reach approximately 3.2 million new cases per year by 2050.

These numbers reflect the magnitude of breast cancer incidence, its impact on women worldwide, and the ongoing and urgent need to take measures towards prevention and proper therapeutic management [2].

The goal of oncological radicality remains essential and obligatory, but breast neoplasm surgery must also consider the aesthetic component due to the psychological impact that a major surgical intervention, such as radical mastectomy, has on patients. In the early stages, breast cancer can be treated without such a procedure being necessary.

Therefore, in today's times, the gold standard of breast cancer surgery is represented by conservative treatment. It involves the resection of the primary tumor with oncological safe margins and sentinel lymph node biopsy, followed by adjuvant radiotherapy [2,3].

The surgical management of the axilla has always been a controversial topic. For a very long time, it was believed that achieving good loco-regional control involves a complete axillary lymph node dissection, with this method being the gold standard of that era.

In recent years, due to the observed increase in morbidity and decrease in the quality of life for these patients, the method has been abandoned and replaced by sentinel lymph node biopsy.

The technique of identifying the sentinel lymph node (SLN) is based on the theory of the lymphatic spread of cancers, with the neoplastic invasion occurring in a lymph node that drains lymph from the site of the tumor [4,5] .

The prime objective of this study is the identification of factors that can prevent unnecessary lymphadenectomy.

MATERIALS AND METHODS

Based on the purpose and objectives of the research, the research design and methodology have been defined, with its main characteristics being: retrospective - spanning over an 8-year interval - January 1, 2014 - December 31, 2021, single-center, descriptive and correlational, non-interventional.

The eligibility criteria were as follows:

- Diagnosis of breast cancer
- The presence of axillary management after a positive sentinel node biopsy
- Female sex
- Stage IIA-IIIB:
  - o IIA (T2N0M0, T1N1M0)
  - o IIB (T2N1M0, T3N0M0)
  - o IIIA (T3N1M0)
  - o IIIB (T4N1M0)
- Single tumor, excluding multifocal and bilateral cases
- The presence of all required data
- Age > 18 years
- M0
- Any Histopathological type

RESULTS

The working hypothesis considers patients who underwent the sentinel lymph node technique, and the result was positive. In this situation, studies have shown that up to 1/3 of cases present a negative result of the axillary lymphadenectomy. To assess the risk factors for a positive axillar biopsy, we made a comparison between group E – a positive result of axillary lymphadenectomy (34 cases), and group D – a negative result of axillary lymphadenectomy (15 cases) (Table 1).

Table 1: Comparative data of groups (E vs. D)

Study lot	D	E
SNB	✓	✓
SNB results	+	+
ALND	✓	✓
ALND results	-	+
Case numbers	15	34

Age

There was no statistical correlation between the age of the patients and the presence of axillary invasion in patients with a positive sentinel lymph node, even though there was a mean difference of 5.85 years - p=0.175 (Table 2 and Figure 1).

Table 2: Comparative statistical data regarding age (E vs. D)

	Age	
	E	D
Valid	34	15
Missing	0	0
Mean	63.324	57.467
Std. Deviation	14.367	12.118
Minimum	28.000	29.000
Maximum	89.000	78.000

	t	df	p	Mean difference	SE difference
Age	1.376	47	0.175	5.857	4.258

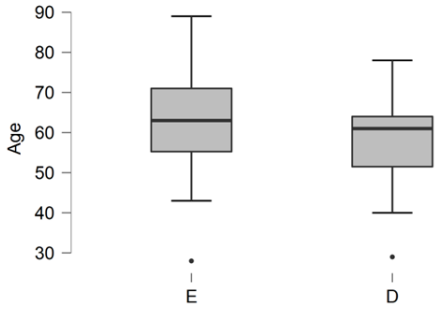

Figure 1: Comparative statistical data regarding age (E vs. D)

BMI

The Body Mass Index (BMI) value calculated for the two groups showed a higher average in the case of patients with axillary invasion, with a difference of 3.58 and moderate statistical significance - p=0.05 (Table 3 and Figure 2).

Table 3: Comparative statistical data regarding BMI (E vs. D)

	BMI	
	E	D
Valid	34	15
Missing	0	0
Mean	28.696	25.107
Median	28.100	24.900
Std. Deviation	6.187	4.608
Minimum	18.970	19.760
Maximum	45.650	33.650

	t	df	p	Mean difference	SE difference
BMI	2.009	47	0.050	3.589	1.786

Bio-clinical analysis

Comparative assessment of routine laboratory test values collected upon admission highlights relatively small differences between the groups discussed in this hypothesis.

The only parameter that demonstrated moderate statistical significance was the white blood cell count - with a mean difference of 1.44 and p=0.065 (Table 4 and Figure 3). Tumor markers did not show statistically significant differences.

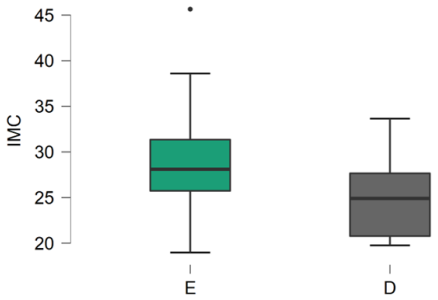

Figure 2: Comparative statistical data regarding BMI (E vs. D)

Table 4: Comparative statistical data regarding bio-clinical analysis (E vs. D)

	Hb		Ht		WBC		ESR		INR		Fibrinogen	
	E	D	E	D	E	D	E	D	E	D	E	D
Valid	34	15	34	15	34	15	34	15	34	15	34	15
Mean	12.515	12.647	40.529	40.067	8.726	7.280	31.971	29.800	1.189	1.130	434.029	378.600
Std. Deviation	1.259	1.148	3.518	4.559	2.550	2.253	18.909	16.618	0.313	0.215	186.974	174.482
Minimum	8.50	9.90	35.00	31.00	5.00	3.90	8.00	3.00	0.830	0.840	201.00	205.00
Maximum	15.30	13.80	48.00	47.00	14.70	10.90	101.00	57.00	1.940	1.60	835.00	823.00

Quadrant

The location of the tumor did not correlate with the extent of the axillary invasion, even though there were differences in the distribution of cases concerning this parameter, the statistical significance was not relevant (Tables 5 and 6).

Table 4: Comparative statistical data regarding bio-clinical analysis (E vs. D) – cont.

	t	df	p	Mean difference	SE difference
Hb	-0.347	47	0.730	-0.132	0.380
Ht	0.387	47	0.701	0.463	1.196
WBC	1.893	47	0.065	1.446	0.764
ESR	0.384	47	0.703	2.171	5.659
INR	0.657	47	0.514	0.059	0.089
Fibrinogen	0.975	47	0.334	55.429	56.830

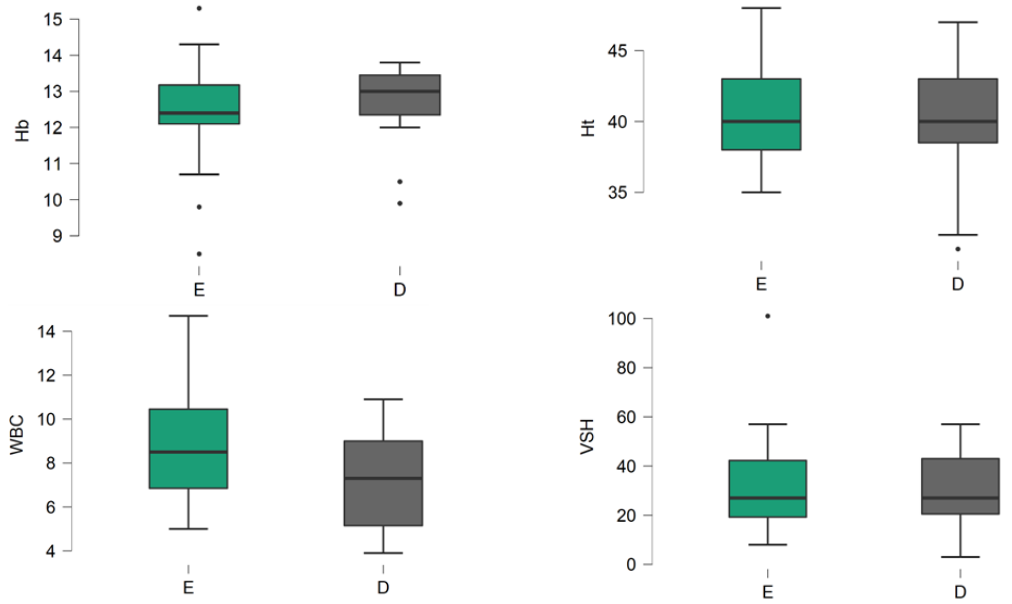

Figure 3: Comparative statistical data regarding bio-clinical analysis (E vs. D)

