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ORIGINAL ARTICLE

Stressors and Coping Mechanisms of Divorced Women

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Abstract

Background/Aim: The high divorce rate in Indonesia has an impact on the number of divorced women. In Indonesia, women with divorced status are still stigmatised, whereas the stigma and perception of divorced women in the community are still skewed. The formation of a negative stigma from the community makes women who experience divorce face many problems that can increase stress and make women psychologically disturbed. To overcome the conflict experienced, individuals have a coping mechanism as a way to defend themselves against changes that occur both within themselves and from outside themselves. The purpose of this study was to explore the sources of stress and coping mechanisms for stress in divorced women in Cirebon Regency, West Java, Indonesia.
Methods: This research was qualitative research with a phenomenological approach. The research participants amounted to 8 women with a divorce status of less than 2 months who experienced mild and moderate stress in dealing with the new status of divorce in Cirebon Regency. The selection of research participants used a convenience sampling technique. Data collection methods using in-depth interview techniques and administering questionnaires perceived stress scale (PSS) to determine a person's level of psychological stress. Data analysis in this study used the Colaizzi method. This study identified two themes, namely the sources of stress of divorced women and coping mechanisms for stress built in divorced women.
Results: Sources of stress included social status, negative stigma from society, the process of raising children without a husband, economic factors, fear of facing life in the future and relationships with ex-husbands and their families. These various stresses did not prevent divorced women from continuing their life process. This was because of the coping mechanisms made by divorced women so that the stress they experience did not harm their lives. These coping mechanisms included self-management, enjoying the process, diverting problems, telling other people about problems, strengthening spirituality and self-reflection.
Conclusion: Divorced women struggle with numerous stressors. It is necessary to strengthen positive coping mechanisms and to overcome stigmas and prejudices in society towards divorced women.

Key words: Stressor; Coping mechanism; Divorced woman.

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Introduction

The hope of everyone who undergoes a marriage is basically to have a happy, eternal and harmonious family. But in the process of its journey, household relationships do not always run smoothly because

marriage brings together two individuals from different backgrounds. This is not an easy thing because these differences often present conflicts. Conflicts can come from outside or from within their households.

Marital conflicts that commonly arise (eg difficulty accepting differences, economic problems, children's problems, feelings of jealousy, division of roles, duties and responsibilities, not in line with the original purpose of marriage and the presence of a third person) trigger continuous and unresolved disputes so that the couple chooses to divorce as the best solution.

According to the Statistics Indonesia report, the number of divorce cases in Indonesia reached 516,334 cases in 2022 (113,643, 102,065 and 85,412 in West, Central and East Java, respectively). This figure increased by 15.31 % compared to 2021 which reached 447,743 cases. The number of divorce cases in the country in 2022 was the highest in the last six years.[1]

West Java province is the province with the highest divorce rate in 2022, Cirebon Regency is the district in West Java with the third highest divorce rate after Indramayu Regency and Garut Regency. In Cirebon Regency, the divorce rate has increased every year.[2]

Divorce is an event that ends a marriage legally, which is not planned and desired by the married couple. The impact of divorce is not only experienced by women, men also experience psychological impacts in dealing with divorce, but in divorced women, the stigma received is much greater than that of men. The stigma built by society against divorced women occurs because there are still assumptions that demean women and elevate the status of men.[3] The results of research by the independent age organisation prove that divorced women feel more loneliness, sadness and even depression than men.[4]

Divorced women are often viewed negatively by some Indonesians. When a woman sues the man for divorce for reasons such as not providing for the family, the woman is often blamed, with society judging that the woman cannot manage finances properly. When in the end women work to help to meet family needs, the community considers that the woman cannot take care of her husband properly. So, if the husband has an affair, the woman is again to blame. The divorcée status attached to women makes women even ostracised by the community. This happens because there are still some people who think that divorced women are women who cannot take good care of their households.[5]

Psychologically, divorced women will experience sadness, shame, trauma and mental shock, especially due to the factor of infidelity by their husbands, these women will experience a personal crisis in the form of negative emotions, namely anger, disappointment, crying easily, irritability and more closing themselves. Stressors and anxiety that arise can be an obstacle to living life in the future. Anxiety is an emotion and subjective experience of a person. Another definition of anxiety is a condition that makes people uncomfortable and is divided into several levels. So anxiety is related to feelings of uncertainty and helplessness.[6] When feeling anxious, individuals feel uncomfortable or afraid or may have a premonition of impending doom even though they do not understand why these threatening emotions occur. Anxiety is an internal warning device that signals danger to the individual. Anxiety will increase in families when one of their family members experiences a life-threatening illness.[7] To deal with this stressful situation, individuals must adapt to stressors. The stress response is also called coping mechanisms, each individual has a different coping mechanism in dealing with a problem depending on the level of stress, parenting and individual experience managing stressors.

Coping mechanisms are the way individuals manage and deal with situations that cause stress to move forward to achieve life goals. According to Stuart and Sundeen[8] coping mechanisms are classified into:

- Adaptive coping mechanisms. Adaptive coping mechanisms are coping mechanisms that support the functions of integration, growth, learning and achieving goals. The categories are talking to others, solving problems effectively, relaxation techniques, balanced exercise and constructive activities. Adaptive coping mechanisms include talking to others and seeking information about the problems faced, in addition to the effort also praying, doing physical exercise to reduce the tension of the problem, making various alternative actions to reduce the situation and feeling confident that everything will return

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to stability, taking lessons from past events or experiences.

- Maladaptive coping mechanism. Maladaptive coping mechanisms are coping mechanisms that inhibit the function of integration, break down growth, reduce autonomy and tend to control the environment and avoidance. Maladaptive coping mechanism behaviours include aggression and withdrawal. Aggression behaviour (attacking) against targets or in the form of objects, goods or people or even themselves. The withdrawal behaviour includes using alcohol and drugs, daydreaming and fantasy, sleeping a lot, crying and switching to other activities.

The purpose of this study was to explore the sources of stress and coping mechanisms for stress of divorced women in Cirebon Regency, West Java, Indonesia.

Methods

Design

The research method in this study used a qualitative descriptive method, which is an approach carried out in research that focuses on phenomena that occur or are natural and are carried out by going to the field.[9] The approach used was a case study approach, namely the search for cases or phenomena with data collection involving detailed information sources.[10]

Population and samples

The selection of participants in this study was carried out by purposive sampling. Initial data collection through in-depth interviews with divorced women who were divorced for less than 2 months in the Cirebon Regency area, a total of 8 participants.

Instruments and data collection

The data collection method used in-depth interview techniques and administered a perceived stress scale (PSS) questionnaire to determine a person's psychological stress level. Data were analysed by using the Colaizzi method, namely 4 stages: (1) collecting data from interviews in the form of primary and secondary data and making data transcripts by listening repeatedly to the recordings; (2) researchers read repeatedly the existing data transcripts so that researchers could find significant data meaning and underline important statements of participants; (3) determining categories and grouping into themes; (4) writing reports.[11]

Results

Based on the results of the interviews conducted, main characteristics of respondents are presented in Table 1.

Table 1: Characteristics of respondents

No	Age	Length of divorce	Number of children	Job	Stress level
1	38	1 month, 20 days	3	Factory employee	Mild
2	43	2 month	3	Former migrant worker	Moderate
3	35	1.5 month	2	Online merchant	Moderate
4	39	1 month	4	Household assistant	Moderate
5	45	2 month	2	Self-employed	Moderate
6	31	2 month	1	Self-employed	Mild
7	46	1 month, 25 days	3	Teacher	Mild
8	37	2 month	2	Factory employee	Moderate

It was found that 3 (37.5 %) participants experienced mild stress and 5 (62.5 %) participants experienced moderate stress after being assessed from the perceived stress scale (PSS) questionnaire to determine a person's psychological stress level.

From the thematic results of the participants, two themes were obtained which included: (1) sources of stress of divorced women and (2) coping mechanisms of divorced women against stress.

Source of stress

Divorced women experienced stress caused by several sources including: (1) social status, (2) negative stigma of society, (3) the process of parenting without the assistance of the husband, (4) economic factors, (5) low self-esteem, (6) fear of facing future life, (7) relationship with ex-husband and his family.

Social status

After a divorce, individuals will face new challenges in their lives, one of the challenges faced by women after a divorce is a new social status. The divorcée status that women hold

will change their social status in the community, which is a source of stress for divorced women. Participants felt ashamed and inferior to their new status. Three participants said they felt ashamed of their new status so they preferred to stay at home.

The following is the participant's statement:

> *... "I have been a divorcée since 2 months ago, I am ashamed of my divorcée status, I feel humiliated, I avoid gatherings and going out unless I have to buy something at the stall, I feel that everyone is talking about me*"...(P2)

> *... "Sometimes I still can't believe what happened, on the one hand, I'm relieved to divorce my husband, I've been fighting with him, huufftt... but yes, I'm not ready for the status of a divorcée. Shocked yes, embarrassed, especially Sist. I feel like everyone is blaming me for getting divorced"...(*P6)

From the above statement, it can be concluded that the source of stress that arises in divorced women less than 2 months is social status. Their new status makes them embarrassed to socialise with the community.

Negative stigma of society

The stigma attached to a divorced or widowed woman is not only associated with her social and economic status but also with her sexuality. Ethnographic research on the marital experiences of men and women shows that divorced women and divorcées bear a much heavier burden of stigma than men.

The following are two participants' statements regarding sources of stress about community stigma:

> ... "*What do you think about divorcées? You are a college student. I'm ashamed if here the name divorcée is still seen as ugly, I don't feel good hearing the word "divorcée" the impression is of a woman who is not right, an odd woman, a woman who is feared and will seduce their husbands, even though who also wants to divorce, I want to have a family that is harmonious forever, but I'm not strong enough to survive."...* (P8)

> ... "*I have been able to accept my status because this divorce is my wish so by divorcing I am even free, I am relieved. I'm not strong enough with his behaviour. During this time I worked selling online, while COD I also went around, people thought that this divorce was because of my bad behaviour, they thought that I liked to walk around, so now the mothers around me are labelling me badly. I'm sad, so I'm lazy to go out, lazy to meet people around."...* (P3)

Lyn Parker, professor and one of the lecturers at the University Of Western Australia Stigmatization in her paper entitled "The Stigmatization of Widows and Divorcées (Janda) in Indonesian Society", said that the stigmatisation of divorcées can be analysed from the institution of marriage. The institution stigmatises that ideally a woman should be married, outside of that, it is wrong.[12]

Divorcées are more closely associated with stigma than widows whose husbands died. A dead widow is more respected, especially if she decides not to marry because she is considered to still maintain her honour. Something that is highly valued by Indonesian society. Such labelling ultimately leads to women preferring to stay in bad marriages.[13]

Parenting without husband's assistance

Having the role of being a single parent will experience changes that can cause problems, which previously only played the role of father or mother after divorce must play multiple roles. The following are the words of two participants:

> ... "*I'm afraid, I'm afraid I can't raise my children, I have to work, take care of my children too, can I give the best for my children. I'm afraid I won't be able to do my best, I feel sorry for them."...* (P1)

> ... "*Child custody rights are indeed in me, I'm happy about that, but I'm also confused about how to pay for the children in the future, the father is already separated like this and doesn't necessarily want to give regularly, especially if he has remarried. I am afraid that my income is not sufficient for the needs of the children, especially since they are still young, they still need their father's figure."...* (P6)

One of the psychological well-being of children is determined by family harmony. A harmonious family will be able to carry out its functions and roles in building children's character optimally. However, social reality shows that not all families can condition and maintain a harmonious and comfortable household for children who eventually choose the path of divorce. Concerns

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about the child development process are one of the sources of stress for divorced women.

Economic factors

The status of being a single mother makes divorced women, especially those who do not work, have to think quickly about how to work and be responsible for the needs of their children. In Indonesian society, for average husband when divorced and custody moves to the wife's hands, the obligation to provide for his children also ends.

> ... "*How will I send my child to school, especially since this teteh is already in high school and wants to go to college, there is a fear that I can't pay for it, especially since I'm just a factory employee, it's enough for a day - just a day, I can save a little - a little, hopefully, the father will want to help the Children's school.*"... (P8)

> ... "*I'm still trying to accept it, but it's still hard, especially for the children's needs, I'm confused. I don't work, do I have to go to Arabia again for the children's future? My salary money from my time in Arabia was spent by my husband on unclear matters. Where will I get my daily needs from?*"... (P2)

This is also supported by research Leung, namely the feeling of anxiety experienced by single-parent mothers (single mothers) is the difficulty of finding work and thinking about the needs of daily life with children and panic thinking about the future of their children. Those who become single mothers, either due to death or divorce, apparently experience problems, especially economic problems.[14]

Low self-esteem

A person's self-acceptance is related to a positive self-concept, where with a positive self-concept, a person can accept and understand facts that are so different from himself. Self-acceptance is a positive attitude towards oneself, can accept one's situation calmly. In divorced women, it takes time to be able to accept themselves with their new social status and divorced women need time to adapt and accept their current situation.

> ... "*This is how it feels to be a divorcée, I'm still shocked, I still don't believe in my current status, I failed to get married, I'm still shy to go out and meet with people around me, I'm also ashamed of my friends who work with me, Alhamdulillah, they really support me, but I'm still ashamed.*"... (P7)

> ... "*I'm just inside the house, I'm embarrassed to go out, the children go out if they want to buy something, I'd rather buy something in a far place where people don't know me, I'm embarrassed that I'm already a divorcée. I feel that people look at me differently.*"... (P5)

It takes different time for each individual to be able to accept his or her situation, of course, the support of the surrounding environment such as family, children and the surrounding community to support and assist newly divorced women so that they can accept the circumstances that occur and have self-acceptance and increase their self-esteem.

Fear of facing life ahead

Women when divorced will think about many things far ahead, not only thinking about themselves, but also thinking about the psychology of children, child development, child education and how to raise children without a partner. Here are the participants' statements:

> ... "*The decision I took is not right, sometimes I still think about how I live with my children in the future without my husband, I am also afraid to get married again, there is a lot of scary news on TV-TV. I feel sorry for my children, but if I raise my children, how will their education be, how will they feel living without their father, afraid of being bullied.*"... (P6)

> ... "*Although economically, God willing, I can send my children to high school, they still need a father figure, I am afraid that their psychology will be disturbed, even now I am still devastated by this incident. I feel sorry for the children, they have started to mature and they have started to have a sense of shame about this incident. I'm afraid of lowering their concentration on learning, worried that I won't be able to control their relationships too.*"... (P7)

Single mothers more often feel anxiety and fear about the future after divorce, especially when they have children who have entered school age. It is women who more often feel anxiety and fear in facing the future after divorce.[15] Anxiety is a feeling of tension associated with fear, worry, feelings of guilt, feelings of insecurity and the need for certainty.[16]

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Relationship with ex-husband and his family

Another impact of divorce that is a source of stressors arising from divorce is the breakup of ties and communication between divorced couples, children and families. Divorce does not always end well, in fact, divorce always triggers a conflict between the parties' families. As stated by the participant:

> ... "*Yes, that's what worries me, I had a big fight with my husband before the divorce and until now there has been no communication, we blocked each other's WA numbers, I don't want to start contacting him, I am still irritated and hate him.*"... (P4)

> ... "*There is no communication yet... Yes, there is, I still keep his number, I don't know how our relationship will go in the future, there are children who still need us parents and I am afraid that the children will be cut off from their father, I am afraid that the children will be neglected, especially if he is married and has another child, how will the future be, but I still hate not wanting to meet first.*"... (P3)

The divorce between husband and wife does not just happen, some factors cause conflict between husband and wife in the household and when these conflicts cannot be resolved then the couple decides to divorce as a solution after divorce parents should still have a good relationship to be together in the process of raising children, but sometimes divorce has an impact on the relationship with the ex-husband after divorce.

Coping mechanisms

Coping mechanisms carried out by participants included: (1) self-management, (2) diversion of problems, (3) telling problems to others, (4) strengthening spirituality and (5) self-reflection.

Self-management

Stress management is about how an individual acts by involving thinking activities, emotions, plans and how to solve problems. Here are the statements of the participants:

> ... "*Stress, anxiety, irritation, sadness don't need to be asked mbak, I want to go far away to release the burden, but I remember I have children, how are they without me. Now I am still trying to continue to accept more, calm myself, convince myself that this is the best, my children always strengthen me.*"... (P7)

> ... "*If you say I'm sincere or not with this divorce, I'm still not sincere, but yes, how else, if I stay down like this, how will my children be, I'm trying to continue living life while introspecting myself, maybe this divorce is my fault too, so just try to live a little - a little to be sincere.*"... (P1)

> ... "*Being viewed negatively by the community because of my divorcée status hurts, yes, I used to dress up and go out to deliver orders and CO and I like to be talked about, but now I don't feel good about going out, but I try to accept maybe it's a form of input for me so that my appearance is improved again, but I still have to work, it's for feeding my children too.*"... (P3)

> ... "*I am trying to accept my new social status, learning to adapt to being a single parent, I must be optimistic and confident that I can educate my children without my husband.*"... (P8)

Self-stress management begins with identifying the sources of stress that occur in life. Then the next step is to choose an effective problem-solving strategy. In general, there are two ways, namely: a) change the situation (avoid the source of the problem) and b) change our reaction to the source of stress.

From these 2 ways, if we look at the first way, which is to change the situation, not everything can be changed as we want because sometimes the source of stress comes from outside and is difficult for us to change. The second way to deal with the source of stress is to change our reaction to the source of stress. It is not easy to see the positive value of the bad things we experience. But sometimes, when we try to accept unpleasant situations that cannot be changed, it is the first step to being able to see the positive side of what we experience. From the statements of the three respondents above, one of the coping mechanisms used by divorced women in dealing with their sources of stress is self-management.

Problem diversion

From the results of the interview, it was found that the participants said they needed to do several things to be able to divert attention to stress so that they could temporarily forget about the stress that they could then carry out activities better. One participant with mild stress diverted stress by taking care of her favourite flowers in the garden.

> ... "*I like taking care of the flowers, by taking care and watering the flowers, I can forget my stress*

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for a while, it's calmer taking care of the flowers so I get distracted."... (P1)

Then one participant with mild stress diverted by watching a funny Korean drama movie.

... *"I watch Korean"s Films Mbak, I choose funny ones - funny for entertainment if the movie is sad I will get sad again, so just look for funny ones."...* (P6)

One participant with mild stress said that she relieves stress by cooking.

... *"My hobby is cooking, I better divert it by cooking, making cakes. It's good enough to temporarily forget the stress."...* (P7)

Five participants with moderate stress divert stress by keeping themselves busy with online selling activities, exercising, cleaning the house, taking a bath with warm water and hanging out with friends.

... *"Instead of being stressed out, I'm getting more and more excited about selling online, posting merchandise and going if someone CODs, it's quite distracted for a while."...* (P3)

... *"My hobby is gymnastics with the mothers of this complex, but I am still shy to get together, I do gymnastics alone at home, the important thing is that my emotions and stress come out together with my sweat he..he..he."...* (P8)

... *"I divert it, if I get down, I can't do anything. I'm busy working and cleaning the house. The important thing is not to stay daydreaming."...* (P5)

..."*Take a warm bath, I give spices and flowers, my mind is also calm."...* (P2)

..."*After cleaning up at Bu Kuwu's house, I gathered with my friends at my house, made salad, ate together, Alhamdulillah the arrival of close friends relieved my sad problems."...* (P4)

Telling problems to others

One way that is often used as a coping mechanism for participants is to share and tell problems with others, some tell it to parents, family and children because their children are adults and some are friends. The following is the participant's statement:

..."*I tell my mom to relieve myself. I can't keep it to myself, sometimes I tell my close friends too."...* (P1)

..."*To my parents, often to my mother, to be relieved, often calmed by my mother."...* (P3)
..."*To my mother, how yes, I can't keep it to myself, when I tell her it's free. My mom always calms me down and encourages me."...* (P8)

..."*Tell my close friend, my bestie. She always listens to me whenever I need her."...* (P4)

..."*I always tell my mother, that I need to be calmed down, I need to be advised by my parents. But I also often talk to my children, coincidentally my children are already adults, they can share with me."...* (P7)

..."*I go to my cousin when I tell him stories, I'm close to him, he's nice to talk to, he always wins me over and listens to me."...* (P2)

Strengthening spirituality

When experiencing stress and anxiety after divorce, all participants, both those who experienced mild and moderate stress, took a spiritual approach to overcoming anxiety, by praying a lot, "istighfar", performing worship, believing that Allah would not abandon them and taking lessons from the events that happened to them. The following are the participants' statements:

..."*Always praying and praying, asking to be strengthened. Never get tired of asking God."...* (P1)

..."*I pray a lot, istighfar, my heart becomes calmer."...* (P3)

..."*Humans can only try and pray, yes mbak, with this incident I tried to calm myself by praying, I believe God will not leave me, just ask to be strengthened mbak."...* (P5)

..."*After Wudhu, istighfar, I pray, ask Gusti Allah, it makes my heart calm, I just surrender everything to Gusti Allah."...* (P2)

..."*Praying, reciting the Quran, praying is all I can do, asking Allah to strengthen me, asking to go through these times with patience and sincerity."...* (P4)

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...*"Just take lessons from this incident, nothing lasts forever and we must be prepared for any event, I believe this is the best from Allah."...* (P7)

Self-reflection

Self-reflection is a brooding activity to do self-introspection and look back at things that have happened in life, analyse habits, experiences and decisions that have been taken. In the participants, it was found that the self-reflection carried out was by self-introspection, making lessons and life experiences and remaining grateful that God still provided health. The following is the participant's narrative:

...*"contemplating, self-introspection, looking for self-deficiencies, finding how to solve problems so that I don't keep thinking about it."...* (P2)

...*"trying to introspect myself, what I've done, what my attitude is like, it's not entirely his fault."...* (P1)

...*"What yes, just a lot of introspection, maybe I haven't been able to be a good wife, maybe a lot of my attitude also makes him uncomfortable, it can calm me down a little bit."...* (P7)

...*"Self-introspection, making lessons and life experiences to be even stronger. Let me be free."...* (P5)

Discussion

In this study, two themes were generated from the results of interviews identified from the research objectives, namely sources of stress and coping mechanisms. The impact of divorce on women will be felt more than on men from before and after legalised divorce.

Sources of stress

The number of divorces increases in Indonesia every year making divorce cases a common thing. But for victims of divorce, this event has a psychological impact that is not only felt by divorced couples but the impact is also felt by children and families of both parties. Stress can be felt by both women and men but women have a higher risk of stress than men because of hormonal and genetic differences that cause women to experience stress more easily than men.[17]

In divorced women, some stressors have a negative impact and can interfere with the lives of divorced women carrying out their lives after being divorced, from the results of interviews, several sources of stress were found in 8 divorced women who were divorced for less than 2 months and had mild and moderate levels of stress.

The first source of stress is social status, experiencing changes with a new social status after divorce is one source of stress in women, divorced women need time to adapt to accepting the new status. Norms prevailing in society, state that the life of a woman who holds the status of a divorcée greatly affects psychologically due to the views of the community.[18]

Stress in responding to the stigma from society regarding widowhood is also a source of stress for divorced women. Divorcée status in women is often viewed negatively by some people. Although the cause of divorce is the man, society always views divorced women negatively. The divorcée status attached to women, makes women ostracised by society.[19] This happens because there are still some people who think that divorced women are women who cannot maintain their households properly.[4]

The process of raising children without the assistance of a husband is also a source of stress for divorced women, being a single mother, a woman who becomes a single parent requires a very heavy struggle because she has to raise children and meet the needs of family life and burdensome is the assumptions of the surrounding environment that put her as a single parent, it is very influential for the life of a single parent family, especially very influential on child development. The task of a parent, especially a mother, will become heavier if she has to be a single parent.[20]

Economic factors are one of the heaviest sources of stress experienced by divorced women, divorced women will immediately think about how to live and raise children without a husband, including financial matters, ex-husbands usually only provide for children as much as they can and even then, not all ex-husbands are responsible, mostly after the breakup of marriage, husbands lose responsibility in providing for their children. That is why divorced women make economic factors one of the sources of stress. After the divorce process, there will be a decrease in economic

problems for both men and women, according to research conducted in India divorced women will experience a greater economic impact than men.[21]

Divorced women tend to experience low self-esteem or low self-esteem problems, this is due to the negative stigma about divorcée status, divorced women feel embarrassed and need time to be able to accept themselves and failure to maintain a harmonious marriage relationship makes women become low self-esteem which will affect their social life in the future if not handled immediately.[22]

The next source of stress in divorced women is fear of facing life in the future. In divorced women, accustomed to living together with their husbands but when women lose their husbands, there will be a heavy burden of life that becomes their responsibility, so they have a very noble and important position because they will continue and maintain the continuity of the family.[16]

Relationships with ex-husbands and their families become a source of stress for divorced women because every divorced couple certainly has different reasons and goes through different processes before deciding to divorce, couples who divorce well, for example, for reasons of differences in principles and not hurting each other when divorcing, there is no reason not to stay on good terms after divorce. But if the divorce is based on conflict that hurts each other, it will create a source of stress for divorced women to keep in touch with their ex-husband and his family. It takes time for a divorced woman to restart a good relationship with her ex-husband if the divorce is based on a hurtful conflict, but it must be considered again if there are children in the marriage then restarting a good relationship with the ex-husband needs to be a concern.

All respondents in this study, both those with mild and moderate stress, still needed time to be able to re-establish communication with their ex-husband, the divorce that occurred by all participants was triggered by unfavourable conflict. They need time to heal the hurt and disappointment experienced.[23] Divorced married couples who have children need to make efforts so that they can maintain interactional relationships after divorce such as: trying to establish a relationship of friendship both by the husband and the wife in addition to establishing a relationship of friendship there is also trying to forgive the actions taken by the husband or wife made during the marriage.[3]

Coping mechanisms

Coping mechanisms are any individual efforts directed at stress management, including direct problem-solving efforts and defence mechanisms used to protect themselves.[24] These efforts are made by individuals both cognitively and behaviourally to deal with a problem they face. From the results of this study, the efforts made by divorced women who were divorced for less than two months with each different level of stress, the coping mechanisms they carried out were self-management, diversion of problems, telling problems to others, strengthening spirituality and self-reflection.

Self-management in this study is a coping mechanism carried out by participants by organising themselves so that problems were faced, not to be avoided. Existing problems were faced and solved such as starting the process of accepting oneself with a new social status, starting to increase their self-esteem by developing their abilities and starting to try to forgive their ex-husband to maintain communication for the children.[25] Andiyani[26] states that problem solving is one of the coping mechanisms commonly applied and is effective coping for divorced women. Problem solving in the study was carried out in ways such as using different strategies to solve problems, participants did so by involving thinking activities, emotions, plans and how to solve problems.

Problem diversion is also one of the efforts to reduce participants' stress, among others, by doing things or activities that can forget for a moment about the stress experienced, namely by channelling into hobbies such as caring for flowers, cooking, watching favourite movies, doing sports, soaking in warm water with aromatherapy, cleaning the house and occupying themselves with activities. Self-diversion is a negative coping mechanism because this coping is temporary and does not focus on solving the problem so it causes difficulties for oneself and inhibits learning functions.[27]

Telling problems experienced to others is a common thing that most participants do with all levels of stress. By telling the problem, participants hope to feel relieved and reduce the

stress experienced. This coping strategy is by the theory of Folkman and Lazarus, which reveals that telling problems to others is part of the coping mechanism of seeking social support.[28]

Spiritual strengthening is also one of the steps to overcome stress for participants. Performing worship, praying, believing that Allah will always help His servants and not worrying so much about the problems faced are examples of steps applied by participants.[15] Always praying for patience with the flexibility to accept the reality that exists in him is included in the prayer and patient coping mechanism, namely surrendering to Allah SWT.[25] Spiritual strengthening affects reducing stress, with individuals surrendering to God, it will make the heart calm and stressors decrease and individuals will be more able to accept the circumstances that befall them when they remember God.[29]

Self-reflection is also a coping strategy of the participants, namely by introspecting themselves, making lessons and life experiences and remaining grateful that God still gives them health. Spirituality has a positive relationship with individual resilience in dealing with stress, spirituality can strengthen mental and calm individual feelings so that they can continue to live life in the future, the belief that God will always be with them in facing trials makes divorced women able to strengthen themselves in the problems experienced.[30]

Conclusion

Sources of the stress of divorced women were social status, negative stigma of society, the process of parenting without the assistance of the husband, economic factors, low self-esteem, fear of facing future life, relationship with ex-husband and his family, while coping mechanisms of divorced women were self-management, enjoying the process, diversion of problems, telling problems to others, strengthening spirituality and self-reflection. Divorced women struggle with numerous stressors. It is necessary to strengthen positive coping mechanisms and to overcome stigmas and prejudices in society towards divorced women.

Ethics

The study was approved by the Health Research Ethics Committee Universitas Muhammadiyah Semarang, Indonesia (decision No 467/EA/KEPK-UNIMUS-2023, dated 18 October 2023).

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Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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ORIGINAL ARTICLE

The Value of Dynamic Contrast-Enhanced MRI and Diffusion-Weighted Sequence in the Evaluation of Endometrial Lesions

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Abstract

Background/Aim: Endometrial abnormalities represent a diagnostic challenge due to overlapping imaging features with normal endometrium. Aim of this study was to assess accuracy of dynamic contrast-enhanced and diffusion-weighted magnetic resonance imaging (MRI) in evaluation of endometrial lesions in comparison with T2 and to assess local staging validity and degree of myometrial invasion in malignancy.

Methods: Forty patients with abnormal vaginal bleeding or sonographic thickened endometrial were recruited. MRI examination of pelvis was performed using 1.5 T scanner with a pelvic array coil. Conventional T1-and T2, dynamic contrast-enhanced (DCE) sequences and diffusion-weighted image (DWI) were performed.

Results: Mean age of patients was 53.2 years and 60 % of patients complained of post-menopausal bleeding. Irregular margin, type III enhancement curve, a high signal in T2WI and DWI and low signal of apparent diffusion coefficient (ADC) were significantly associated with malignancy. The optimum ADC threshold value for distinguishing benign from malignant endometrial lesions was 0.905 × 10^{-3} mm^2/S, with 95.5 % sensitivity and 92.9 % specificity. DWI was most sensitive to malignant endometrial lesions, followed by DCE (89.6 %, 98.4 %) and T2 (86.7 %, 91.4 %). DWI and DCE staging correlated with FIGO staging ($p = 0.0001$ and $p = 0.019$, respectively). DWI had the best sensitivity for myometrial invasion (95.6 %), followed by DCE (91.9 %) and T2WI (90.1 %). All three sequences had 89.7 % specificity.

Conclusion: DWI and DCE MRI were superior to conventional MRI at distinguishing malignant from benign endometrial lesions and can improve myometrial invasion depth evaluation and therapy planning when combined with morphological T2WI. ADC cutoff at a high b value improved MRI diagnostic sensitivity and specificity.

Key words: MRI; Dynamic contrast enhancement; Diffusion-weighted images; Endometrial lesions; Benign; Malignant; Myometrial invasion.

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Introduction

Endometrial abnormalities represent a significant diagnostic challenge for radiologists. This may be attributed to the potentially overlapping imaging features of the normal endometrium influenced by the menstrual phase in addition to variable benign and malignant endometrial lesions

including submucosal fibroid, endometrial polyp, endometrial hyperplasia and endometrial neoplasms. Ultrasonography (US) and endometrial biopsy have been the gold standard for identifying endometrial abnormalities prior to surgery. Sonographic observations of endometrial thickness, heterogeneity and a focal endometrial lesion are non-specific, with probable interference between malignant and benign illnesses and must be considered in conjunction with the patient's age, symptoms and hormonal state.[1] Endometrial curettage or biopsy should be performed in these circumstances for a definitive diagnosis.[2] However, since these tests are typically performed blindly, it is not always possible to provide an accurate diagnosis.[3]

The International Federation of Gynaecology and Obstetrics (FIGO) approach, which is used to stage endometrial cancer (EC) was recently revised.[4] The most important morphologic prognostic indicator is the depth of myometrial invasion, which correlates with the tumour grade, the presence of lymph node metastases and overall patient survival. Magnetic resonance imaging (MRI) can reliably identify the depth of myometrial invasion, whereas endometrial biopsy can determine the histologic grade. The myometrial extension is a reliable index of lymphatic spread. The prevalence of lymph node metastases increases from 3 % with superficial myometrial invasion to 46 % with deep myometrial invasion.[5] Patients with lymph node metastases have a significantly higher recurrence rate and a lower 5-year survival rate than those without lymph node metastases.[6] More advanced diseases, such as cervical stromal invasion or adnexal involvement, can be accurately assessed by MRI. Additional information from an MRI staging assessment (eg uterine size, tumour size, presence of ascites or adnexal illness) may aid in determining whether surgery should be trans-abdominal, transvaginal or laparoscopic.[7]

Diffusion-weighted and dynamic multiphase contrast-enhanced MRI sequences have been found to increase MR imaging accuracy in measuring the depth of myometrial invasion. These sequences can be used to monitor tumour response to therapy and differentiate tumour recurrence from post-treatment alterations.[7] The purpose of this study was to evaluate the diagnostic performance of dynamic contrast-enhanced MRI and diffusion-weighted sequence in the evaluation and characterisation of endometrial lesions in comparison with morphological T2-weighted image, as well as the degree of myometrial invasion in malignant-appearing lesions in these sequences.