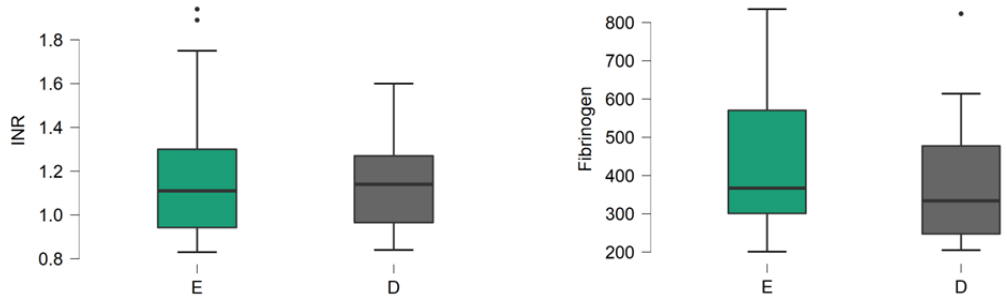

Figure 3: Comparative statistical data regarding bio-clinical analysis (E vs. D) – cont.

Table 5: Comparative statistical data regarding the tumor location (E vs. D)

Frequencies for Quadrant					
Hypothesis 2	**Quadrant**	**Frequency**	**Percent**	**Valid Percent**	**Cumulative Percent**
E	C	3	8.824	8.824	8.824
	SE	20	58.824	58.824	67.647
	SI	3	8.824	8.824	76.471
	IE	7	20.588	20.588	97.059
	II	1	2.941	2.941	100.000
	Missing	0	0.000		
	Total	34	100.000		
D	C	2	13.333	13.333	13.333
	SE	12	80.000	80.000	93.333
	SI	0	0.000	0.000	93.333
	IE	1	6.667	6.667	100.000
	II	0	0.000	0.000	100.000
	Missing	0	0.000		
	Total	15	100.000		

Chi-Squared Tests			
	Value	**df**	**p**
X^2	3.922	4	0.417
N	49		

Tumor size

The distribution of tumor sizes highlights higher values within group E - with axillary lymph node invasion, with a mean difference of 8.7 mm and a statistical significance of p=0.04 (Table 7 and Figures 4 and 5).

Immunohistochemistry

From the perspective of evaluating the presence of hormone receptors in the immunohistochemical assessment of breast resection specimens, only estrogen receptors have been identified as a risk factor for axillary invasion beyond the sentinel lymph node. The calculated risk is 7.33 times with a statistical significance of p=0.005 (Tables 8 - 10).

Subtype

From the perspective of molecular subtypes, the division into Luminal A, Luminal B, and Triple Negative did not show a statistically significant correlation with the presence of axillary lymph node invasion in patients with a positive sentinel lymph node. In the case of the HER2 sub-type, the risk of axillary lymph node invasion was nearly 10 times higher - p=0.084 (Tables 11-14).

Table 6: Distribution of cases concerning the localization in the interior of the exterior quadrant of the tumor (E vs D)

Ext 1 vs Int 0 quadrant		**Hypothesis 2**		
		0	**1**	**Total**
0	Count	7.000	2.000	9.000
	% within row	77.778 %	22.222 %	100.000 %
	% within column	20.588 %	13.333 %	18.367 %
1	Count	27.000	13.000	40.000
	% within row	67.500 %	32.500 %	100.000 %
	% within column	79.412 %	86.667 %	81.633 %
Total	Count	34.000	15.000	49.000
	% within row	69.388 %	30.612 %	100.000 %
	% within column	100.000 %	100.000 %	100.000 %

Table 6: Distribution of cases concerning the localization in the interior of the exterior quadrant of the tumor (E vs D) – cont.

	Log Odds Ratio	95% Confidence Intervals		p
		Lower	Upper	
Odds ratio	0.522	-1.183	2.227	
Fisher's exact test	0.512	-1.331	2.926	0.702

Table 7: Comparative statistical data regarding the tumor size (E vs. D)

	Size	
	E	D
Valid	34	15
Mean	21.235	12.533
Std. Deviation	14.078	11.154
Minimum	4.000	1.000
Maximum	58.000	47.000

	t	df	p	Mean Difference	SE Difference
Size	2.115	47	0.040	8.702	4.115

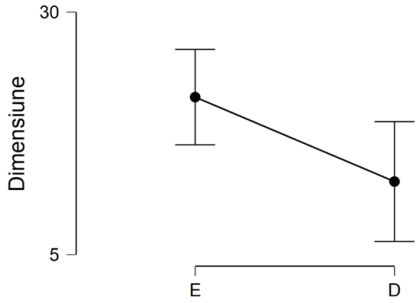

Figure 4: Comparative statistical data regarding tumor size (E vs. D)

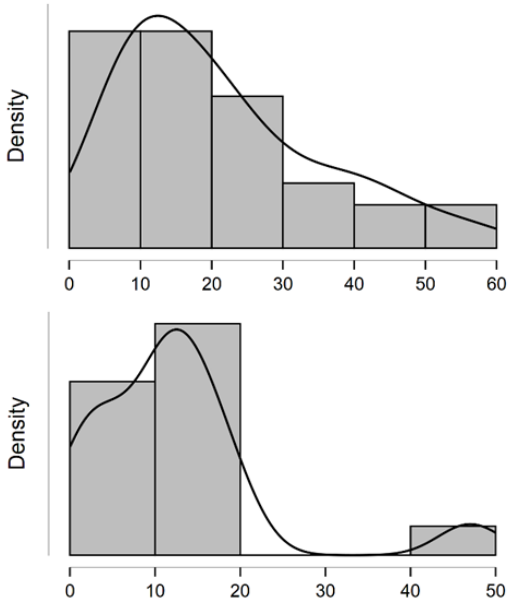

Figure 5: Distribution of cases concerning the tumor size (E left vs. D right)

Table 8: Distribution of cases concerning the presence of ER receptors (E vs D)

ER+		Hypothesis 2		Total
		0	1	
0	Count	22.000	3.000	25.000
	% within row	88.000 %	12.000 %	100.000 %
	% within column	64.706 %	20.000 %	51.020 %
1	Count	12.000	12.000	24.000
	% within row	50.000 %	50.000 %	100.000 %
	% within column	35.294 %	80.000 %	48.980 %
Total	Count	34.000	15.000	49.000
	% within row	69.388 %	30.612 %	100.000 %
	% within column	100.000 %	100.000 %	100.000 %

	Odds Ratio	95% Confidence Intervals		p
		Lower	Upper	
Odds ratio	7.333	1.724	31.186	
Fisher's exact test	7.019	1.498	46.390	0.005

Table 9: Distribution of cases with the presence of PR receptors (E vs D)

PR+		Hypothesis 2		Total
		0	1	
0	% within row	72.000 %	28.000 %	100.000 %
	% within column	52.941 %	46.667 %	51.020 %
1	Count	16.000	8.000	24.000
	% within row	66.667 %	33.333 %	100.000 %
	% within column	47.059 %	53.333 %	48.980 %
Total	Count	34.000	15.000	49.000
	% within row	69.388 %	30.612 %	100.000 %
	% within column	100.000 %	100.000 %	100.000 %

	Odds Ratio	95% Confidence Intervals		p
		Lower	Upper	
Odds ratio	1.286	0.381	4.344	
Fisher's exact test	1.279	0.321	5.206	0.762

Table 10: Distribution of cases with the presence of PR receptors (E vs D)

HER2+		Hypothesis 2		Total
		0	1	
0	Count	22.000	10.000	32.000
	% within row	68.750 %	31.250 %	100.000 %
	% within column	64.706 %	66.667 %	65.306 %
1	Count	12.000	5.000	17.000
	% within row	70.588 %	29.412 %	100.000 %
	% within column	35.294 %	33.333 %	34.694 %
Total	Count	34.000	15.000	49.000
	% within row	69.388 %	30.612 %	100.000 %
	% within column	100.000 %	100.000 %	100.000 %

	Odds Ratio	95% Confidence Intervals		p
		Lower	Upper	
Odds ratio	0.917	0.254	3.308	
Fisher's exact test	0.918	0.198	3.851	1.000

Table 11: The distribution of cases concerning the Luminal A subtype (E vs D)

Luminal A		Hypothesis 2		Total
		0	1	
0	Count	21.000	7.000	28.000
	% within row	75.000 %	25.000 %	100.000 %
	% within column	61.765 %	46.667 %	57.143 %
1	Count	13.000	8.000	21.000
	% within row	61.905 %	38.095 %	100.000 %
	% within column	38.235 %	53.333 %	42.857 %
Total	Count	34.000	15.000	49.000
	% within row	69.388 %	30.612 %	100.000 %
	% within column	100.000 %	100.000 %	100.000 %

	Odds Ratio	95% Confidence Intervals		p
		Lower	Upper	
Odds ratio	1.846	0.541	6.302	
Fisher's exact test	1.823	0.455	7.538	0.363

DISCUSSION

The literature describes four parameters as independent factors of lymph node metastasis, namely lymphovascular invasion, primary tumor size, multiple foci, and tumor localization.

An independent prognostic factor for disease-free survival (DFS) that has been recently analyzed in literature for patients with breast carcinoma is the degree of lymphovascular invasion [6].

Additionally, lymphovascular invasion is associated with a higher frequency of axillary lymph node metastases [7]. To use this data as a tool in determining the indication for sentinel lymph node biopsy (SLNB) or axillary lymphadenectomy, more extensive studies are needed to specifically determine the degree of invasion and the cutoff value that necessitates radical intervention.

Table 12: The distribution of cases concerning the Luminal B subtype (E vs D)

Luminal B		Hypothesis 2		Total
		0	1	
0	Count	26.000	10.000	36.000
	% within row	72.222 %	27.778 %	100.000 %
	% within column	76.471 %	66.667 %	73.469 %
1	Count	8.000	5.000	13.000
	% within row	61.538 %	38.462 %	100.000 %
	% within column	23.529 %	33.333 %	26.531 %
Total	Count	34.000	15.000	49.000
	% within row	69.388 %	30.612 %	100.000 %
	% within column	100.000 %	100.000 %	100.000 %

	Odds Ratio	95% Confidence Intervals		p
		Lower	Upper	
Odds ratio	1.625	0.428	6.171	
Fisher's exact test	1.608	0.331	7.329	0.500

Table 13: The distribution of cases concerning the Luminal B subtype (E vs D)

Her2		Hypothesis 2		Total
		0	1	
0	Count	27.000	15.000	42.000
	% within row	64.286 %	35.714 %	100.000 %
	% within column	79.412 %	100.000 %	85.714 %
1	Count	7.000	0.000	7.000
	% within row	100.000 %	0.000 %	100.000 %
	% within column	20.588 %	0.000 %	14.286 %
Total	Count	34.000	15.000	49.000
	% within row	69.388 %	30.612 %	100.000 %
	% within column	100.000 %	100.000 %	100.000 %

	Odds Ratio	95% Confidence Intervals		p
		Lower	Upper	
Odds ratio	0.118	0.006	2.214	
Fisher's exact test	0.000	0.000	1.466	0.084

A series of studies have demonstrated that younger age is a risk factor for positive axillary lymph nodes (ALN) [8–11]. This has been attributed to more biologically aggressive tumors in this younger age group. The average age of menopause is around 50 years, so most literature establishes 50 years as a cutoff point in exploring the relationship between age and clinicopathological factors in invasive breast cancer [8,12]. The current study did not show correlations between the risk of axillary lymph node spread and age, BMI, and the biological status of breast cancer patients, with results similar to those described in the studies.

Table 14: The distribution of cases concerning the Triple-negative subtype (E vs D)

Triple negative		Hypothesis 2		Total
		0	1	
0	Count	28.000	13.000	41.000
	% within row	68.293 %	31.707 %	100.000 %
	% within column	82.353 %	86.667 %	83.673 %
1	Count	6.000	2.000	8.000
	% within row	75.000 %	25.000 %	100.000 %
	% within column	17.647 %	13.333 %	16.327 %
Total	Count	34.000	15.000	49.000
	% within row	69.388 %	30.612 %	100.000 %
	% within column	100.000 %	100.000 %	100.000 %

	Odds Ratio	95% Confidence Intervals		p
		Lower	Upper	
Odds ratio	0.718	0.127	4.051	
Fisher's exact test	0.723	0.063	4.803	1.000

Histologic grade and tumor size (often over 5 centimeters in size) are also important predictors of lymph node metastasis. The probability is high if a patient has a large tumor and a high histologic grade. Our presented data show that patients with larger tumor sizes and a high histologic grade are more likely to have axillary invasion, a finding that aligns with those from previous studies [1,6,13–15]. Patients with small invasive breast cancer might benefit from SLNB.

The inclusion of extranodal extension in the evaluation protocol is necessary to achieve precise oncologic staging. Although attempts have been made to establish cut-off values for the circumferential and perpendicular diameters of invasion extension, these have not yielded significant predictive values. Therefore, more extensive studies are needed to specifically determine the dimensions of invasion from which axillary lymph node metastatic burden can be predicted, subsequently guiding the appropriate therapeutic approach [16,17].

Estrogen receptor status was another factor that showed a significant correlation with axillary lymph node involvement, with a calculated risk of axillary invasion beyond the sentinel lymph node being 7.33 times higher in HER+ patients. Various studies have evaluated the role of hormonal receptor status in predicting axillary lymph node metastasis (ALNM) with contradictory results. Capdet et al. [18,19], in their study of 1416 patients with early breast cancer, found no association between hormonal status and lymph node involvement. In contrast, Lee et al. [20] reported a high incidence of ALNM regardless of tumor size in the Korean population with T1-2 breast carcinoma. Finally, in a recent study conducted by Holm-Rasmussen et al. [21], patients with ER-negative tumors had a reduced risk of ALN involvement at the time of diagnosis compared to other patients.

Large integrative analyses are necessary to explain why ER-negative tumors have a low rate of axillary metastases and a high rate of distant metastases. Conversely, HER2-positive tumors have an axillary metastasis rate closely linked to tumor size and a high rate of distant metastases [22,23].

Among the limitations of the study, the retrospective nature of its design, the small number of tracked patients, and the lack of information regarding the use of chemotherapy, radiotherapy, or hormone therapy are identified.

Systemic therapies, including hormonal therapy, cytotoxic chemotherapy, and/or radiation therapy, remain critically important, especially in the treatment of breast cancer with positive lymph nodes [24–28]. Therefore, the results of this study will contribute to decision-making regarding the addition of axillary lymph node dissection (ALND), systemic therapy, and/or radiotherapy in the case of SLN metastases efforts to determine the cost of QALY in Romania [26,27,28].