Methods

This was a prospective analytic study carried out in the Radiology Department of Oncology Teaching Hospital/Medical City Complex in Baghdad, Iraq during the period from 1 December 2020 to December 2021.

Included patients were referred from the gynaecological department in Baghdad teaching Hospital/Medical City with abnormal vaginal bleeding (post-menopausal, peri-menopausal, pre-menopausal bleeding, postcoital bleeding and/or abnormal vaginal discharge), abnormal endometrial pathology or endometrial thickening by transvaginal and/or abdominal ultrasound. Endometrial thickening was defined as an increase of the endometrial thickness over 5 mm in post-menopausal women and an increased thickness of endometrium non-compatible with the expected thickness in proliferative or secretory phases in reproductive women) decided by a clinician according to clinical and sonographic findings). Patients with previous surgical history for uterine/endometrial pathology and EC treated previously with suspected recurrence or adenomyosis were excluded. Patients with general contraindications for MRI and those who did not fit within the MRI gantry, had severe motion artifact and degraded MRI and did not have diffusion-weighted image (DWI) or dynamic contrast-enhanced (DCE) or histopathology report were not included in the study.

Clinical and demographic information was recorded for each patient using a prepared case sheet. The final diagnosis of the patients was based on the histopathological results of the material obtained after surgery and/ or dilation and curettage (D and C).

MRI protocol

The patients were asked to fast for about 4 to 6 h before the examination as part of preparation. Pelvic MRI was performed using a (*Siemens Healthineers: MAGNETOM Aera*, Erlangen,

Germany, 1.5 T) with both conventional MRI sequences and dynamic contrast enhancement (CE) MRI, in addition to the DWI sequence in pre-contrast images. All the patients were imaged in the supine position using a pelvic array coil, with the arms along their body and were instructed not to move during the examination. Axial, coronal and sagittal localiser images were captured, then fast spin echo (FSE) T1-weighted (TR 500 ms, TE 10 ms, matrix 320,512, slice thickness: 5 mm with an interslice gap of 1–2 mm, FOV 300 mm and a flip angle of 90 degrees) in the axial, oblique and sagittal plane followed by FSE T2-weighted images (TR 1400 ms, TE 93 ms, matrix 256,512, slice thickness: 5 mm with an interslice gap of 3-4 mm, FOV 200-240 mm and a flip angle of 90 degrees) in the axial oblique and sagittal plane. Image acquisition was optimised and perpendicular to the endometrium for T2WI. To get axial oblique images of a tilted uterus, "double oblique images" at an angle in both the sagittal and coronal planes produce a "true oblique" that is orthogonal to the endometrium. These pictures were taken perpendicular to the parasagittal T2-weighted MR imaging and were centred on the imaging plane in which the endometrium was clearly seen.

CEMRI procedure

After manually injecting 0.1 mmol/kg gado-pentetate dimeglumine (*Magnevist*) intravenously (iv), volumetric interpolated breath-hold examination (VIBE) was used for 4–5 min of sagittal dynamic MR imaging. Images were taken at 30, 70, 120 min and 4-5 min following iv contrast administration. In the first 30 s after contrast material administration, "the arterial phase" revealed the sub-endometrial zone, which enhanced earlier than the myometrium and corresponds to the inner junctional zone (JZ). Early myometrial invasion detection requires identifying this zone.[8] In "the venous phase" (70 s), the tumour-inner myometrium contrast peaks, revealing the superficial layer. "The parenchymal phase" (120 s) (equilibrium phase) which is considered excellent for assessing the tumour against myometrial and deep myometrial invasion was carefully examined since the difference in enhancement between the tumour and the outer myometrial muscle layer peaks at this phase. Four to five min after contrast material delivery, "the delayed phase", allowed cervical stromal invasion measurement in sagittal planes with the same settings as unenhanced T1-weighted spin-echo imaging. A high-signal-intensity (SI) mass in the endo-cervical canal or disruption of the typical low-signal cervical stroma indicated uterine cervix infiltration.

Diffusion-weighted image (DWI)

All examinations included a fat-suppressed single-shot echo-planar DWI sequence of the pelvis employing tridirectional motion-probing gradients with b-values of 0, 400 and 800 s/mm^2 (with an inline reconstruction of the apparent diffusion coefficient (ADC) map and the following parameters: TR/TE 2100–2500/76–82 ms; slice thickness 6–8 mm; FOV 350 mm with 75–80 % rectangular FOV; matrix 144 x 192; 3 signal averages; receiver bandwidth 1300 Hz/voxel.

Interpretation

Two senior radiologists blinded to the pathology analysed images on the workstation. T2-weighted images were evaluated for tumour signal intensity in relation to the surrounding endometrium and myometrium. An EC tumour appeared as a diffuse or focal soft tissue mass within the endometrial cavity with heterogeneous intermediate SI relative to the hyperintense normal endometrium and hypointense myometrium. The JZ was visible as a band of low signal intensity immediately subjacent to the endometrial stripe. In cases with a focal endometrial lesion, the maximal endometrial thickness on sagittal T2-weighted images and the maximal focal endometrial abnormality diameter in any plane was recorded. The minimal transverse diameter cutoff value of 10 mm indicated enlarged pelvic and/or para-aortic lymph nodes. The T1WI was checked for haemorrhage and necrosis inside the endometrial cavity or related to the lesion to be avoided while calculating DWI ADC values or DCE enhancement curves. Lesions were then examined visually on DWI/ADC, compared to the myometrium. The lesions either had low signal intensity on diffusion images and high signal in the accompanying ADC maps (facilitated diffusion) or high signal intensity on diffusion images and low signal in the ADC maps (restricted diffusion). Compared to normal outer myometrium, DWI was higher and the ADC signal was lower. The ADC value was automatically calculated after manually drawing the region of interest (ROI) within the lesion's most restricted part. Three ADC measurements per lesion were averaged and expressed in a value of 10^{-3} mm^2/s. Similarly, an ROI was manually created on the lesion's most enhancing area in the dynamic phase. Type I, II and III enhancement curves were created from the time

CONTENT

– SI curve. Each subject had two parallel/similar curve measurements. According to delayed phase enhancement, persistent Type I curves (continuous increase in signal intensity on each successive contrast-enhanced image), plateau Type II curves (initial increase in signal intensity followed by a flattening of the enhancement curve) and washout Type III curves (rapid rising curve and rapid wash out).

Statistical analyses

Data was analysed using Statistical Package for Social Sciences (SPSS) version 26. Continuous data were presented in simple measures of frequency, percentage, mean, standard deviation and range (minimum-maximum values). The significance of the difference in different percentages (qualitative data) was tested using the Pearson Chi-square test. Statistical significance was considered whenever the p-value was equal to or less than 0.05.

Results

A total of 40 female patients were recruited in this study. The mean age of the patients was 53.2 ± 9.5 years and 65 % were middle age (41-60 years) (Table 1). The majority (92.5 %) were married and 57 % were postmenopausal. The main presenting complaint was vaginal bleeding, only 15 % had postcoital bleeding (Table 1). Half of the patients underwent hysterectomy and the other half had curettage.

According to histopathological results of the endometrial lesions, there were 25 benign endometrial lesions (19 patients had benign endometrium hyperplasia and 6 patients had benign polyp) and 15 malignant endometrium lesion (10 patients with adenocarcinoma, 2 patients had squamous cell carcinoma, 1 patient for each of adenosquamous carcinoma, endometrioid carcinoma and sarcoma).

Association between MRI appearance and endometrial lesion nature

The majority of the benign endometrial lesion had a type I enhancement curve whereas the type III curve was the dominant type in the malignant lesions, p = 0.0001 (Table 2, Figure 1E and J). Regarding signal intensity in T2WI, there was a statistical association between high T2

Table 1: Demographic characteristics of participants

Demographic characteristics	N	%
Age		
≥ 40 years	4	10.0
41-60 years	26	65.0
> 60 years	10	25.0
Mean ± SD	53.2 ± 9.5	
Marital status		
Married	37	92.5
Single	3	7.5
Menopausal status		
Postmenopausal	23	57.5
Premenopausal	17	42.5
Clinical presentation		
Postcoital bleeding	6	15.0
Perimenopausal bleeding	11	27.5
Postmenopausal bleeding	23	57.5
Surgery type		
D and C	20	50.0
Hysterectomy	20	50.0
Total	**40**	**100.0**

D and C: dilation and curettage;

Table 2: The association of the MRI signals, enhancement curve and lesion morphology with the type of the endometrial lesion

Signal	Endometrium lesion n (%)		p-value
	Benign (n = 25)	Malignant (n = 15)	
Enhancement curve			
I	20 (80.0)	2 (13.3)	**0.0001**
II	3 (12.0)	1 (6.7)	
III	2 (8.0)	12 (80.0)	
T2 signal			
High	3 (12.0)	6 (40.0)	**0.0390**
Intermediate	6 (24.0)	6 (40.0)	
Low	10 (40.0)	1 (6.7)	
Mixed	6 (24.0)	2 (13.3)	
DWI signal			
High	6 (24.0)	12 (80.0)	**0.0030**
Intermediate	12 (48.0)	2 (13.3)	
Low	7 (28.0)	1 (6.7)	
ADC signal			
High	20 (80.0)	1 (6.7)	**0.0001**
Intermediate	0 (0.0)	2 (13.3)	
Low	5 (20.0)	12 (80.0)	
Endometrial lesion morphology			
Lesion outline			
Regular	24 (96.0)	5 (33.0)	**0.0001**
Irregular	1 (4.0)	10 (67.0)	
Focal or diffuse endometrial lesion			
Focal	7 (28.0)	5 (33.0)	0.7210
Diffuse	18 (72.0)	10 (67.0)	

DWI: diffusion-weighted image; ADC: apparent diffusion coefficient;

CONTENT

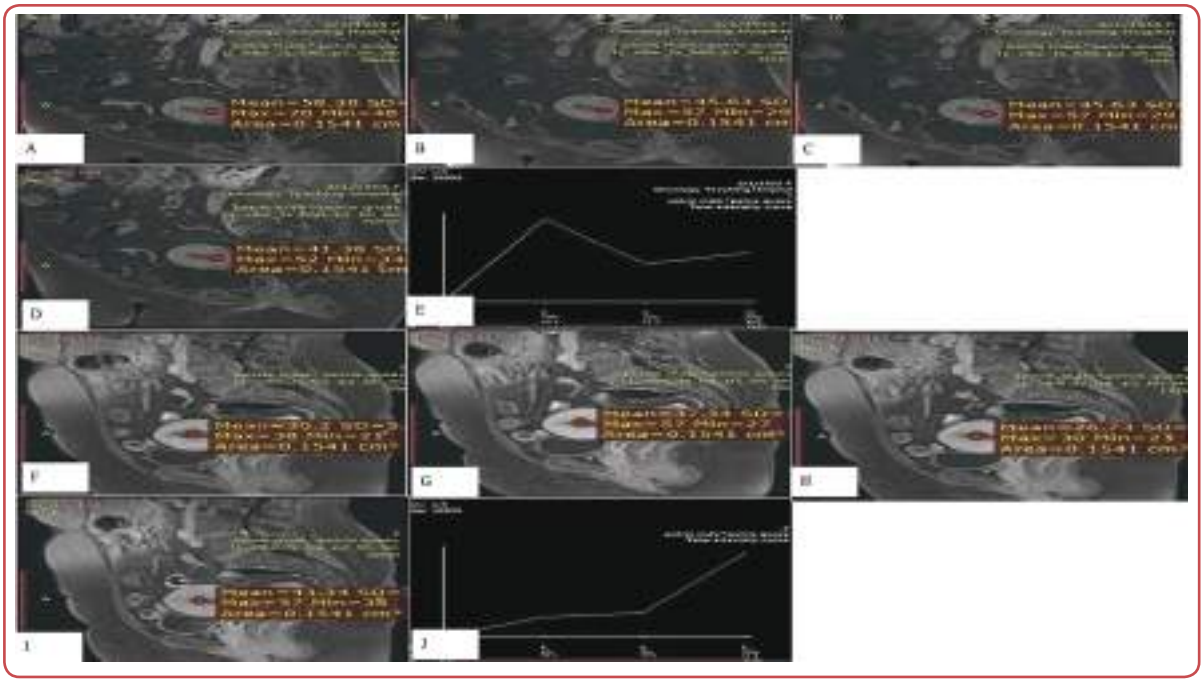

Figure 1: A-E, A 69 years old female presented with vaginal bleeding; sagittal dynamic contrast-enhanced MRI showed (A) arterial enhancement; (B) venous phase; (C) parenchymal phase; (D) delayed phase showed early enhancement of the mass and (E) post-processing time enhancement curve shows type III curve. Histopathology by total abdominal hysterectomy revealed endometrioid cancer stage Ia. F-J, 51 years female with vaginal bleeding dynamic contrast-enhanced MRI; (F) early arterial enhancement; (G) late arterial phase; (H) parenchymal phase and (I) delayed phase MR showed mild and late enhancement of the mass and (J) post-processing time enhancement curve shows type I curve. Histopathology by dilatation and curettage revealed benign endometrial hyperplasia.

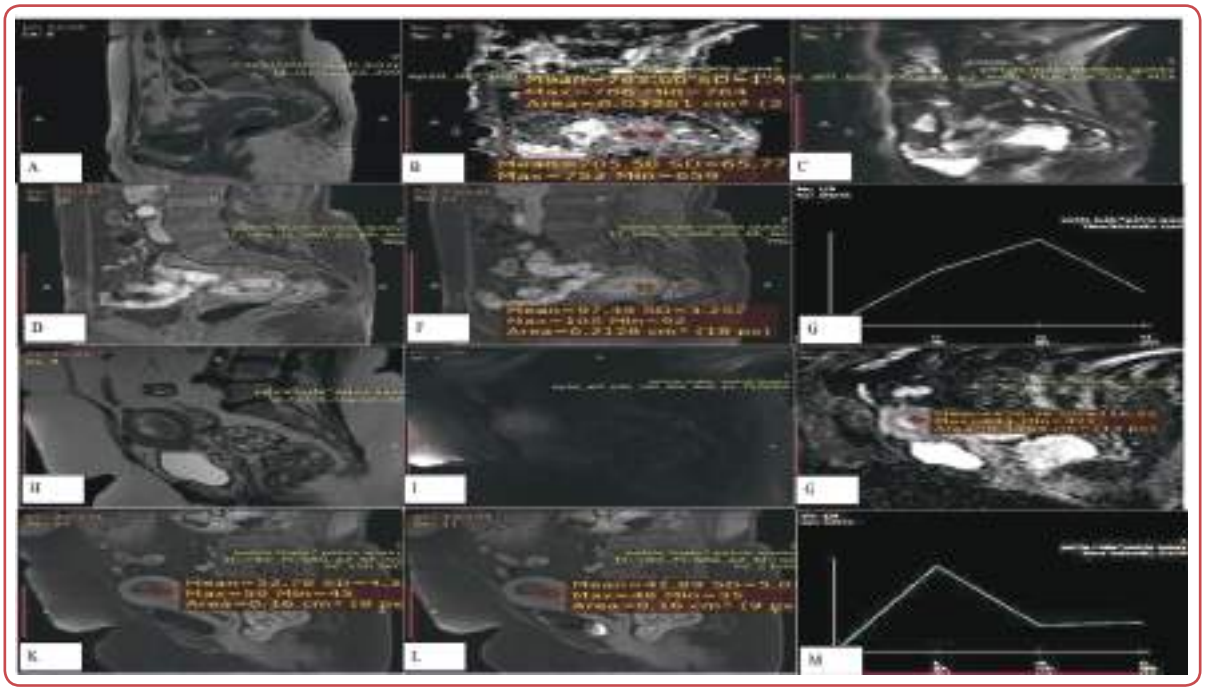

Figure 2: A-G, a 70 years old female with MRI stage TIa (A) T2 WI sagittal MRI the junctional zone is disrupted at the posterior wall with an extension of abnormal signal intensity more than 50 % of the myometrial wall; (B and C) DWI and ADC map shows restriction diffusion with ADC value = 0.76 x10^{-3} and also shows disruption of the junctional zone and extension through less than 50 % of myometrial wall (D and F) dynamic contrast series shows extension more than 50 % of the myometrial wall; (E) post-processing time-intensity curve shows type III. The final histopathological stage confirmed a TIa (less than 50 % myometrial invasion). H-M, a 50 years old woman with MRI stage TIb (H). Sagittal T2 shows mixed signal endometrial mass, with deep myometrial invasion; (I) diffusion WI (b = 800 s/mm^2) showed a homogenous hyper-intense restricted endometrial mass with a sign of deep myometrial invasion; (G) ADC shows the low signal intensity of endometrial mass, ADC value = 6.5 x 10^{-3}; (K and L) dynamic contrast series shows deep myometrial invasion; (M) post-processing time-intensity curve shows type III enhancement curve. Final histopathology confirmed Stage Ib endometrial cancer.