CONCLUSIONS

Regional lymph node staging is necessary for tumor staging and surgical planning. Previous studies have identified several important factors in predicting axillary lymph node metastasis (ALNM), but the predictive power has not been satisfactory.

With advancements in surgical techniques, sentinel lymph node biopsy (SLNB) is now widely used in early breast cancer and has become a standard procedure in clinically node-negative patients. The benefits of SLNB include fewer surgical complications than complete axillary lymph node dissection (ALND) and effectiveness and accuracy in long-term follow-up.

Conflicts of interest and sources of funding

The authors declare no conflict of interest. This research received no external funding.

Authors' contribution

Conceptualization, R.A-M. and D.N.P.; methodology, O.A.; software, S.N.; validation, A.B., F.S. and M.C.D.; formal analysis, D.I.; investigation, R.A-M.; resources, D.N.P.; data curation, O.A.; writing—original draft preparation, S.N.; writing—review and editing, A.B.; visualization, F.S.; supervision, M.C.D.; project administration, D.I.; funding acquisition, R.A-M. All authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

The study was conducted following the Declaration of Helsinki, and approved by the Institutional Review of University Emergency Hospital of Bucharest.

Patient consent for publication

Informed consent was obtained from all subjects involved in the study.

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ORIGINAL ARTICLE

Hepatic Steatosis is the Most Common Finding in Abdominal Ultrasonography Performed in Patients with Psoriasis before Biological Agent Treatment

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Abstract: *Psoriasis has been associated with metabolic syndrome and nonalcoholic fatty liver disease. We wanted to investigate the abdominal ultrasonography results of psoriasis patients before biological agent treatment. Between April 2019 and February 2022, abdominal ultrasonography results of psoriasis patients which were performed before biological agent treatment were reviewed retrospectively. This study included 90 patients, 45 females, and 45 males with a mean age of 48.91±13.43 years. The most common ultrasonography finding was increased echogenicity of the liver parenchyma due to steatosis (58.9%) which was followed by an increase in liver size (25.6%), kidney cysts (17.8%), gallstones (8.9%) and increase in spleen size (7.8%). Hepatic steatosis was more frequent in older patients (p<0.001), patients with diabetes (p=0.007), and patients who were previously treated with biological agents (p=0.007). 70.4% of the patients with moderate or severe hepatic steatosis were female. Herein, hepatic steatosis was detected in 53 (58.9%) patients. Nonalcoholic fatty liver disease which presents as hepatic steatosis in early stages has an asymptomatic course and poor prognosis in psoriasis patients. We suggest that abdominal ultrasonography should be performed in psoriasis patients before biological agent treatment, especially in females, patients with advanced age, patients who were previously treated with biological agents, and patients with diabetes.*

Keywords: *abdomen; biological agents; hepatic steatosis; psoriasis; ultrasonography*

INTRODUCTION

Psoriasis is a systemic inflammatory disorder that primarily affects the skin and may present with various comorbidities such as psoriatic arthritis, nonalcoholic fatty liver disease, diabetes, metabolic syndrome, and inflammatory bowel disease [1]. Tumor necrosis factor-α (TNF-α), interleukin-17 (IL-17), and IL-23 are the cytokines mainly responsible for the inflammation in psoriasis [2]. Chronic inflammation has been implicated in the development of accompanying comorbidities. On the other hand, non-alcoholic fatty liver disease which is an important comorbidity of psoriasis due to its risk of progressing to cirrhosis has been associated with the development of severe psoriasis since it may lead to inflammatory cytokine production from the liver [3]. Biological agents affect specific pathways of the immune system that are implicated in the etiopathogenesis of psoriasis. Several biological agents have been developed and successfully used for the treatment of psoriasis. It has been suggested that biological agents were more effective and safer than conventional systemic treatments of psoriasis which lead to a broad immunosuppressive activity [4]. However, some safety concerns have been raised due to the widespread and long-term use of biological agents. Hepatotoxicity, reactivation of hepatitis B and latent tuberculosis infection, exacerbation of inflammatory bowel disease, and invasive fungal infections have been reported in psoriasis patients treated with biological agents. Side effects vary on each biological agent received [5]. For instance, administration of TNF-α inhibitors for long periods may lead to weight gain which has a negative impact on nonalcoholic fatty liver disease in patients with psoriasis [6].

Furthermore, it is controversial whether biological agent treatment increases the risk of malignancy in patients with psoriasis or not. Fiorentino et al. reported that the risk of malignancy might be increased in patients with psoriasis treated with TNF-α inhibitors for longer than a year [7]. On the

other hand, Mastorino et al. stated that the use of biological agents affecting TNF-α, IL-17, IL-12, and IL-23 for the treatment of psoriasis was safe even in patients with a past medical history of malignancy [8]. It has been suggested that abdominal ultrasonography might be performed in patients with psoriasis before the initiation of treatment with biological agents to exclude intraabdominal malignancy such as lymphoma and it might be repeated during the treatment [9]. However, abdominal ultrasonography is not included as a pretreatment test to be performed in psoriasis patients before the initiation of biological agents in major guidelines such as Joint American Academy of Dermatology – National Psoriasis Foundation (AAD-NPF) guidelines of care for the management and treatment of psoriasis with biologics and EuroGuiDerm guideline on the systemic treatment of psoriasis vulgaris [10, 11].

Within this study, we aimed to evaluate abdominal ultrasonography results of patients with psoriasis which were performed before the initiation of biological agent treatment**.**

MATERIALS AND METHODS

Gazi University Ethics Committee approval was obtained before this study (approval number: 2022-264). Between April 2019 and February 2022, medical records of patients with psoriasis who underwent abdominal ultrasonography before biological agent treatment were evaluated retrospectively. Psoriasis type, disease duration, psoriasis treatment, accompanying psoriatic arthritis, and systemic diseases were reviewed. All patients with psoriasis aged 18 years and over with complete data have been included in the study.

Statistical analysis was performed using SPSS version 20.0. Quantitative variables were represented as mean ± standard deviation or median and interquartile range, categorical variables were represented as counts and percentages. Kolmogorov-Smirnov test was used to determine whether variables were normally distributed or not. Differences in variables between the two groups were evaluated by chi-square test, independent sample t-test, and Mann-Whitney U test. P values less than 0.05 were considered statistically significant.

RESULTS

This study included 90 patients, 45 females and 45 males. The mean age of the patients was 48.91±13.43 years (range: 19-75 years). 79 (87.8%) patients had psoriasis vulgaris, 8 (8.9%) patients had generalized pustular psoriasis, and 3 (3.3%) patients had palmoplantar psoriasis. The mean disease duration was 19.35±11.93 years (range: 2-53 years). The median Psoriasis Area and Severity Index (PASI) was 7.2 (11.4) [12]. Psoriatic arthritis was detected in 34 (37.8%) patients. 34 (37.8%) patients were treated with ustekinumab, 29 (32.2%) patients were treated with IL-17 inhibitors such as secukinumab and ixekizumab, 23 (25.6%) patients were treated with TNF-α inhibitors such as adalimumab, infliximab and certolizumab pegol, 4 (4.4%) patients were treated with IL-23 inhibitors such as risankizumab and guselkumab. All patients previously received conventional treatments such as acitretin, cyclosporine, and methotrexate. 15 (16.7%) patients were previously treated with phototherapy. 37 (41.1%) patients were previously treated with biological agents whereas 53 (58.9%) patients were biologic-naive. None of the patients stated excessive alcohol consumption. Past medical history was unremarkable in 48 (53.3%) patients. However, 23 (25.6%) patients had hypertension, 17 (18.9%) patients had diabetes, 4 (4.4%) patients had chronic obstructive pulmonary disease, 4 (4.4%) patients had asthma, 4 (4.4%) patients had hypothyroidism, 4 (4.4%) patients had chronic hepatitis B virus infection, 3 (3.3%) patients had chronic kidney disease, 3 (3.3%) patients had coronary artery disease, 1 (1.1%) patient had nephrolithiasis, 1 (1.1%) patient had villous adenoma of the colon. In addition, 1 (1.1%) patient had a history of testicular seminoma, 1 (1.1%) patient had cervical cancer, and 1 (1.1%) patient had urothelial carcinoma.