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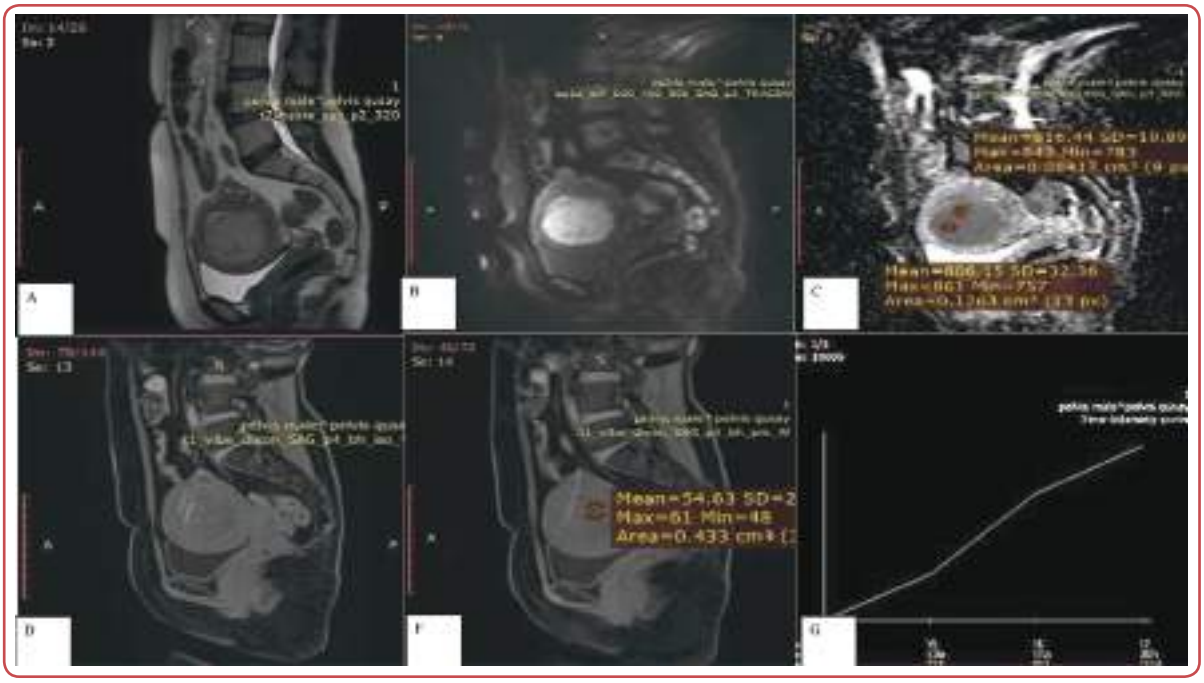

Figure 3: A 50-year-old woman with MRI Stage Ib endometrial cancer. (A) Sagittal T2 shows mixed signal endometrial mass, with deep myometrial invasion; (B) Diffusion WI (b = 800 s/mm^2) depicted a homogenous hyper-intense restricted endometrial mass with a sign of deep myometrial invasion; (C) ADC show the low signal intensity of endometrial mass with a value of 6.5 x 10^{-3}. (D and F) dynamic contrast series showed deep myometrial invasion (G) post-processing time-intensity curve shows type III enhancement curve. Final histopathology confirmed Stage IB endometrial carcinosarcoma.

signal and malignant endometrium in 40 % of the participants while low signals were associated with a benign lesion in 40 % of them, p = 0.039 (Figure 1). Similarly, the high signals in DWI were found mostly among malignant lesions, p = 0.003, (Figures 1 and 2). While high ADC signals were found among benign lesions (80 %) compared to 80 % of malignant lesions with low signal p = 0.0001 (Table 2). The mean ADC value of the benign lesions was 1.137 ± 0.16 mm^2/s ranging between 0.9 to 1.6 which was significantly higher than that of malignant lesions 0.746 ± 0.12 mm^2/s ranging between 0.6-1.0 (p = 0.025). The lowest ADC value (0.5 x 10^{-3}) was seen in carcinosarcoma followed by endometrial carcinoma (Figure 3 G).

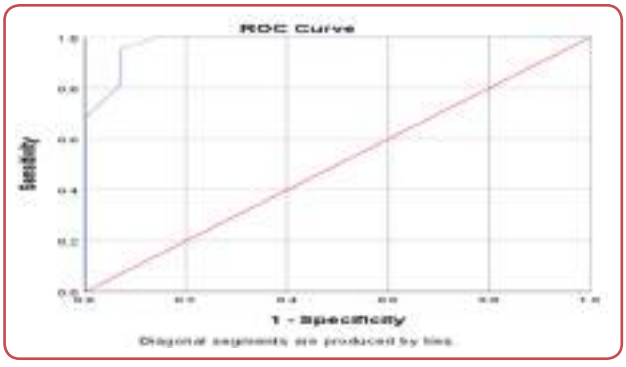

Figure 4: ROC curve comparative diagnostic value of ADC value parameter for discriminating between benign and malignant endometrium lesions

Regarding the lesion's morphology, there was a significant association between the lesion outline lesion nature. About (67 %) of malignant endometrial lesions had an irregular outline compared to (96 %) of benign endometrial lesions which depicted a regular outline with a significant difference (p = 0.0001). No significant association was observed between the benign or malignant lesion in terms of diffusion type.

The utility of ADC value in differentiating malignant and benign lesions was tested using the ROC curve. A value of 0.905 × 10^{-3} mm^2/s was suggested to be the optimal cutoff value with a sensitivity of 95.5 % and specificity of 92.9 % as shown in Figure 4.

Diagnostic performance of T2, DWI and DCE MRI in distinguishing between benign and malignant endometrial lesions

DWI had the highest sensitivity (93.3 %) in distinguishing benign from malignant endometrial lesions with the highest negative predictive value (NPV) of 96.2 % followed by DCE and T2 with a sensitivity of 89.6 % and 86.7 %, respectively, while the specificity of DCE, DWI and T2 were 98.4 %, 94.8 % and 91.4 %, respectively as shown in Table 3.

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When T2 and DWI were combined, their sensitivity was increased to 95.2 % and the specificity reached 92.7 % with an NPV of 94.3 %. Similarly, when T2+DCE were combined an increase in the sensitivity, specificity, positive productive value and NPV was observed (Table 3).

When it comes to the depth of myometrium invasive, DWI had the highest sensitivity (95.6 %) followed by DCE and T2 with a sensitivity of 91.9 % and 90.1 %, respectively, while the specificity, positive predictive value (PPV) and NPV were very close (Table 3).

Table 3: The sensitivity, specificity, positive predictive value and negative predictive value of MRI sequences in distinguishing benign from malignant endometrial lesions

MRI imaging	Sensitivity	Specificity	PPV	NPV
Endometrium malignancy				
T2	86.70 %	86.70 %	86.70 %	86.70 %
DWI	93.30 %	93.30 %	93.30 %	93.30 %
DCE	89.60 %	89.60 %	89.60 %	89.60 %
T2+DWI	95.20 %	95.20 %	95.20 %	95.20 %
T2+DCE	92.10 %	92.10 %	92.10 %	92.10 %
Myometrium invasive				
T2	90.10 %	90.10 %	90.10 %	90.10 %
DWI	95.60 %	95.60 %	95.60 %	95.60 %
DCE	91.90 %	91.90 %	91.90 %	91.90 %

PPV: positive predictive value; NPV: negative predictive value; DWI: diffusion-weighted image; DCE: dynamic contrast-enhanced;

Table 4: The association of the MRI staging with the histopathological staging among malignant endometrial lesions (n = 15)

MRI stage	Histopathological T stage of malignant lesions (n = 15)				p-value
	TI (n = 3)	TIa (n = 7)	TIb (n = 3)	TII (n = 2)	
Stage in T2					
I	0 (0.0)	1 (14.3)	0 (0.0)	0 (0.0)	0.332
Ia	2 (66.7)	1 (14.3)	0 (0.0)	0 (0.0)	
Ib	0 (0.0)	4 (57.1)	3 (100.0)	2 (100.0)	
No	1 (33.3)	1 (14.3)	0 (0.0)	0 (0.0)	
Stage in DWI					
I	2 (66.7)	0 (0.0)	0 (0.0)	0 (0.0)	0.0001
Ia	0 (0.0)	7 (100)	0 (0.0)	0 (0.0)	
Ib	0 (0.0)	0 (0.0.0)	3 (100.0)	2 (100.0)	
No	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	
Stage in DCE					
I	0 (0.0)	1 (14.3)	0 (0.0)	0 (0.0)	0.019
Ia	2 (66.7)	3 (42.9)	0 (0.0)	0 (0.0)	
Ib	0 (0.0)	2 (28.6)	3 (100.0)	0 (0.0)	
II	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)	
no	1 (33.3)	1 (14.3)	0 (0.0)	0 (0.0)	
Stage T2 + DWI					
I	3 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.0001
Ia	0 (0.0)	7 (100.0)	0 (0.0)	0 (0.0)	
Ib	0 (0.0)	0 (0.0)	3 (100.0)	2 (100.0)	
Stage T2 + DCE					
I	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0.008
Ia	2 (66.7)	4 (57.1)	0 (0.0)	0 (0.0)	
Ib	0 (0.0)	2 (28.6)	3 (100.0)	0 (0.0)	
II	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)	
No	0 (0.0)	1 (14.3)	0 (0.0)	0 (0.0)	

DWI: diffusion-weighted image; DCE: dynamic contrast-enhanced;

MRI staging and assessment of myometrial invasion in malignant-looking lesion

The association of staging on T2 and histopathology was not significant. There was a strong significant association between histopathology staging and DWI and DCE, p = 0.0001 and 0.019, respectively. On DWI there was 100 % concordance of TIa, TIb and equivalent histopathology stage (Table 4). Combined T2+DWI and T2+DCE showed strong associations pathological staging (p = 0.0001 and 0.008, respectively). Representative cases are illustrated in Figure 2.

Discussion

MRI features relating to both morphology and signal characteristics in conventional MRI sequences are beneficial in the assessment of endometrial lesion's nature and in the early staging of known cancers but their accuracy remained uncertain.[8] To authors' knowledge, this is the first study in Iraq that evaluated the efficiency of dynamic contrast-enhanced MRI in conjunction with MRI DWI in the assessment of endometrial lesions and assessed its validity in EC staging.

In agreement with other studies, the main referring cause in this study was vaginal bleeding (85 %) and more than half were post-menopausal women. Approximately two-thirds of these cases were histologically confirmed benign lesions. Regular outline morphology and high ADC signal were the most distinct characteristics seen in 96 % and 80 % of benign lesions respectively compared to 33 % and 6.7 % of the malignant lesions. It is widely agreed that the most distinguishing characteristics of benign and malignant endometrial lesions are an irregular outline and a disruptive myometrial-endometrial interface.[9]

High DWI signals and low ADC signals were features of malignancy in presented study, indicating that endometrial malignancy appeared restricted in DWI. Malignant lesions also appear dense in DWI because water molecules cannot pass through their enhanced cellularity. These findings were almost identical to those mentioned by Mansour et al.[10] Endometrial sarcoma had the lowest ADC value (0.5 x 10^{-3}) in presented study, followed by carcinoma consistent with Fujii et al findings.[11] Qualitative parameters of dynamic contrast-enhanced MRI can be easily applied in daily practice and reflect the kinetic properties of the lesion which include the enhancement curves. Findings in this study revealed that type I enhancement curves were present in the vast majority of benign endometrial lesions (80 %), while type III washout curves were present in only two benign lesions (8 %). Eighty percent of malignant lesions, in contrast, exhibited a type III washout enhancement curve. Al-Shimaa et al[12] reported that the majority of benign endometrial lesions had a type I curve (17/22, 77.2 %), whereas only one lesion displayed a type III curve (4.5 %).

Presented findings indicated that DWI (93.3 %) was more sensitive than T2WI (86.7 %) and DCE (89.6 %) in distinguishing benign from malignant lesions. This was the same conclusion of study by Gharibvand et al[13] and Masroor et al,[14] the latter of whom reported that DWI is more sensitive to small lesions than T2. Compared to T2WI (91.4 %) and DWI (94.8 %), DCE had the highest specificity (98.4 %). This was higher than Ahmed et al[12] results who reported 91.6 % sensitivity and 88.6 % specificity for DCE and 85.7 % and 95.5 % for DWI.

Variable ADC cut-off values have been suggested to distinguish between benign and malignant endometrial lesions. Presented results revealed that a cutoff value of 0.905 × 10^{-3} mm^2/s had a sensitivity of 95.5 % and specificity of 92.9 %. Latif et al[15] suggested a higher cutoff value of 1.21 × 10^{-3} mm^2/s with a lower sensitivity of 89.5 % and higher specificity (95.5 %). Similarly, Kececi et al[16] and Elsammak et al[1] suggested (1.10 x 10^{-3} mm^2/s) and (1.19 x 10^{-3} mm^2/s) respectively with 85.7 -88.9 % sensitivity and 92.8-100 % specificity. These differences could be attributed to differences in technical parameters that might influence the ADC value such as different MRI machines, or different b values that were used.

In terms of local staging, the current study revealed a significant correlation between DWI and DCE and FIGO staging. The use of combined T2 and DWI significantly increased the accuracy of the MRI staging. In agreement with that, several studies concluded that the combination of T2WI and DWI can replace DCE imaging as the patient's primary imaging modality with EC.[17-19]

Regarding myometrial invasion, presented study found that DWI was more sensitive (95.6 %) to

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myometrium invasion similar to other studies.[17] Lower DCE sensitivity in the evaluation of myometrial invasion may be attributable to thinning inner myometrium, which makes the differentiation between inner and outside myometrium less apparent because most of presented patients were postmenopausal. The diagnostic accuracy of combined DWI+T2WI and DCE-T2WI was superior to that of T2WI alone, consistent with the findings of Gil et al.[20]

Conclusion

DWI and DCE MRI differentiated malignant endometrial lesions better than conventional MRI. Qualitative time-intensity enhancement curves and ADC cutoff thresholds for high b values in pelvic MRI help distinguish benign and malignant endometrial lesions with good diagnostic sensitivity and specificity. A combination of DCE or DWI with morphological T2WI showed improved diagnostic performance in assessing myometrial invasion depth and played a crucial role in preoperative assessment and local staging of EC, enabling proper therapy planning.

Ethics

The study was approved by the Scientific Council of the Arab Board of Medical Specialisation for diagnostic radiology (registration No EAC- 20453, dated 1 September 2020). Written informed consent was obtained from patients prior to their participation in the study and for publishing of the anonymised data. The study was organised and implemented based on the adherence to the Ethical Principles for Medical Research Involving Human subjects (The Declaration of Helsinki, 8th Revision, 2013).

Acknowledgement

None.

Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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ORIGINAL ARTICLE

Quantitative Evaluation of Mitochondrial Dynamics During Maintenance of Cellular Bioenergetics Using *ImageJ*

Arpit Mehrotra,[1] Abhilasha Sood,[1] Diksha Kanwar[2]

Abstract

Background/Aim: Mitochondria are one of the most dynamic organelles essential for maintaining cellular energy demands, including execution of several vital cellular processes. This feature is attributed to rapid adaptation in morphological features which dictates their functionality. Depending on the cellular status, mitochondria can be rod shaped, branched, spherical, inter-connected or can exist as a network. Aim of this study was to analyse mitochondrial morphological appearance under normal vs stress condition in mitochondria.

Methods: The study evaluated mitochondrial morphology under normal and experimentally generated cellular stress condition by utilising *ImageJ* software, a versatile image analysis tool. Live-cell imaging technique was employed to capture high-resolution images of mitochondrial dynamics in SH-SY5Y cells and subsequent ultra-structural changes were evaluated using transmission electron microscopy. The images were later processed using *ImageJ* software, with inbuilt plugins designed for image processing.

Results: The present study identified alterations in mitochondrial morphology ranging from elongated, rod and interconnected mitochondria indicative of healthy mitochondrial network in controls to punctate, large/rounded and fragmented mitochondria in stress induced treated condition. Moreover, transmission electron microscopy confirmed significant abberation of mitochondrial structure with disapperance of outer mitochondrial membrane, decrease in matrix space and increase in mitochondrial size, with concomittant decrease in the cristae length and simultaneous increase in cristae lumen width in treated sections.

Conclusion: The study implicates existence of a mutual association between mitochondrial morphology and execution of cellular functions occurring during several pathological conditions, including neurodegenerative disorders. Furthermore, by utilising such a tool for quantitative analysis, a deeper understanding of mitochondrial dynamics and potential advancement in development of mitochondria-targeted drugs is suggested.

Key words: *ImageJ*; Mitochondria; Mitochondrial dynamics; Mitochondrial network; Mitochondrial morphology; Oxidative stress.

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Introduction

The eukaryotic cell is a notable entity which encompasses an intricate interplay between different organelles dedicated for precise functioning *via* tightly regulated processes. Amongst such

organelles, mitochondrion is a pivotal player involved in maintaining cellular bioenergetics, regulation of apoptosis and its associated diverse signalling pathways.[1] Mitochondria are highly dynamic entity, wherein incessant fusion and fission processes, movement along the cytoskeleton network and selective degradation through mitophagy occurs. Collectively, such plethora of mechanisms is referred to as "mitochondrial dynamics" and they characterise an essential aspect of mitochondrial biology.[2] Maintenance of such mitochondrial dynamics is fundamentally significant for regulation of cellular homeostasis. Moreover, by regulating their morphological features and distribution within the cellular environment, mitochondria possess the ability to rapidly provide bioenergetic demands, with concomitant response to diverse environmental factors. Any dysregulation or failure in maintenance of mitochondrial dynamics has been implicated in several pathological conditions, including neurodegenerative disorders.[3] In recent years, a significant advancement in microscopy techniques coupled with state-of-the art image analysis tools such as *ImageJ* software have provided investigators potent analytical tools to quantify mitochondrial dynamics at unprecedented levels of detail and precision. This has indeed provided new opportunities for investigating the molecular mechanisms, helping in regulation of mitochondrial dynamics and their associated functions.[4]

The morphological appearance of a mitochondrion can vary in shapes depending upon the metabolic requirement of the cell at particular localisation, such as: it could be rod, branched, spherical or highly interconnected in the form of a network. This existence of mitochondrial morphology is influenced by constantly occurring fission and fusion events at mitochondrial metabolic sites. These regular events are required to replace damaged mitochondria or to form new mitochondria whenever there is an increased cellular energy demand.[5] Fission leads to initiation of apoptosis, whereas fusion involves mixing of mitochondria leading to the formation of reticular mitochondrial networks, associated with increased bioenergetic demand. Depending upon energy requirements there are hundreds to thousands of mitochondria per cell in humans. Mutation in mitochondria has been shown to cause advancement of several neurological disorders, like Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease (HD) etc.[6,7] For instance, the first report of a direct involvement of mitochondrial dysfunction in progression of PD pathology came from brain samples of PD patient. Later, a significantly reduced activity of mitochondrial complex-I in the brain tissue of PD patients was also confirmed.[8,9] Rotenone, a naturally occurring pesticide and insecticide extracted from the roots of plants was first used as a potent inhibitor of mitochondrial complex-I in pre-clinical PD research. Rotenone is highly lipophilic and readily crosses all biological membranes including the blood-brain barrier and leads to the production of free radicals, thus causing oxidative damage.[10]

This research article aimed to provide a succinct overview of mitochondrial morphological appearance under normal vs stress condition in mitochondria, thereby emphasising on the significant role played by mitochondrial dynamics in maintaining normal cellular physiology or rather progression of a pathological condition. Furthermore, the study analysed usage of *ImageJ* software based quantitative analysis in studying such mitochondrial dynamics, highlighting its application in measuring mitochondrial morphological features.

Methods

Cell culture

For the evaluation of mitochondrial dynamics, human neuroblastoma SH-SY5Y cells (ATCC CRL-2266, USA) were used. The cells were cultured in DMEM/F12 media (*Thermo Fischer Scientific*, USA) with 10 % heat-inactivated foetal bovine serum (*Thermo Fischer Scientific*, USA) and a mixture of 0.1 % penicillin + streptomycin antibiotics (*Merck*, Mumbai, India). Cells were seeded on sterile glass coverslip and maintained at 37 °C in 5 % CO_2 incubator for the microscopy experiments.[11]

Confocal microscopy

For visualising mitochondrial morphology, *MitoTracker™ Red CMXRos* dye (*Thermo Fischer Scientific*, USA) was used. Neuronal cells (SH-SY5Y) were cultured in respective culturing media on coverslips till the day of experiment, labelled with *MitoTracker™ Red CMXRos* dye and placed on *Attoflour Cell Chamber* (*Thermo Fisher Scientific*, USA) for live cell viewing under the Nikon A1 plus Ti confocal microscope (*NIKON*, Japan). Im-

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aging was done using 60X oil objective lens, with a numerical aperture of 1.45. The *MitoTracker™ Red CMXRos* dye was excited (571 nm excitation) with argon and a helium-neon laser using confocal microscope.[12] Images were captured using *NIS elements* software and analysed using *ImageJ* software (*NIH*, USA).

Transmission electron microscopy

Transmission electron microscopy was done as previously described.[13] The protocols followed were approved by the Institutional Animal Ethics Committee of the University and were in accordance with the guidelines for humane use and care of laboratory animals. Tissue was fixed in 4 % glutaraldehyde solution overnight and later post fixed in osmium tetraoxide solution, dehydrated in a graded ethanol, cleared in propylene oxide at room temperature and finally embedded in an EPON mixture. The 0.5 µm sections were cut using ultra microtone, mounted on Nickel grids and were stained using uranyl acetate and lead citrate. Sections were examined and photographed using *Hitachi H-7500* transmission electron microscope (*ZE155906*, Munich, Germany). The captured images were analysed using *ImageJ* software (*NIH*, USA).

Image processing and analysis using *ImageJ*

A widely accepted method of scoring mitochondrial morphological features was followed in the present study. The analysis of mitochondrial networking was done with the help of *ImageJ* software. Initially, captured images were processed for various parameters by using toolbar kit containing, both build-in and customised plugin/s.[5]

Statistical analysis

All values were expressed as mean ± standard error of mean (SEM) and data was analysed using one way analysis of variance (ANOVA) followed by Student t-test for comparisons between the groups using SPSS 17 software. Values with $p < 0.05$ were considered as statistically significant.

Results

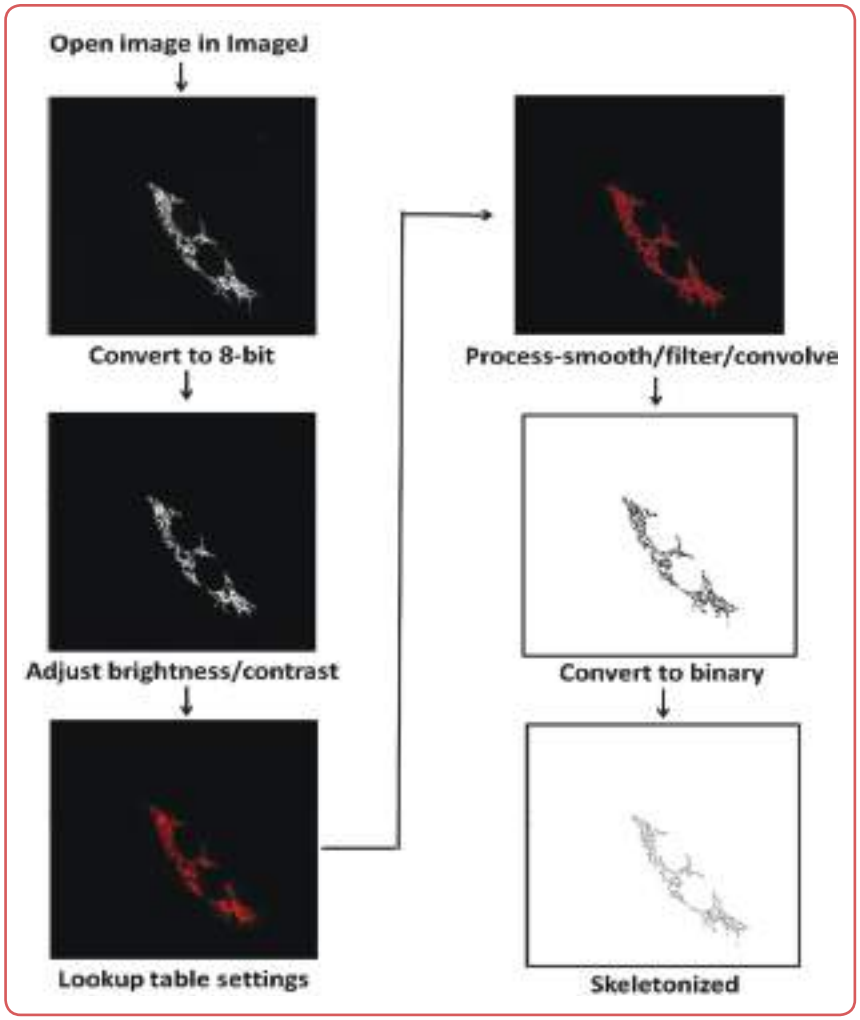
Evaluation of altered mitochondrial dynamics using *ImageJ*

The raw images of control cells were captured using confocal microscope and processed using *ImageJ* software in order to investigate mitochondrial dynamics in control conditions (Figure 1). Processing and analysis of raw images showed significant disruption in mitochondrial networks in treated cells as compared to controls (Figure 2A). The stress-induced rotenone treated cells showed higher punctate morphology followed by large/rod shape with very few mitochondria showing network connectivity, however, control cells showed significantly greater network connectivity. In Figure 2B, the high density of green dots represented a significant perturbation in mitochondrial networking in treated cells, as shown by punctate appearance. Whereas, white dots (representing large/round appearance) showed mitochondria were not well connected to each other due to broken mitochondrial networking with concomitant appearance of very few connected mitochondrial network (represented by red dots) in treated cells.

These mitochondrial observations suggest that mitochondrial network has been significantly interrupted due to hampered dynamics in treated cells. Moreover, due to rotenone treatment the large mitochondrial network broke into small network leading to significantly increased appearance of punctate, large/round and rod observations (Figure 2C). This is in alignment with low score obtained in treated cells as compared to controls due to significantly reduced presence of healthy mitochondrial network (Figure 2D). These results were further supported by correlation analysis performed between the two parameter- punctate vs rod and punctate vs large/round with respect to mitochondrial observations obtained in control and treated groups, respectively (Figure 3). It was found that there was 94 % correlation/similarity in punctate vs rod for controls, whereas it reduced to 82.5 % in treated cells. Similarly, a correlation analysis for punctate vs large/round also showed the same trend, wherein 82 % of correlation was observed in controls, however, this was increased to 94.6 % in case of treated cells.

Accessing mitochondrial ultrastructural changes using *ImageJ*

In order to strengthen the above findings with mitochondrial morphological appearance, the *ImageJ* software was extensively used to measure ultrastructural changes in mitochondrial length, mitochondrial width, mitochondrial cristae length, mitochondrial cristae lumen width in the raw images of control and treated sections,



The original raw image is processed through several filters and plugins, using ImageJ software and then evaluated for desired observations, scale bar = 50 nm.

Figure 1: Processing of a raw image from control cells for analysing mitochondrial dynamics

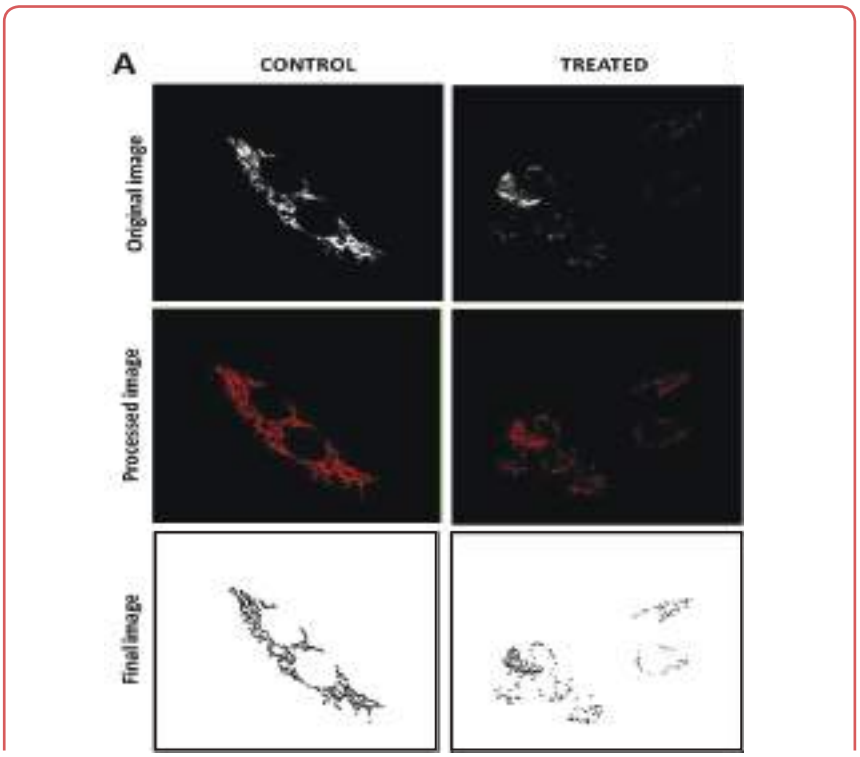


Figure 2: Evalution of changes in mitochondrial dynamics in control vs treated cells.

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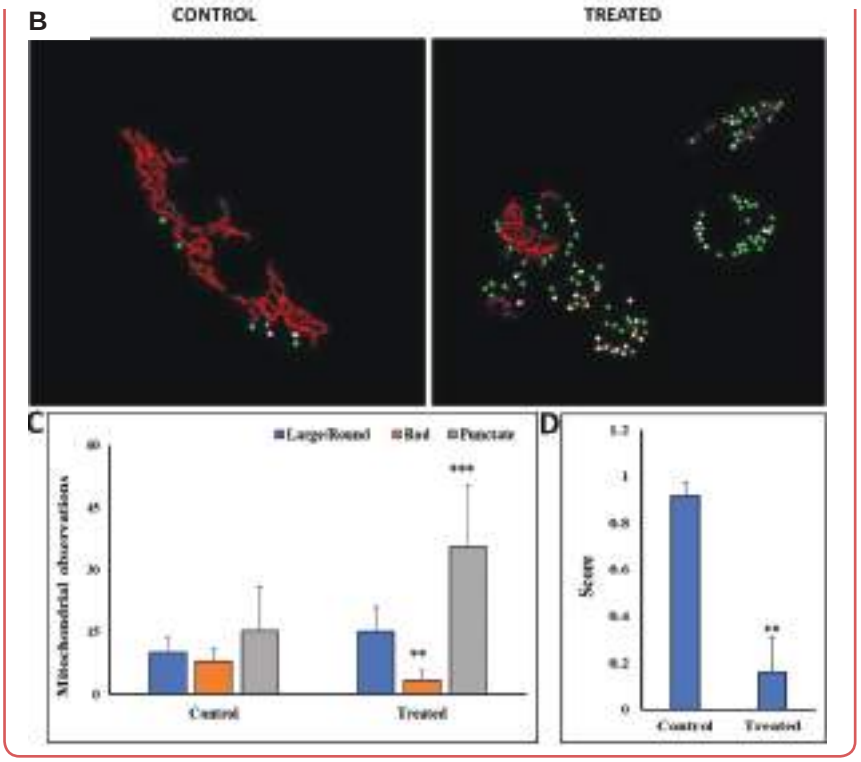


Figure 2: *(continued from previous page)*

The raw images were processed for in-depth analysis of fragmented mitochondrial network using ImageJ software (A). A healthy-intact mitochondrial network was observed in controls in comparison to fragmented mitochondrial network 48 h after retenone treatment (B). The images shows the number of punctate, rod, large/round in control and treated cells represented by green, blue and white colour respectively (C). The graphical data representation shows reduced number of rod and increased number of punctate, large/round mitochondrial network with reduced mitochondrial score in rotenone treated cells (D), scale bar = 50 nm.