Abdominal ultrasonography revealed an increase in liver size in 23 (25.6%) patients (the mean craniocaudal length in the midclavicular line: 16.69±0.96 mm, range: 15.5-20 mm). Among the patients with increased liver size, 17 (73.9%) were female and 6 (26.1%) were male (p=0.013). Increased liver size was observed in 14 (60.9%) patients with psoriatic arthritis and in 9 (39.1%) patients without arthritis (p=0.008) (Table 1). Increased liver size was more common in patients with hypertension (10, 43.5%) compared to patients without hypertension (13, 19.4%) (p=0.023). Increased liver size was observed in 6 (35.3%) patients with diabetes and 17 (23.3%) patients without diabetes (p=0.309).

The mean psoriasis duration was 22.15±12.47 years in patients with increased liver size and 18.39±11.68 years in patients with normal liver size (p=0.185). Among the patients with increased liver size, the mean craniocaudal length was 17.03±1.10 mm in those who were biologic-naive and 16.25±0.48 mm in those who received biological agents previously (p=0.037). All patients with increased liver size showed increased echogenicity of the liver parenchyma due to steatosis. An increase in echogenicity of the liver parenchyma due to steatosis was reported in 53 (58.9%) patients [mild: 26 (28.9%), moderate: 14 (15.6%), severe: 13 (14.4%)]. The mean age of the patients with and without increased echogenicity of the liver parenchyma due to steatosis was 53.62±11.71 years and 42.16±12.99 years, respectively (p<0.001).

Table 1: Patients with increased liver size and increased echogenicity of the liver parenchyma

	Increased liver size		P value
Patient characteristics (n, %)	Female (17, 73.9)	Male (6, 26.1)	0.013
	With psoriatic arthritis (14, 60.9)	Without psoriatic arthritis (9, 39.1)	0.008
	With hypertension (10, 43.5)	Without hypertension (13, 19.4)	0.023
Craniocaudal length (mm, mean±SD)	Biologic-naïve (17.03±1.10)	Previous biologic (16.25±0.48)	0.037
	Increased echogenicity of the liver parenchyma due to steatosis		
Patient characteristics (n, %)	Previous biologic (28, 75.7)	Biologic-naïve (25, 47.2)	0.007
	With diabetes (15, 88.2)	Without diabetes (38, 52.1)	0.007
Moderate/severe (n, %)	Female (19, 70.4)	Male (8, 29.6)	0.021

SD: Standard deviation. Increased liver size was more common in patients with psoriatic arthritis and hypertension. Moderate or severe increase in the echogenicity of the liver parenchyma due to steatosis was more common in female patients.

Among the patients with increased echogenicity of the liver parenchyma due to steatosis, 28 (75.7%) received biological agents previously and 25 (47.2%) were biologic-naive (p=0.007). Increased echogenicity of the liver parenchyma due to steatosis was detected in 15 (88.2%) patients with diabetes and in 38 (52.1%) patients without diabetes (p=0.007). Among the patients with a moderate or severe increase in the echogenicity of the liver parenchyma due to steatosis, 19 (70.4%) were female and 8 (29.6%) were male (p=0.021). In addition, heterogeneous liver parenchyma was reported in 5 (5.6%) patients, hypoechoic areas in the liver which might be compatible with focal fatty sparing in 4 (4.4%) patients, liver cyst in 3 (3.3%) patients, hyperechoic liver lesions which might be compatible with hemangioma in 3 (3.3%) patients and Chilaiditi syndrome was reported in 1 (1.1%) patient.

Moreover, gallstones were detected in 8 (8.9%) patients (the mean diameter: 15±7.05 mm, range: 3-20 mm) and gallbladder polyps in 3 (3.3%) patients (the mean diameter: 4.5±0.5 mm, range: 4-5 mm). Among the patients with gallstones and gallbladder polyps, 7 (63.6%) were female and 4 (36.4%) were male (p=0.141). The mean age was 53.73±13.32 years in patients with gallstones and gallbladder polyps and 48.24±13.40 years in those who had neither (p=0.094). Gallbladder wall thickening (4 mm) and echogenicities in the gallbladder wall (cholesterolosis) were detected in 1 (1.1%) patient. An increase in the transverse diameter of the gallbladder (42 mm) and accompanying gallbladder wall thickening (6 mm) were detected in 1 (1.1%) patient. Furthermore, kidney cysts were detected in 16 (17.8%) patients (the mean diameter: 26.5±15.8 mm, range: 8-50 mm). Increased echogenicity of kidney parenchyma was detected in 5 (5.5%) patients. The mean ages of the patients with and without kidney cysts were 56.81±7.54 years and 47.20±13.85 years, respectively (p=0.008). Kidney stones were detected in 4 (4.4%) patients (the mean diameter: 7.5±5.8 mm, range: 3-16 mm). Lobulated kidney contour was detected in 4 (4.4%) patients. Dilated renal collecting system was detected in 3 (3.3%) patients, focal caliectasis was detected in 1 (1.1%) patient, increase in kidney size was detected in 1 (1.1%) patient, decrease in kidney size was detected in 1 (1.1%) patient and a renal angiomyolipoma was detected in 1 (1.1%) patient. Abdominal ultrasonography also revealed increase in spleen size in 7 (7.8%) patients (the mean craniocaudal length: 13.87±1.05 mm, range: 13-16 mm), accessory spleen in 2 (2.2%) patients, splenic cyst with a diameter of 7 mm in 1 (1.1%) patient, situs inversus totalis in 1 (1.1%) patient, calcifications in prostatic parenchyma in 1 (1.1%) patient, heterogeneous prostatic parenchyma in 1 (1.1%) patient, an ovarian cyst measured 33x27 mm in size in 1 (1.1%) patient, uterine myoma with a diameter of 11 mm in 1 (1.1%) patient, increased bladder wall thickness (6 mm) in 1 (1.1%) patient, echogenic debris within the bladder which might be compatible with urinary tract infection in 1 (1.1%) patient (Table 2).

Based on ultrasonography findings, abdominal computed tomography (CT) was performed in 3 (3.3%) patients for further evaluation of kidney cysts and abdominal magnetic resonance imaging (MRI) in 2 (2.2%) patients for hypoechoic liver lesions and liver cysts. Renal scintigraphy was performed in 1 (1.1%) patient for the dilation of the renal collecting system.

DISCUSSION

Within this study, the most common abdominal ultrasonography finding in patients with psoriasis was increased echogenicity of the liver parenchyma due to steatosis (58.9%). Hepatic steatosis was more frequent in older patients, patients with diabetes, and patients who were previously treated with biological agents (p<0.001, p=0.007, p=0.007, respectively). These results may be related to the fact that patients previously treated with biologic agents were older than biologic-naive ones (p=0.021). In addition, increased echogenicity of the liver parenchyma due to steatosis was moderate or severe in 50.9% of the patients and

most of these patients (70.4%) were female. An increase in liver size (25.6%) was the other common abdominal ultrasonography finding followed by kidney cysts (17.8%), gallstones (8.9%), and an increase in spleen size (7.8%). The increased liver size was statistically significantly more prevalent in female patients, and patients with psoriatic arthritis and hypertension (p=0.013, p=0.008, p=0.023, respectively).