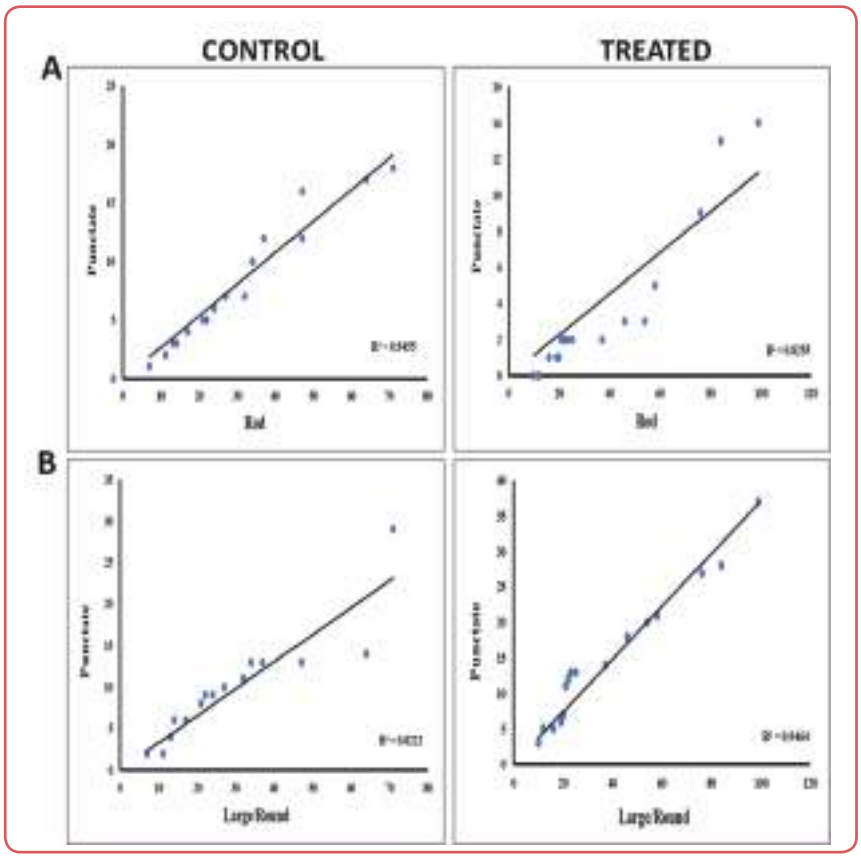
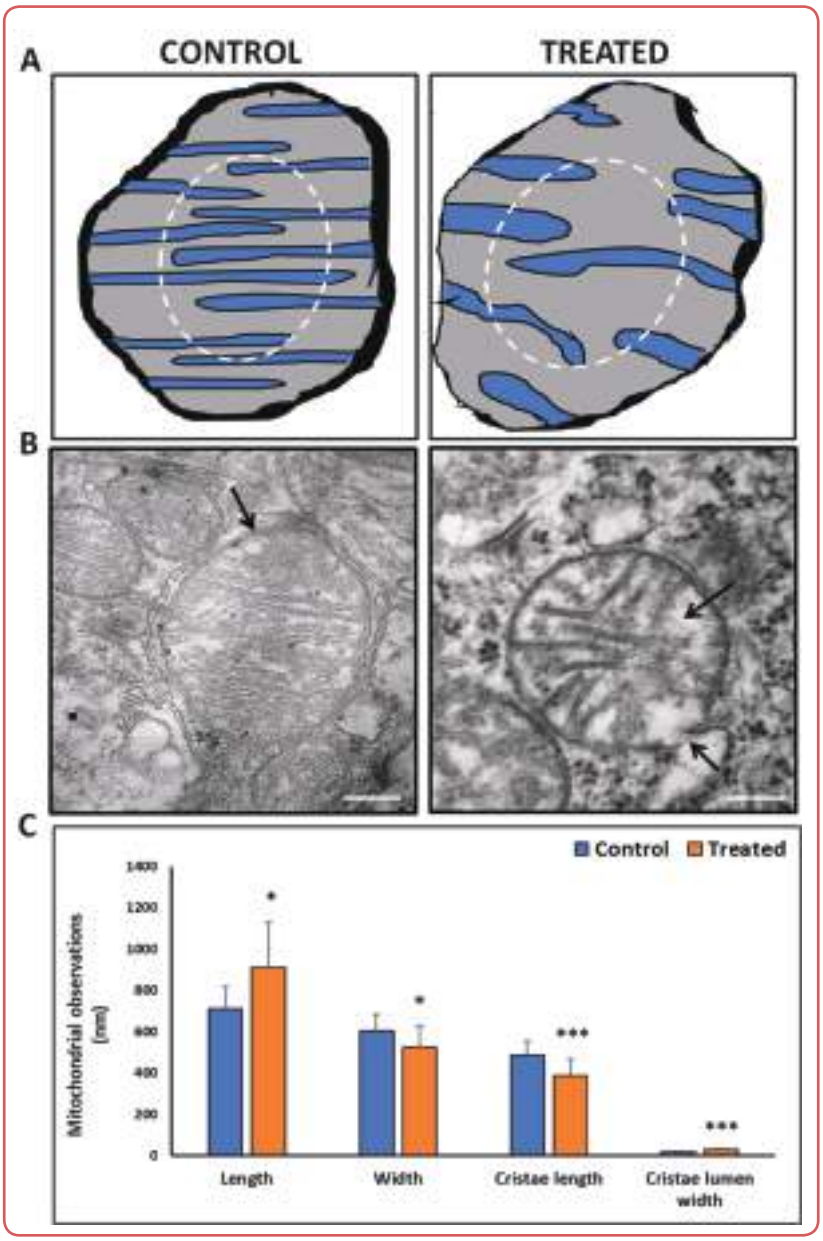


Figure 3: *Correlation analysis for evaluating changes in mitochondrial dynamics*

Correlation analysis for mitochondrial observations between punctate vs rod (A) and punctate vs large/rod (B), in control and treated cells respectively.

CONTENT

using TEM (Figure 4). The hypothetical model showed the difference between cristae length and cristae lumen width in control and treated conditions respectively, wherein presence of intact mitochondrial membranes in control and swollen mitochondrial apperance upon rotenone treatment is shown with dotted circle in Figure 4A. Transmission electron microscopy showed significant abberation of mitochondrial structure with disapperance of outer membrane of mitochondria, decrease in matrix space and increase in the size of mitochondria in treated sections as compared to controls (Figure 4B). Moreover, this was accompained by enlarged and disrupted cristae due to rotenone induced swelling, with concomittant decrease in the cristae length and simultaneous increase in cristae lumen width (Figure 4C). The results were further supported by the correlation analysis performed between two parameters, that is: mitochondrial length vs mitochondrial width (Figure 5A) and mitochondrial cristae length vs



*The hypothetical model representing appearance of mitochondrial morphological features during control and treated conditions (A). Ultra-structural changes in mitochondrial morphology were found to be altered in treated conditions (as shown by arrow) in comparison to controls in images captured at 60,000X (B). Such changes were quantified using ImageJ for calculating the mitochondrial length, mitochondrial width, mitochondrial cristae length and mitochondrial cristae lumen width, as represented by graphical data (C). * Statistically significant at $p < 0.05$, *** Statistically significant at $p < 0.001$; scale bar = 100 nm.*

Figure 4: Ultra-structural appearance of a mitochondrion using TEM

CONTENT

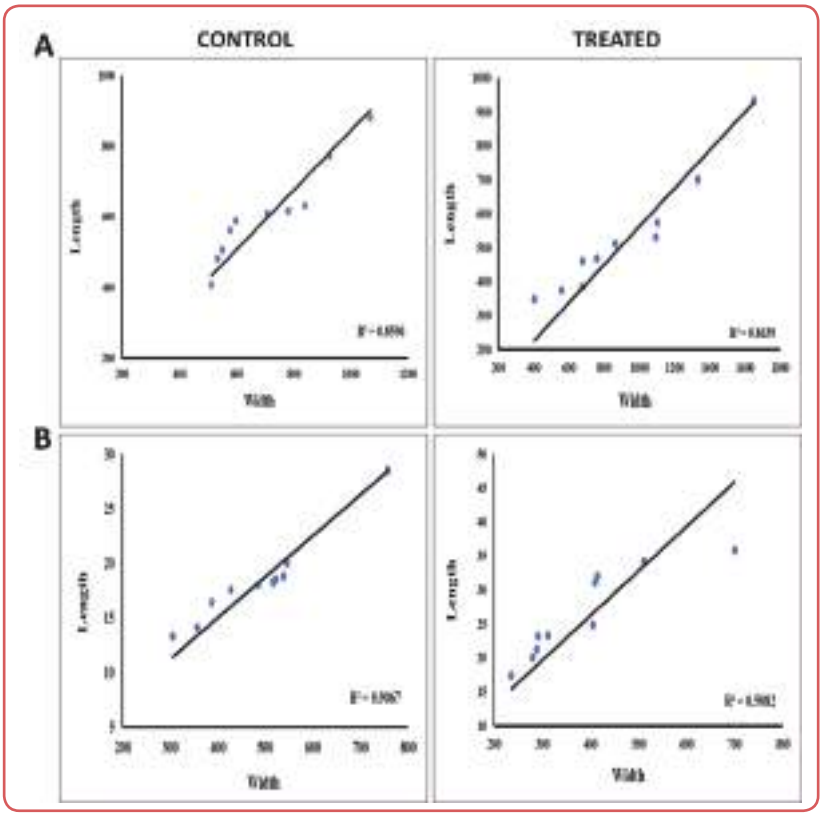


Figure 5: Correlation analysis for evaluating changes in mitochondrial morphology

The correlation analysis showed existence of ~85 % similarity in mitochondrial length vs mitochondrial width (A) for both the conditions, whereas correlation analysis for mitochondrial cristae length vs mitochondrial cristae lumen width was found to be red.

mitochondrial cristae lumen width (Figure 5B) in both control and treated groups, respectively. It was found that there was 85 % correlation in mitochondrial length vs mitochondrial width for control group, whereas it was increased to 86 % in treated group.

Similarly, a correlation analysis for mitochondrial cristae length vs mitochondrial cristae lumen width also showed the same trend, wherein 90 % of correlation was observed in control group. However, the values were reduced to 50 % in case of treated group.

Discussion

The processed images were evaluated for desired observations, such as mitochondrial morphology and mitochondrial network. The evaluation of mitochondrial dynamics changes was performed using *MitoTracker™ Red CMX Ros* dye in control and rotenone treated (to generate mitochondrial stress condition) SH-SY5Y cells. Networks are mitochondrial structures which have single node and are characterised by connected branches. Whereas, individual structures are commonly punctate, rods, or large/round in appearance. Rod structures show that, the mitochondrial network has been interrupted or broken, but may be connected at places and are unbranched. Punctate structures are small dot like and highlights that the mitochondria are destined for degradation, whereas large/round structures reflect the mitochondria are completely disconnected from each other. The punctate and large/round appear more in fragmented mitochondrial network, however the number of rods are more in non-fragmented healthy mitochondrial network.[5] Rotenone is a plant derived toxic compound and a mitochondrial complex-I inhibitor, which has been extensively used to study mitochondrial dysfunctions in pre-clinical or cellular models of neurodegenerative diseases.[14, 15] Whereas, the human neuroblastoma cells (SH-SY5Y) are known to exhibit impairment in mitochondrial functioning upon rotenone treatment due to alteration in several mitochondrial proteins associated in maintaining mitochondrial dynamics.[16, 17] Such an analysis demonstrated

CONTENT

that, due to presence of significantly increased amount of large/round and punctate mitochondrial observations, with reduced rod appearance suggests compromised mitochondrial dynamics and existence of significantly impaired mitochondrial networking in treated cells.

Advances in live cell imaging in the past decade have revealed the dynamic existence of mitochondria and its associated network in a cellular environment. In order to preserve their functional homeostasis, these dynamic organelles are known to form elongated tubules (termed as mitotubes) that constantly divide and fuse and such morphological existence is due to maintenance of equilibrium between fusion and fission processes. Whenever this equilibrium is compromised, mitochondria are expected to lose their characteristic morphological features.[18] In the present study, rotenone induced stress resulted in significant appearance of punctate and fragmented mitochondrial morphology and this finding was further supported by establishment of a positive correlation among such characteristic features in treated cells. In case of normal cellular status, mitochondria are reported to form an elongated and interconnected network, thereby resisting mitophagy with concomitant increase in ATP generation. However, mitochondria exhibit a fragmented network accompanied by smaller fragments (*punctae* or round) in case of failed homeostasis.[19] The mitochondrial cristae morphology was found to be altered in treated sections due to presence of significantly increased cristae length and cristae lumen width in comparison to controls. These changes are suggested to occur due to enhanced reactive oxygen species (ROS) mediated ensuing of mitochondrial swelling in cristae upon stress generated by rotenone treatment.[20]

Overall, such changes are well reported to cause mitochondrial dysfunctioning due to failure in maintenance of mitochondrial biogenesis, less proportion of active mitochondria to total mitochondria, enhanced ROS production, with simultaneous reduction in ATP generation and eventually leading to apoptosis.[21] However, the appearance of normal mitochondrial structure, with intact morphological features was observed in controls.

Conclusion

Mitochondrial dynamics regulating or maintaining mitochondrial structure are widely documented to be involved in smooth functioning of mitochondrial metabolism. Any change or imbalance in the mitochondrial dynamics may be involved in progression of pathological condition. Therefore, it becomes obligatory to analyse any imperfections observed in mitochondrial network which could impart more extensive understanding of mitochondria induced changes observed during such diseases, using high end software tools. One such software employed in the present study was *ImageJ*, which improved the outcome of the dataset from a raw image of mitochondrion by passing through several stages of processing and quantified later. With the help of a special plug-in installed with *ImageJ*, a detail analysis of morphological features of mitochondrial networks in control and treated cells was obtained, thus helping to conclude a clear difference between an intact and fragmented mitochondrial network. In conclusion, the investigations involving elucidation of mitochondrial dynamics using such software based approaches could be utilised in the area of targeted drug development.

Ethics

The ethical approval for the study was obtained from the Institutional Animal Ethics Committee (IAEC), Panjab University, Chandigarh, India (decision No IAEC/11/19, dated 22 October 2019).

Acknowledgement

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Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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ORIGINAL ARTICLE

Perception and Utilisation of Information and Communication Technology (ICT) in the Management of Diabetes in Children: Insights From Health Students in Indonesia

Alva Cherry Mustamu[1, 2]

Abstract

Background/Aim: The management of diabetes in children necessitates active involvement from diverse stakeholders, encompassing parents, lecturers, medical experts, nutritionists and technology-facilitated information and communication technology (ICT). In this context, medical students play a crucial role in the management of diabetes in children. Aim of this study was to delineate the perceptions and utilisation of ICT in the management of diabetes in children among students in Indonesia.

Methods: This study employed the cross-sectional method, enlisting participants from bachelor and diploma programs in midwifery, nursing and nutrition at the Health Polytechnic of the Ministry of Health, Indonesia. Data were gathered through the distribution of questionnaires *via* the *WhatsApp* platform. The questionnaire encompassed respondent demographics, knowledge about diabetes in children, perceptions and utilisation of ICT in the management of paediatric diabetes, factors influencing ICT perception and utilisation and the efficacy of ICT implementation in paediatric diabetes management. The collected data were subjected to univariate and multivariate analyses, employing structural equation modelling with partial least squares.

Results: The analytical findings revealed a noteworthy correlation between perception factors and the effectiveness of managing diabetes in children. Nevertheless, ICT utilisation and knowledge about paediatric diabetes did not exhibit significant individual influences on the effectiveness of diabetes management in children. These findings underscored the significance of a comprehensive understanding of diabetes, a favourable perception towards ICT utilisation and engagement in activities involving ICT to enhance the effectiveness of paediatric diabetes management.