Table 2: Abdominal ultrasonography findings in patients with psoriasis before biological agent treatment

	Patients n (%)	Mean size±SD (mm)	Range (mm)
Increased liver parenchyma echogenicity	53 (58.9)		
Increased liver size	23 (25.6)	16.69±0.96	15.5-20
Kidney cysts	16 (17.8)	26.5±15.8	8-50
Gallstones	8 (8.9)	15±7.05	3-20
Increased spleen size	7 (7.8)	13.87±1.05	13-16
Increased kidney parenchyma echogenicity	5 (5.6)		
Heterogeneous liver parenchyma	5 (5.6)		
Kidney stones	4 (4.4)	7.5±5.8	3-16
Lobulated kidney contour	4 (4.4)		
Hypoechoic areas in the liver	4 (4.4)		
Liver cyst	3 (3.3)		
Liver hemangioma	3 (3.3)		
Gallbladder polyps	3 (3.3)	4.5±0.5	4-5
Dilated renal collecting system	3 (3.3)		
Accessory spleen	2 (2.2)		
Gallbladder wall thickening	2 (2.2)		
Echogenicities in the gallbladder wall	1 (1.1)		
Focal caliectasis	1 (1.1)		
Echogenic debris within the bladder	1 (1.1)		
Increased kidney size	1 (1.1)		
Decreased kidney size	1 (1.1)		
Renal angiomyolipoma	1 (1.1)		
Increased bladder wall thickness	1 (1.1)		
Splenic cyst	1 (1.1)		
Uterine myoma	1 (1.1)		
Ovarian cyst	1 (1.1)		
Heterogeneous prostatic parenchyma	1 (1.1)		
Calcifications in the prostatic parenchyma	1 (1.1)		
Chilaiditi syndrome	1 (1.1)		
Situs inversus totalis	1 (1.1)		

SD: Standard deviation. An increase in echogenicity of the liver parenchyma due to steatosis was the most common abdominal ultrasonography finding in patients with psoriasis.

Data on abdominal ultrasonography findings in patients with psoriasis treated with biological agents are scarce. Raudonis et al. reported that hepatic and pancreatic steatosis, hepatomegaly, kidney cyst, splenomegaly, gallbladder polyp, and pancreatic stiffness were commonly detected findings in abdominal ultrasonography which were annually performed in patients with psoriasis treated with infliximab, etanercept, and ustekinumab. Among them, hepatic steatosis (12.7%) and pancreatic steatosis (12.7%) were the most common [13].

Nonalcoholic fatty liver disease is a metabolic disorder that may present with steatosis, and nonalcoholic steatohepatitis and may result in cirrhosis or hepatocellular carcinoma [14-16]. Advanced age and diabetes are the risk factors for the development of nonalcoholic fatty liver disease, whereas the

effect of gender on disease remains controversial [17]. In addition, nonalcoholic fatty liver disease is more common in psoriasis patients with a prevalence of up to 65% compared to the general population [15]. Furthermore, poor prognosis has been reported in patients with nonalcoholic fatty liver disease accompanied by psoriasis [14,15]. Dermatologists are recommended to perform abdominal ultrasonography for screening fatty liver in patients with psoriasis, particularly in those who have metabolic syndrome even without elevated liver enzyme levels [6]. Dermatologists must determine accompanying nonalcoholic fatty liver disease in patients with psoriasis to choose appropriate treatment options. Dermatologists should also encourage patients for lifestyle changes or refer them to a gastroenterologist if needed [16]. For instance, Campanati et al. suggested that weight gain during anti-TNF-α treatment might increase the risk of hepatic fibrosis in psoriasis patients with nonalcoholic fatty liver disease [18]. Medications that may contribute to steatosis should be omitted in these patients. However, no specific treatment has been reported or primarily recommended for the treatment of psoriasis patients with nonalcoholic fatty liver disease [16]. On the other hand, TNF-α inhibitors such as adalimumab, etanercept, and infliximab may lead to hepatotoxicity. Among them, infliximab was the most commonly implicated one in the development of adverse effects on the liver such as jaundice, hepatitis, acute liver failure, cholestasis, and autoimmune hepatitis [19]. Even mild and rare, hepatotoxicity has been detected in patients who received ustekinumab [20].

Abdominal ultrasonography may be useful in patients with psoriasis since psoriasis has been associated with nonalcoholic fatty liver disease or intraabdominal malignancies such as hepatocellular carcinoma, gastric cancer, bladder cancer, lymphoma of the stomach and colon adenocarcinoma [21,22]. Intraabdominal malignancy and infections have also been reported in patients who received biological agents for the treatment of psoriasis. For instance, Nosotti et al. reported a 59-year-old male patient with psoriasis treated with adalimumab and diagnosed with B cell lymphoma with gastric, splenic, hepatic, abdominal lymph node, and vertebral involvement. Nosotti et al. suggested that the risk for the development of abdominal lymphoma should be kept in mind in patients with psoriasis under biological agent treatment. Nosotti et al. recommended performing abdominal ultrasonography before and during biological agent treatment for psoriasis [9]. Fortaleza et al. reported a 43-year-old male patient with psoriasis who developed splenic tuberculosis under infliximab treatment. Fortaleza et al. stated that close follow-up of patients treated with TNF-α inhibitors on the risk for extrapulmonary tuberculosis and disseminated tuberculosis was crucial [23]. Bud et al. reported peritoneal tuberculosis in a 32-year-old patient who received adalimumab for the treatment of psoriasis vulgaris. The patient had multiple splenic lesions on computed tomography of the abdomen and underwent surgery due to an initial diagnosis of a possible malignancy. Bud et al. suggested that Quantiferon test, abdominal ultrasonography, and computed tomography might be performed to exclude intestinal tuberculosis in patients with gastrointestinal symptoms treated with TNF-α inhibitors [24].

CONCLUSIONS

We suggest that patients with psoriasis should undergo abdominal ultrasonography before biological agent treatment not only to exclude intraabdominal malignancy and infections but also to determine hepatic steatosis. It should be kept in mind that nonalcoholic fatty liver disease has an asymptomatic course and poor prognosis in patients with psoriasis. Since lifestyle modification and weight loss can reverse early stages of nonalcoholic fatty liver disease such as steatosis [25], early detection of hepatic steatosis is crucial in the treatment of patients with psoriasis. Therefore, we suggest that abdominal ultrasonography should be performed in patients with psoriasis before biological agent treatment, especially in females, patients with advanced age, patients who were previously treated with biological agents, and patients with comorbidities such as diabetes. In addition, considering the high frequency of nonalcoholic fatty liver disease in patients with psoriasis, the most appropriate biological agent treatment should be determined in this population and stated in psoriasis treatment guidelines.

Conflicts of interest and sources of funding

The authors declare no conflict of interest. This research received no external funding.

Authors' contribution

Conceptualization, F.T., O.A., M.P., E.A.; methodology, F.T.; software, F.T.; validation, F.T., O.A., M.P., E.A.; formal analysis, F.T.; investigation, F.T., O.A.; resources, F.T., O.A., M.P., E.A.; data curation, F.T., O.A., M.P., E.A.; writing-original draft preparation, F.T.; writing-review and editing, M.P., E.A.; visualization, F.T.; supervision, M.P., E.A.; project administration, F.T., O.A., M.P., E.A. All authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki, and local ethics committee approval for this study was obtained from Gazi University (approval number: 2022-264).

Patient consent for publication

Informed consent was obtained from all subjects involved in the study.