Conclusion: The utilisation of ICT in the management of paediatric diabetes by health students holds significant potential for enhancing effectiveness in management. This necessitates a profound understanding of diabetes, a favourable perception regarding ICT utilisation and active engagement in ICT-related activities as integral components of paediatric diabetes management strategies.

Key words: Type 1 diabetes mellitus; Paediatric; Diabetes management; Technology information and communication; ICT; Perception; Effectiveness.

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CONTENT

Introduction

Type 1 diabetes mellitus is an autoimmune chronic condition characterised by impaired insulin production, leading to elevated blood glucose levels. Despite constituting only 2 % of global diabetes cases, type 1 diabetes exhibits a considerable prevalence, ranging from 5 % to 10 %, impacting an estimated 21–42 million individuals worldwide. In Indonesia, the prevalence of type 1 diabetes has witnessed a significant increase over the past decades, rising from 3.88 to 28.19 cases per 100 million people.[1-4]

The management of type 1 diabetes in children demands focused attention from various stakeholders, encompassing parents, healthcare professionals, nutritionists and technology. Parents play a crucial role by gaining a comprehensive understanding of paediatric diabetes and providing essential emotional support. Healthcare professionals, including doctors and nurses are instrumental in monitoring and controlling blood sugar levels, as well as administering treatment in the event of complications. Nutritionists contribute by designing dietary patterns tailored to children with diabetes and imparting information about essential nutrients and supplements. Concurrently, technology can be leveraged for the supervision and regulation of blood sugar levels, the coordination of dietary routines and the formulation of a customised exercise program suitable for the child's condition.[5, 6]

Several studies indicate that incorporating technology, such as digital blood sugar meters, calorie-counting apps and physical activity monitors, can be beneficial in aiding children with type 1 diabetes in managing their condition. Additionally, technology serves as a valuable tool for formulating exercise programs tailored to suit the specific needs of children with diabetes. Moreover, student education emerges as a pivotal agent of societal change, playing a vital role in disseminating knowledge and outreach initiatives related to the management of diabetes in children.[7, 8]

Results of a study conducted in the United States show that the utilisation of a mobile application for blood sugar monitoring demonstrated a positive impact on enhancing blood sugar control in children diagnosed with type 1 diabetes.[7] Another study conducted in Canada revealed that the utilisation of a calorie-counting app can contribute to enhancing children's comprehension of healthy eating patterns.[9] Similarly, research conducted in Italy demonstrated that engaging children with type 1 diabetes in interactive games can be effective in promoting increased physical activity.[10]

The management of diabetes in children necessitates collaborative efforts and coordination among parents, medical professionals, nutritionists and technology for information and communication. Technology, specifically information and communication technology (ICT), assumes a pivotal role in paediatric diabetes management when it provides essential information and tools. Establishing appropriate dietary patterns and promoting increased physical activity are crucial components for monitoring the health condition of children with diabetes. Moreover, the knowledge possessed by students becomes highly significant in the management of paediatric diabetes. As prospective healthcare professionals, they possess the requisite knowledge and skills essential for paediatric diabetes management. Furthermore, students have access to the latest information regarding diabetes management and the most recent technological advancements applicable to the management of diabetes in children.[11]

As catalysts for societal transformation, health students can offer education and outreach initiatives regarding the management of diabetes in children to various segments of society, encompassing parents, teachers and friends of children with diabetes. Through this approach, the knowledge held by students can play a pivotal role in heightening public awareness concerning the significance of diabetes management in children and fortifying social support networks for those children affected by diabetes.[12-14]

Nonetheless, health students may encounter several potential challenges related to the utilisation of ICT in the management of type 1 diabetes in children. These challenges encompass limitations in knowledge and skills about the latest technological advancements, as well as constraints in accessing timely updates and adequate health facilities.[8, 15]

A preceding study indicated that students generally possess commendable knowledge concerning type 1 diabetes in children; nevertheless, their awareness regarding the latest technological advancements applicable to diabetes management remains somewhat restricted. Additionally,

research suggests that students may encounter challenges in effectively navigating blood sugar monitors and insulin pumps, potentially impeding the optimal management of type 1 diabetes in children.[7, 8, 15]

In the realm of paediatric diabetes management, student knowledge assumes a crucial role in leveraging ICT to enhance blood sugar control and mitigate the risk of complications. The augmentation of knowledge and skills among students in utilising ICT coupled with familial and environmental support, has the potential to significantly amplify the effectiveness of paediatric diabetes management.[16-19]

This research distinguishes itself from previous studies as it focused on exploring the perceptions and utilisation of ICT in managing diabetes in children among Indonesian health students. The researcher's goal was to delve into methodological perspectives and introduce innovative approaches, setting them apart from existing studies. The primary objective of the study was to elucidate the perceptions and utilisation of ICT in the management of diabetes in children among students in Indonesia. The anticipated outcomes aimed to provide additional information and enhance understanding regarding the application of ICT in paediatric diabetes management. Moreover, the research indicated potential benefits for parents, children, healthcare professionals (doctors and nurses) and experts, contributing to the improvement of diabetes management in children.

Methods

This study was a cross-sectional study. The study was carried out at the Health Polytechnic of the Ministry of Health. Data collection was carried out in March 2023 through distributed questionnaires *via WhatsApp*.

Participants

The Health Polytechnic under the Ministry of Health provided bachelor's and diploma programs tailored for health students with interests in nutrition, nursing and midwifery. The study subjects consisted of willing participants who fulfil specific criteria related to their knowledge about the treatment of diabetes in children. The selection of participants was conducted based on their availability and their ability to meet the qualifying requirements pertinent to the subject matter, ensuring a suitable cohort for the study.

Variables

The research outcomes encompassed the effectiveness of managing diabetes in children, with a primary focus on the utilisation of ICT in the management process. Predictors and potential challenges included demographic factors such as type, gender, marital status and semester level, along with knowledge about diabetes in children and perceptions and utilisation of ICT in the management of paediatric diabetes. Additionally, effect modification explored the interaction between these factors, shedding light on their interconnected influences in the context of paediatric diabetes management.

Source of data

Data for this study were gathered using a questionnaire designed through *Google Forms*. The questionnaire comprised several sections, including respondent identity, knowledge about diabetes in children, perceptions and utilisation of ICT in paediatric diabetes management, levels of ICT use in diabetes management for children, factors influencing perception and ICT use and the effectiveness of ICT utilisation in managing diabetes in children. All questions in the questionnaire utilised a Likert scale, with response options ranging from 1 to 5, indicating varying degrees of agreement or disagreement.

Bias

Attempts made to overcome internal bias in a potential study included giving the same opportunity to all student nurses, nutritionists and midwives to become respondents, testing the validity and reliability of the instrument before data collection, delivering an explanation of intent and purpose to the candidate respondent before filling out the questionnaire and using an inside Likert scale question. Method structural equation modelling—partial least squares (SEM-PLS) analysis was also used to control influence factors and troublemaker potential. Ten samples for each variable were used, following the minimum sample rule of regression analysis, or structural equation modelling with partial least squares.
Quantitative variables in this study were managed through statistical analysis methods, encompassing both univariate and multivariate analyses. Univariate analysis examined individ-

ual variables independently, providing insights into their individual characteristics and distributions. On the other hand, multivariate analysis considered the simultaneous relationships between multiple variables, allowing for a more comprehensive understanding of the interplay among them in the context of the study. This dual approach facilitated a nuanced and thorough examination of the quantitative variables within the research framework.

Statistical analyses

Univariate analysis was employed to assess the characteristics of the respondents using statistical software, specifically Jamovi. This analysis allowed for the measurement of individual variables independently, providing a detailed examination of respondent attributes. In contrast, multivariate analysis utilised structural equation modelling with the partial least squares method. The SEM-PLS method was applied to model the relationships between influencing factors, the utilisation of ICT in paediatric diabetes management and its effectiveness in disease management. Additionally, SEM-PLS was utilised to control for influencing factors and potential challenges in the study, offering a comprehensive understanding of the interconnected variables within the research framework.

Data collection were carried out through a *Google Forms* questionnaire, therefore no data were lost.

Results

Demographic characteristics of research participants

The study encompassed 103 respondents who participated in the survey; however, two respondents were unwilling and 32 respondents lacked any information about the management of diabetes in children. Consequently, 69 individuals responded to the survey. The majority of respondents were female (95.7 %), with an average age of 19.7 years. Additionally, the majority were unmarried (98.6 %). Respondents exhibited diverse educational backgrounds, with bachelor's programs in midwifery (34.8 %) and nursing diploma programs (26.1 %) being predominant. Regarding internet usage, respondents tended to use the internet moderately, with an average frequency rating of 3.94 on a scale of 1 to 5. The majority of respondents (94.2 %) reported having partial internet access and facilities. However, a notable proportion (92.8 %) lacked experience in working or undergoing an apprenticeship related to the management of diabetes in children. The data is presented in Table 1.

Table 1: Demographic characteristic of the participants

Variable	Value	
Age	**Mean (SD), min-max**	
Years	19.7 (1.51), 17-24	
Gender	**N**	**%**
Man	3	4.3
Woman	66	95.7
Marital status	**N**	**%**
Single	68	98.6
Married	1	1.4
Study program	**N**	**%**
Nutrition diploma	5	7.2
Midwifery diploma	9	13.0
Nursing diploma	18	26.1
Bachelor midwifery	24	34.8
Bachelor nursing	13	18.8
Frequency of the Internet usage	**Mean (SD), min-max**	
Likert scale (1-5)	3.94 (1.14), 1-5	
Internet access and facilities	**N**	**%**
Available	65	94.2
Not available	4	5.8
Experience	**N**	**%**
No	64	92.8
Yes	5	7.2

Experience: Work or apprentice involved in management of diabetes in children;

Validity and reliability instrument

The research outcomes indicated that all the measured constructs in the study exhibited high reliability, as evidenced by Cronbach's alpha values surpassing 0.7. This observation underscored that the questions within the research instrument exhibited commendable internal consistency, rendering them dependable for measuring the intended constructs. Furthermore, the Pearson moment validity test results demonstrated significant correlations between all constructs and the measured questions. This finding affirmed the validity of the instrument in effectively capturing the desired constructs in the study. The data is presented in Table 2.

Outer loading

The results of the outer loading test were robust, revealing that all examined questions in this study exhibit outer loading values surpassing 0.7. This signified that each question makes a signifi-

Table 2: Validity and reliability tests

Question	Pearsons (r)
Understanding about diabetes in children (Cronbach's α = 0.900)	
I have enough knowledge about diabetes symptoms in children.	0.746
I know the right method to prevent complications of diabetes in children.	0.866
I understand the possible complications that happen as a consequence of diabetes in children.	0.913
I understand how to manage diabetes in children, including arrangements for eating and drinking as well as recommended exercise.	0.861
I always look for the latest information about diabetes in children so that I can give optimal care.	0.839
Perceptions about the use of technology, information and communication (ICT) in the management of diabetes in children (Cronbach's α = 0.952)	
I feel ICT is very helpful in the management of diabetes in children.	0.839
I'm sure that ICT can help monitor blood sugar levels in children with diabetes.	0.950
I believe that ICT can help arrange diets in children with diabetes.	0.924
I feel that using ICT can help parents better understand the management of diabetes in children.	0.951
I argue that using ICT can help increase the quality of life in children with diabetes.	0.924
Utilisation rate of ICT in the management of diabetes in children (Cronbach's α = 0.897)	
I often use mobile applications to monitor the blood sugar levels of children with diabetes.	0.815
I feel comfortable using ICT in the management of diabetes in children.	0.884
I have been using social media to look for information related to the management of diabetes in children.	0.867
I feel helped by the existence of ICT in the eating patterns of children with diabetes.	0.781
I often look for the latest information about the management of diabetes in children through websites or applications.	0.869
Influencing factors: perception and utilisation of ICT in the management of diabetes in children (Cronbach's α = 0.938)	
I believe that using ICT is very helpful for managing diabetes in children.	0.916
I feel ICT is very helpful in monitoring blood sugar levels in diabetic children.	0.880
I feel I can easily access information about ICT for managing diabetes in children.	0.866
I feel I have sufficient skills in using ICT for the management of diabetes in children.	0.917
I feel ICT can help reduce the risk of complications in children with diabetes.	0.903
Effectiveness of using ICT in the management of diabetes in children (Cronbach's α = 0.958)	
I believe that using ICT can help control blood sugar in children with diabetes.	0.919
I believe that using ICT can help reduce the risk of complications in children with diabetes.	0.949
I feel comfortable using ICT in the management of diabetes in children.	0.948
I'm sure that using ICT can improve the quality of life for a child with diabetes.	0.896
I will recommend to the families of patients with diabetes that they use ICT during their care.	0.916

cant contribution to the intended construct. Outer loading values exceeding 0.7 suggested that the questions effectively represent the measured constructs and contributed substantially to the measurement of those constructs. Consequently, the outcomes of the outer loading test affirmed that the research instrument employed in this study possessed strong validity. The robust relationship between each question and its measured construct instilled confidence in the instrument's capability to reliably measure the desired constructs within the context of managing diabetes in children. The data is shown in Figure 1.

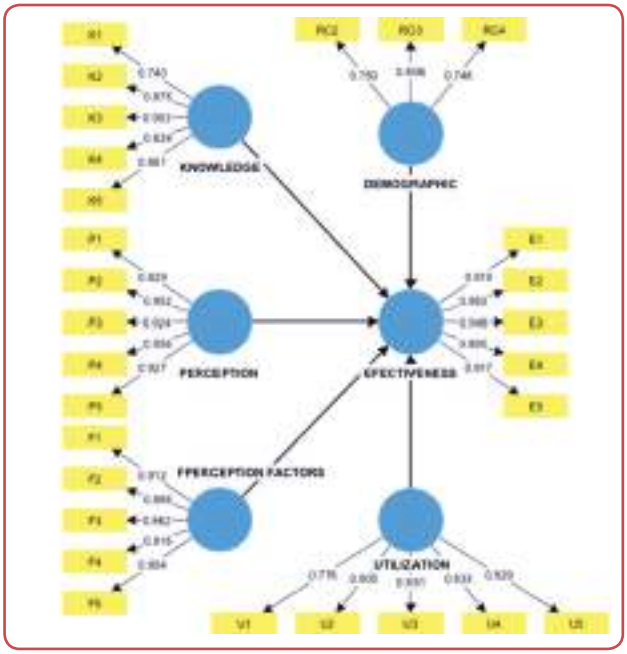

Figure 1: Outer loading test: the research instrument employed possessed strong validity

Criteria quality

The R-square quality indicates that the model utilised in this study exhibited a commendable ability to elucidate the variation in the dependent variable based on the independent variables employed. This suggested that the model was robust and reliable in analysing and predicting

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the connections between the variables involved in the management of diabetes in children. The high R-square value signified the proportion of variability in the dependent variable that was explained by the independent variables, underscoring the model's effectiveness in capturing and understanding the relationships within the context of paediatric diabetes management. The data is presented in Table 3.

Table 3: R-square quality criteria analysing the model ability to analyse and predict the connections between the variables involved in the management of diabetes in children

Variable	R-square	Adjusted R-square
Effectiveness	0.816	0.801

Validity and reliability construct

The results of the validity and reliability tests indicated that the research instrument utilised in this study demonstrated good reliability and validity. The reliability assessment, conducted using Cronbach's alpha method, revealed that all constructs exhibited values exceeding 0.7, indicating a commendable level of internal consistency. Demographic constructs, including gender, marital status and semester level, as well as utilisation, knowledge, perception factors, perception and effectiveness, exhibited high reliability with Cronbach's alpha values of 0.665, 0.898, 0.900, 0.939, 0.953 and 0.958, respectively.

These findings affirmed that the questions employed in the research instrument align consistently with the intended constructs. Additionally, the results of the construct validity, assessed through the Rho a and Rho c methods, also demonstrated high values across all constructs. The Rho a values range between 0.668 and 0.961, while the Rho c values range between 0.812 and 0.968. This underscored that each question within the construct maintained a significant relationship with the construct under measurement, highlighting coherence among the questions within each construct.

Furthermore, the average variance extracted values substantiated the construct validity, with average variance extracted (AVE) values ranging from 0.590 to 0.858. The elevated AVE values affirmed that the variables within each construct effectively explained the variation in the respective constructs. The data is presented in Table 4. Based on the presented table, it was deduced that the research instrument employed in this study was a reliable and valid tool for measuring the intended constructs. This instilled confidence in the results of measurements and analyses conducted using such instruments, thereby providing a basis for trust in the interpretation and generalisation of research findings.