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ORIGINAL ARTICLE

Essential Oils and their Components in Combating Fungal Pathogens of Human Skin-lipophilic Yeast Malassezia furfur

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Abstract: *The most common problem of modern medicine is the development of increasing antifungal drug resistance nowadays including fungal infections of the skin and its appendages caused by dermatomycetes, yeast-like fungi, and molds so, there will be a need for alternative antifungal treatment. Natural substances with high antifungal properties like essential oils and their components show potential in this regard. Citrus lemon and Citrus sinensis essential oils were screened against Malassezia furfur by disc diffusion method and Minimum Inhibitory Concentration was determined by Modified Microdilution method. Citrus lemon (lemon) and Citrus sinensis (orange) show excellent inhibitory effects on the lipophilic, yeast-like fungus Malassezia furfur which causes Pityriasis versicolor, a chronic superficial fungal disease of the skin. The diameter of the inhibition zone was found to be 70 mm and 60 mm respectively. Malassezia furfur was found to be resistant against Clotrimazole & Ketoconazole. The minimum inhibitory concentration (MIC) of lemon and orange oil against Malassezia furfur was found to be 0.6 µl/ml and 0.8 µl/ml. These findings concluded that the use of Citrus lemon (lemon) and Citrus sinensis (orange) oil showed remarkable and excellent antifungal activity, and can be used as a traditional herbal medicine for the control of Pityriasis versicolor infection of the skin after undergoing clinical trials.*

Keywords: *Pityriasis versicolor, skin, Malassezia furfur, Citrus lemon and Citrus sinensis oils*

INTRODUCTION

Pityriasis versicolor (PV) is a superficial mycosis, that affects the superficial layer of stratum corneum [1]. The causative organism is Malassezia furfur, a yeast-like lipophilic fungus. Previously the mycelial form was called either Pityrosporum ovale or Pityrosporum orbiculare [2]. In 1951, Gordon isolated the organism M.furfur and renamed it P.orbiculare. It was recognized that M.furfur is the correct name and that P.orbiculare and P.ovale are synonyms [3]. The disease is most prevalent in early adulthood and small children are rarely affected [4-5]. PV is common in the post-pubertal age where sebaceous glands are active and in individuals who sweat more [6]. The prevalence in colder climates is less than 1% [7]. M. furfur is a component of normal skin flora in more than 90% of adults living in tropical areas [8]. Infectious diseases account for a high proportion of health problems in developing countries including India. Microorganisms have developed resistance to many antibiotics and as a result, immense clinical problem in the treatment of infectious diseases has been created [9]. The resistance of the organisms increased due to the indiscriminate use of commercial antimicrobial drugs commonly used for the treatment of infectious diseases. This situation forced the researchers to search for new antimicrobial substances from various sources including medicinal plants [10-11]. A survey of the literature reveals that many essential oils possess antifungal activity. Therefore, we need to search for plant-derived antifungal drugs which are safe and without side effects. Herbal medicines may be in the form of powders, liquids, or mixtures, which may be raw or boiled, ointments, liniments, and incisions [12]. Essential oils have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases [13]. Essential oils have been shown to possess antibacterial, antifungal, antiviral, insecticidal, and antioxidant properties

[14-15]. Numerous essential oils have been tested for both in vitro as well as in vivo antifungal activity and some pose much potential as antifungal agents. Studies on the antimicrobial, especially antibacterial and antifungal activity of lemon grass oil and its components were reported [16-18]. Orange oil was composed principally of d-limonene, at 94%, and myrcene at about 3%. The essential oils of lemon and orange exhibited antifungal activity against common molds Aspergillus niger, A.flavus, Penicillium chrysogenum, and P.verrucosum. In the present study, the antifungal activities of lemon and orange oils were investigated to discover the medicinal potential of these oils for future application as an antifungal agent for Pityriasis versicolor disease.

MATERIALS AND METHODS

Extraction and Preparation of Oil

In the summer season, extraction of oil from the peels of lemon (Citrus lemon) and orange (Citrus sinensis) was carried out by standard hydrodistillation method, Clevenger's apparatus, and all operations were carried out at room temperature. The fresh peels of lemon and orange were washed to remove soil and sliced. Sliced pieces of fresh peels of lemon and orange (250 g) were placed in a separate flask, together with distilled water (1 L). After 5-6 hours, oil was collected from the apparatus, anhydrous with sodium sulfate for removal of water traces, then this 100% pure essential oil was dispensed into dark bottles and stored at $4^{O}C$ until used. Essential oil was ready to use for disc diffusion and determination of MIC.

Screening of essential oil using Disc Diffusion method

Oil was screened for its antifungal activity against M.furfur by disc diffusion method [19]. Standard size Whatman No.1 filter paper discs, 6.0 mm in diameter, sterilized by dry heat at 140°C in an oven for one hour were used to determine antifungal activity. SDA medium for the disc diffusion test was prepared. After sterilization, it was poured into sterilized Petri plates and allowed to solidify. Then one day old, fresh culture of yeast will be used for inoculum preparation. A suspension that was just turbid (~0.5 McFarland standard) by visual inspection was prepared by suspending yeast in 0.9% NaCl solution and the homogeneous suspension was used for inoculation. Using a sterile cotton swab, yeast cultures were swabbed on the surface of sterile Sabourauds dextrose agar plates. Sterilized filter paper discs were soaked in neat, undiluted (100%) concentration of oils. Using ethanol-dipped and flamed forceps, oil-saturated discs of 100% concentration were aseptically placed over Sabourauds dextrose agar plates seeded with the respective test microorganism. The antibiotic discs of Clotrimazole and Ketoconazole (10 mcg/disc) were also aseptically placed over the seeded Sabouraud's dextrose agar plates as a standard drug for comparison of antifungal activity of lemon and orange oils. The plates were incubated at 37ºC for 24 hours. The diameter of the inhibition zones was measured in mm. Three replicates were kept in each case and average values were calculated. The activity of oils was measured by the following formula:

AI (Activity index) = Inhibition zone of sample/Inhibition zone of standard

Determination of Minimum inhibitory concentration using Microdilution method

The modified microdilution method by Provine and Hadley (2000) was followed to determine MIC [20]. Media used for MIC was semisolid agar media (Brain Heart Infusion Agar) aliquots of semisolid agar media (Bacto Agar; Difco Laboratories) at a pH of approximately 7.4 were poured into a 16-by 125-mm glass tube and autoclaved. A suspension that was just turbid (~0.5 McFarland standard) by visual inspection was prepared by suspending the selected fungi in 0.9% NaCl solution, vortexing, and homogeneous suspension was used for inoculation. Different concentrations of lemon and orange oils were added in media containing test tubes, afterwards a standard platinum loopful (~0.001 ml, Himedia, Flexiloop) of the inoculum suspension was inserted deep into each tube of medium containing a different concentration of oils, as well as an oil-free control, by a centered down-up motion to form a two-dimensional inoculum. The tubes were then incubated at 30OC for 48 hours to determine the MIC. MIC was read to be the lowest concentration at which there was no visible growth of the organism. Then, by visual inspection, good growth of the respective fungi in an oil-free medium as a control was detected (48 hrs for yeasts). Afterward, the growth in all tubes at different concentrations of lemon and orange oil was compared with that of the oil-free control to determine inhibition.

RESULTS

In our present work, M.furfur was found main etiological agent of Pityriasis versicolor disease hence antimycotic studies were carried out on M.furfur (Figure 1). The conventional treatment of fungal disease is limited, and part of the reason is due to the limited spectrum of the current antifungal drugs, and the expensive treatment, particularly due to the need for prolonged therapy. Thus, new drugs and alternative therapies are necessary, including natural products. We report here the antimycotic study of lemon and orange oil against M.furfur in vitro (Figure 2). The results of the present work on the antifungal activity of lemon and orange oil against M. furfur studied by two methods i.e Disc diffusion and Microdilution

method are presented in Tables 1, 2, and 3.

Table 1: Antifungal activity of lemon and orange oil against Malassezia furfur

Essential Oil	Test Strain	IZ of Essential Oil
C. limon	*Molassezia furfur*	70 mm
C. sinensis	*Molassezia furfur*	60 mm

Table 2: MIC of C. limon oil against Malassezia furfur

Test Strain	Different concentrations of lemon Oil used in µl/ml	Growth visually inspected in different concentrations of oil
Malassezia furfur	0.1	+4
	0.2	+3
	0.3	+3
	0.4	+2
	0.5	+1
	0.6	0
	0.7	0
	0.8	0
	0.9	0
	1	0
	Control without oil	100% growth

Growth was scored in the following manner: *4+, growth comparable to that of the oil free control; 3+, growth approximately 75% that of the control; 2+, growth approximately 50% that of the control; 1+, growth 25% or less that of the control; and 0, no visible growth*

Table 3: MIC of C. sinensis oil against Malassezia furfur

Test Strain	Different concentrations of lemon Oil used in µl/ml	Growth visually inspected in different concentrations of oil
Malassezia furfur	0.1	+4
	0.2	+3
	0.3	+2
	0.4	+2
	0.5	+1
	0.6	+1
	0.7	+1
	0.8	0
	0.9	0
	1	0
	Control without oil	100% growth

Growth was scored in the following manner: *4+, growth comparable to that of the oil free control; 3+, growth approximately 75% that of the control; 2+, growth approximately 50% that of the control; 1+, growth 25% or less that of the control; and 0, no visible growth*

In our study, lemon and orange oils presented higher diameters of inhibition zones. The diameter of the inhibition zone obtained against lemon and orange oil at 100% concentration of pure oil was 70 mm and 60 mm respectively by disc diffusion method (Figure 3). According to our study by comparing with the reference drugs, lemon and orange oil was found to be more effective in inhibiting the growth of M. furfur. Clotrimazole and ketoconazole were found to be resistant to Malassezia furfur (Figure 4). Results of MIC of lemon and orange oils against M. furfur. are summarized in Tables 2 and 3. The results show that the lemon and orange oil exhibited inhibitory action at 0.6 µl/ml and 0.8 µl/ml concentrations against M. furfur. Both oils can be used for the development of natural antifungal agents against Pityriasis versicolor infections.

Figure 1: M. furfur on SDA media with olive oil & Bottle shape yeast cells under microscopic examination

Figure 2: Fruit of Citrus lemon (lemon) & Citrus sinensis (sweet orange)

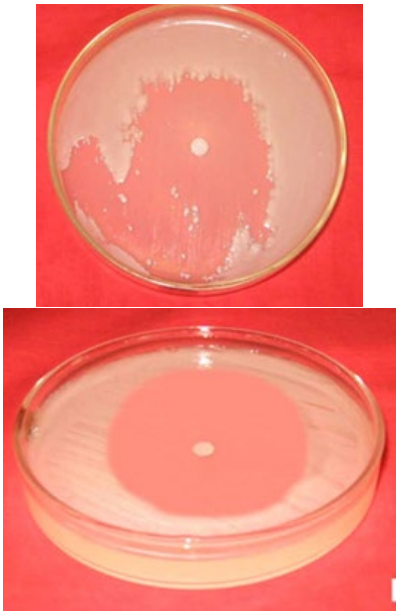

Figure 3: Antifungal activity of C. lemon & C. sinesis against Malassezia furfur

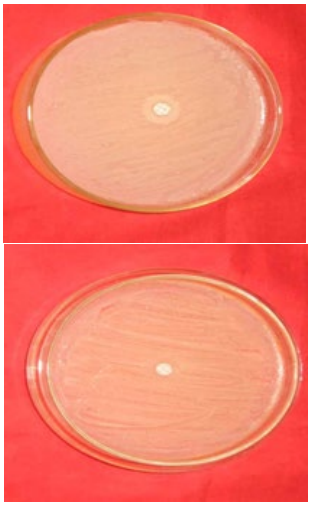

Figure 4: Resistance of Clotrimazole & Ketoconazole against Malassezia furfur

Their mechanism of action appears to be predominantly on the fungal cell membrane, disrupting its structure causing leakage and cell death; blocking the membrane synthesis; and inhibition the spore germination, fungal proliferation, and cellular respiration [22]. Because of the high volatility and lipophilicity of the essential oils, they are readily attached to penetrate the cell membrane to exert their biological effect [23]. The results showed the efficacy of lemon oil on animal skin infections caused by Trichophyton rubrum and Microsporum gypseum. In our study, lemon oil exhibited strong antimycotic activity against M. furfur. In the screening of lemon oil, the diameter of the inhibition zone by the disc-diffusion method was found to be 50 mm. MIC of oil obtained by microdilution method was 0.8µl/ml. Our work is in agreement with the observations of Silvia et al in which screening of lemon grass oil against Candida species was studied and it was found as an effective antifungal agent [24]. Our work also coincides with the previous findings of Ezzat et al which revealed that the essential oil of Citrus lemon at 200 µl concentration showed a diameter of inhibition zone 37 mm against Candida albicans 10261 [25]. According to Rusenova and Parvano Citrus lemon does not show antimicrobial activity by disc-diffusion method against both yeasts, Candida albicans and Malassezia pachydermatis [26]. These results are not in agreement with our studies which showed that antifungal activity by disc-diffusion and minimum inhibitory concentration by microdilution method, lemon oil was found to be effective in inhibiting the growth of M. furfur at a low concentration of oil. Lemon oil is also preferentially more active against Gram-positive bacteria as compared to Gram-negative bacteria. The antimicrobial activity of lemon oil is due to the presence of terpenoids-a-pinene, camphene, b-pinene, sabinene, myrcene, terpinene, b-bisabolene, limonene, trans-a-bergamotene, nerol and neral [27]. Essential oils from citrus offer the potential for all-natural antimicrobials for use in improving the safety of organic or all-natural foods [28]. Shahi et al in 2003 found that the MIC of oil of Citrus sinensis were in the range of 0.7-1 µl/ml against Candida albicans, Aspergillus flavus, A. fumigatus, A. niger, A. ustus, Epidermophyton floccosum, Microporum audouinii, M. canis, M. gypseum, M. nanum, Rhizopus nigricans, Trichophyton tonsurans, T. rubrum, T. mentagrophytes and T. violaceum [29]. The present investigation suggests that essential oils exhibit a narrow or wide range of antifungal activity, which may prove useful in the development of effective antifungal substances. Souza et al (2005) reported MIC of lemon oil at 0.5% concentration against mold strains i.e. Fusarium spp, Penicillium spp, Aspergillus niger, A. flavus, and Rhizopus spp [30]. In our findings, MIC of lemon oil against M. furfur was 0.8 µl/ml i.e. very close to that concentration. The small differences may be due to local environmental and climatic conditions and variations in the choice of microorganism tested.

Our results of orange (Citrus sinensis) and lemon (Citrus lemon) are also similar to the work of Viuda-Martos et al which suggests that both oils were effective in inhibiting the growth of Aspergillus niger, A.flavus, Penicillium chrysogenum and P. verrucosum [18]. The hydrodistilled oil of lemon and orange was also found to be more sensitive against Penicillium digitatum [31]. Antimicrobial activity of Rutaceae family species has been observed on dermatophytes [32] and on some molds [33]. Essential oils of some plants have recently been proven to be successful eco-friendly, bio-control agents [34]. Many authors have reported antimicrobial, antifungal, antioxidant, and radical-scavenging properties of essential oils [35-36].

DISCUSSION

The present results suggest that lemon and orange oils exhibit strong antifungal activity. This is in agreement with the findings of Valero and Frances who reported narrow and wide ranges of antifungal activites. Essential oils derived from many plants are known to possess antifungal activities [21].

CONCLUSIONS

The present study suggests that the oils extracted from the waste product (peels) of C. sinensis and C. lemon hold good promise as an antifungal agent, which could be used in therapeutic remedy against human pathogenic fungi on account of its various antifungal properties, viz. strong fungicidal action, long-shelf life, withstand heavy inoculum density, thermostable, broad range of antifungal activity and absence of any adverse effects. The waste product of C.

sinensis and C. lemon can be used for the development of a potential source of effective and economically viable herbal antifungal against Pityriasis versicolor (fungal skin infection) after undergoing successful clinical trials.

Conflicts of interest and sources of funding

The authors declare no conflict of interest. This research received no external funding.

Authors' contribution

RS contributed to design of experiments, review, discussion, and editing of the manuscript. GS contributed to the review and writing of the manuscript. RS contributed to all editing of the manuscript. ER contributed to the review of the manuscript. MS contributed to the editing and review of the manuscript.

Ethics approval and consent to participate

This article does not contain any studies with human and animal participants performed by any of the authors,

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