Table 4: Validity and reliability construct of the researched instrument

Variable	Cronbach's alpha	Rho a	Rho c	AVE
Demographics	0.665	0.668	0.812	0.590
Utilisation	0.898	0.934	0.923	0.706
Knowledge	0.900	0.926	0.926	0.715
Perception factors	0.939	0.942	0.953	0.803
Perception	0.953	0.961	0.964	0.844
Effectiveness	0.958	0.959	0.968	0.858

AVE: average variance extracted;

The results of the inner model test, as determined by the p-values, lead to the conclusion that there exists a significant association between the constructs of perception factors, perception and effectiveness within the given context. The p-value obtained for both perception factors and perception constructs was < 0.001, signifying a statistically significant connection with the effectiveness construct.

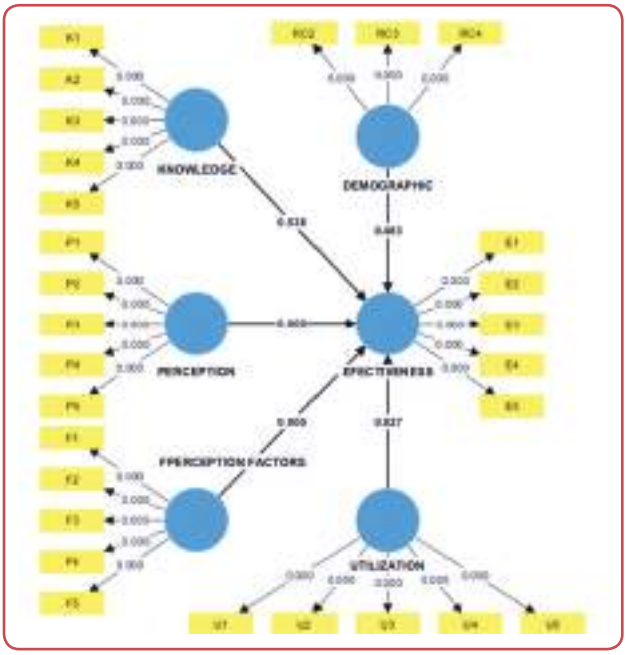

Figure 2: Inner model analysing the association between the constructs of perception factors, perception and effectiveness within the given context

Conversely, the p-value test results for demographic constructs (gender, marital status and semester level), utilisation and knowledge reveal higher values, namely 0.483, 0.827 and 0.538,

respectively. These values suggested that demographic factors (gender, marital status and semester level), technology utilisation and knowledge about diabetes in children did not exert a significant influence on the effectiveness of the management of diabetes in children. The data are presented in Figure 2.

Based on these findings, it can be concluded that factors influencing perception, such as the utilisation of technology for information and communication, along with perceptions regarding technology utilisation, played a pivotal role in enhancing the effectiveness of diabetes management in children. However, demographic factors (gender, marital status and semester level), technology utilisation and knowledge about diabetes in children did not exert a significant influence on the study's effectiveness.

Discussion

The management of diabetes in children is a critical aspect that necessitates an effective strategy.[7, 8, 20, 21] This study endeavours to elucidate the perceptions and utilisation patterns of ICT in the management of paediatric diabetes among students in Indonesia. The research findings underscore that perceptions and adept utilisation of ICT have a substantial impact on the efficacy of diabetes management in children. Specifically, maintaining a positive perception and leveraging technology for information dissemination and communication emerged as pivotal factors contributing to enhanced management effectiveness. Conversely, demographic variables such as gender, marital status and semester level, alongside considerations of technology use and knowledge regarding paediatric diabetes were not found to exert a statistically significant influence on the effectiveness of management practices.

The study revealed several noteworthy findings. Firstly, it highlighted the crucial role of ICT in the management of diabetes in children. The effective utilisation of technology substantially enhances the efficacy of diabetes management, underscoring the necessity of integrating technology into strategies for diabetes care. Furthermore, the study emphasise that factors such as the perception and utilisation of technology wield greater influence than demographics and knowledge. This observation aligns with previous research indicating that knowledge, perception and technology utilisation are pivotal elements in the effective management of diabetes in children.[22]

However, some studies, like that of Berndt et al, found no significant relationship between technology use and diabetes management effectiveness.[23] This discrepancy may be due to variations in research samples and methods. Similarly, research by Ng et al suggests that technology's impact on diabetes management may be limited due to social and psychological factors. Therefore, a holistic approach is needed in addition to technology use.[24]

The utilisation of SEM-PLS as a statistical method for multivariate analysis bestows a significant advantage in this study. This approach enables a comprehensive understanding of the intricate relationships among the variables under investigation. In the context of this study, SEM-PLS not only reveals the correlation between the utilisation of ICT in the management of diabetes in children and the effectiveness of disease management but also assists in identifying factors influencing ICT utilisation and potential challenges within this context. The strength of SEM-PLS is particularly evident in its ability to address complex, non-linear models, a common occurrence in research involving multiple independent and dependent variables.

Additionally, the meticulous handling of data through the use of *Google Forms* as a data server is a pivotal aspect of this study. This approach ensures the preservation of data integrity, preventing any loss or fragmentation of data during the processes of data collection and storage. This not only upholds the veracity of the data but also ensures that analyses are conducted with comprehensive and accurate datasets. Hence, the adoption of SEM-PLS in multivariate analysis and the judicious data management executed *via Google Forms* constitute two crucial facets that underpin the validity and excellence of this research.

This study has several limitations, notably the constrained sample size comprising student midwives, nutritionists and nurses. To enhance the generalisability of the findings, future research endeavours should encompass a more extensive and diverse participant pool. The utilisation of self-report questionnaires introduces potential response bias; hence, incorporating objective measures such as blood sugar levels is imperative for ensuring the reliability of results. To mitigate

these limitations, forthcoming research should consider enlarging the sample size, adopting objective data collection methodologies and evaluating the direct influence of technology on diabetes management.

Presented study proposes the hypothesis that increased utilisation of ICT in diabetes management by midwifery students, nutritionists and nurses leads to better blood sugar control and reduced complications in children with diabetes. Future research should test this hypothesis with larger samples and objective measures, considering various influencing factors in diabetes management. The implications of this research are significant. It highlights the need to enhance the understanding and skills of midwifery, nutrition and nursing students in using ICT for diabetes management. Creating a supportive environment with adequate infrastructure and training is crucial. Moreover, the findings provide a foundation for targeted interventions and policies to improve diabetes management through ICT, benefiting children with diabetes.

Conclusion

This study indicates that the utilisation of information technology in managing diabetes in children by student midwives, nutritionists and nurses holds significant potential to enhance the effectiveness of managing this condition. The findings underscore the crucial relationship between knowledge, perception and the utilisation of information technology, emphasising their pivotal role in managing childhood diabetes. However, there are limitations in this study, such as the limited sample size and reliance on self-reported data by respondents. To enhance future research, it is recommended to involve a more diverse sample representation and employ more objective methods of data collection. Despite its limitations, this research makes a significant contribution to understanding the role of technology in managing childhood diabetes. Its findings can shape more effective clinical practices and policies, emphasising the importance of a holistic approach involving knowledge enhancement, fostering a positive attitude toward technology and actively utilising technology in managing diabetes in children.

Ethics

The study was approved by the Ethics Committee of the Ministry of Health Polytechnic Slide below the supervision of the Ministry of Health of the Republic of Indonesia, decision No DM.03.05/6/050/2022, dated 15 December 2022. Written informed consent was obtained from patients prior to their participation in the study and for publishing of the anonymised data. The study was organised and implemented based on the adherence to the Ethical Principles for Medical Research Involving Human subjects (The Declaration of Helsinki, 8th Revision, 2013).

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Conflicts of interest

The author declares that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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Contributed to the conceptualisation, methodology development, software, validation, formal analysis, investigation, resource management, data curation, original draft writing, revision and visualisation aspects in this research: ACM

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ORIGINAL ARTICLE

The Gut Microbe-Derived Metabolite Trimethylamine N-Oxide in Patients With Systemic Lupus Erythematosus

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Abstract

Background/Aim: Both human and animal studies suggest that the gut microbe-derived metabolite trimethylamine N-oxide (TMAO) is strongly associated with several autoimmune diseases including systemic lupus erythematosus (SLE) and correlates to disease severity. The study aimed to investigate the diagnostic and prognostic validity of TMAO as a potential biomarker in patients with SLE, particularly focusing on lupus nephritis patients and its relation to disease activity.

Methods: A total of 90 patients were included and assigned into either: group I (SLE without nephritis (NN)), group II (lupus nephritis (LN)) and group III (healthy controls). Serum TMAO levels were compared between the study groups and correlated to the clinical, laboratory and histopathological criteria.

Results: Unpredictably, TMAO levels were significantly higher in healthy controls compared to the total SLE population ($p = 0.003$), to LN and NN groups individually ($p = 0.01$). TMAO levels did not significantly vary between (NN) and (LN) patients and only correlated to anti-dsDNA titres ($p = 0.02$) and red blood cells count ($p = 0.02$) among LN patients.

Conclusion: Contrary to previous studies, TMAO levels were found to be higher in healthy controls. A possible confounding effect of the dietary pattern and ingested drugs on the gut microbiome limits the utility of TMAO as a potential marker in different diseases.

Key words: Trimethylamine N-oxide (TMAO); Systemic lupus erythematosus; Lupus nephritis; Gut microbiome; Disease activity.

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease that is characterised by the presence of hyperactive immune cells and aberrant antibody responses to nuclear and cytoplasmic antigens.[1] The "hygiene hypothesis" is a popular hypothesis accounting for the role of the gut microbiome in the pathogenesis of SLE. The hygiene hypothesis states that as incidence of bacterial infection have decreased with increased hygiene standards and practices, both pathogenic and non-pathogenic colonisation of the gut have also decreased.[2] The earliest evidence for a potential role for intestinal microbiota in SLE came from a report by Gul'neva et al in 2007 who described genera that dominated the intestinal microbiota of SLE patients.[3] Later reports revealed that SLE patients had a reduced bacterial diversity in their faecal samples, compared with individuals from the general population.[4]

SLE patients demonstrate a decrease in Firmicutes levels, exhibiting a 2.5-fold decrease in the

ratio of Firmicutes to Bacteroidetes.[1] Butyrate, produced by Firmicutes, plays a role in the differentiation of regulatory T cells in the colon, spleen and lymphatic system that suppress inflammation, the dysfunction of which is a hallmark of SLE and a precursor to cardiovascular incidents.[5] Therefore, a decrease in Firmicutes and consequent decrease in butyrate levels may contribute to inflammation in SLE patients.[6] This hypothesis is further supported by evidence demonstrating that antibiotics, which diminish populations of butyrate-producing Firmicutes such as trimethoprim–sulfamethoxazole, minocycline and amoxicillin have been shown to trigger lupus flares.[2] It is worth noting that *Lachnospiraceae*, butyrate-producing bacterium of the Firmicutes phylum are present in higher numbers in SLE patients than in their healthy counterparts. Therefore, it is possible that *Lachnospiraceae*, or any butyrate-producing bacteria, may not be able to suppress inflammation in SLE cases.[6]

The relative increase in Bacteroidetes in the SLE microbiome results in heightened Toll like receptor 4 (TLR-4) activity that has been associated with spontaneous lupus development.[7, 8] Adjustment of the SLE microbiome *via* dietary intervention has shown attenuation of SLE symptoms. The ingestion of retinoic acid, a metabolite of vitamin A as a dietary intervention restored normal *Lactobacilli* levels in lupus-prone mice.[6] Vitamin A ingestion has also been shown in a very small study to ameliorate lupus nephritis and proteinuria.[9] Finally, increases in *Lactobacilli* in SLE murine models have been shown to suppress pro-inflammatory responses.[6]

Recent advances in "metabolomics" have broadened insights of the metabolites produced or metabolised by the gut microbes, which can serve as important immune regulators or initiators in a wide variety of diseases, including autoimmune diseases.[10] However, there is a scarcity of studies investigating the metabolites of altered gut microbiota in SLE patients where TMA/TMAO have been studied.[11, 12] In humans, trimethylamine (TMA) is synthesised exclusively by gut microbiota from dietary nutrients contained in high-fat foods including choline, L-carnitine and other TMA-containing nutrients. TMA produced in the gut enters the circulation and is further converted into trimethylamine N-oxide (TMAO) by host enzymes like flavin monooxygenase (FMO) in the liver.[13, 14]

Eight species of human commensal bacteria were identified to produce TMA from choline including two different Firmicutes.[15] Both human and animal studies suggest that the gut microbe-derived metabolite TMAO is strongly associated with cardiovascular disease.[16] TMAO has been positively linked to the pathogenesis of several other diseases as well, including chronic kidney disease, type 2 diabetes and obesity in a tissue- and cell-specific pattern.[17-20] Direct exposure to TMAO can augment Ca^{2+} release, enhance platelet activation and form pro-thrombotic state.[21] TMAO activates mitogen-activated protein kinase (MAPK) in vascular endothelial cells and smooth muscle cells, which promotes the expression of inflammatory genes and recruitment of activated leukocytes.[22] Dietary choline and TMAO initiate renal fibrosis and dysfunction in animal models *via* a transforming growth factor-β (TGFβ)–phospho-SMAD3 (P-SMAD3) signalling pathway.[19] The metabolite perturbations related to rheumatic disease remains poorly resolved and merits further studies.

The aim of this study was to investigate the diagnostic and prognostic validity of TMAO as a potential biomarker in lupus nephritis patients and its relation to disease activity.

Methods

Study sample

This was a cross sectional study conducted to assess the role of TMAO as a potential biomarker in patients with SLE, conducted for 1-year duration. The study included 90 subjects who were classified into 3 groups: Group (I): included patients who were diagnosed as SLE without nephritis (NN); Group (II): included patients who were diagnosed as lupus nephritis (LN) and Group (III): included healthy subjects as a control. Male and female SLE patients diagnosed according to 2019 European League Against Rheumatism and American College of Rheumatology classification criteria for SLE[23] and aged > 18 years with willingness to participate in the study were included. Patients who refused the enrolment in the study, patients with overlap syndrome, patients with organ specific or other systemic autoimmune disorders, patients with non-SLE renal affection eg diabetic or hypertensive nephropathy and primary or other

secondary glomerulopathies and pregnant SLE female patients were excluded from the study.

Data collection

The medical records of patients were reviewed using a computerised sheet including all studied data for each patient. The authors have followed Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines. All patients were subjected to the following: complete history taking; thorough clinical examination; SLE international Collaborating Clinics/ American College of Rheumatology Damage Index (SLICC/ACR); Systemic Lupus Erythematosus Disease Activity Index (SLEDAI); laboratory investigations including: complete blood count (CBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), kidney function test, urine analysis, 24-hour urinary protein, antinuclear antibody (ANA), complement (C3, C4) and anti-ds DNA; kidney biopsy; radiology - abdominal sonography with emphasising on kidneys.

The SLICC/ACR Damage Index (SDI) was developed in 1996 to assess an ongoing reflection of disease activity in SLE patients and to measure irreversible damage resulting from SLE disease activity and its treatment.[24] SLEDAI index is composed of 24 features that are attributed to lupus which are listed, with a weighted score. The more serious manifestations (such as renal, neurologic and vasculitis) are weighted more than others (such as cutaneous manifestations). The maximum possible score was 105.[25] Kidney biopsy was performed for all patients fulfilling LN ACR criteria to confirm the diagnosis and to classify the glomerular disease by current International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification.

Renal histopathological examination

The renal histopathology was evaluated according to the WHO classification of LN with assessment of activity and chronicity indices as follows: activity index (AI). This index was assessed as the sum of individual scores of the following items considered to represent measures of active lupus nephritis: glomerular proliferation, leucocyte exudation, karyorrhexis/fibrinoid necrosis (x2), cellular crescents (x2), hyaline deposits and interstitial inflammation. The maximum score was 24 points for the AI.[26] Chronicity index (CI) consisted of the sum of individual scores of the following items considered to represent measures of chronic irreversible lupus nephritis: glomerular sclerosis, fibrous crescents, tubular atrophy and interstitial fibrosis. The maximum score was 12 points for the CI.[27]

Sampling

Urine and venous blood samples were obtained after overnight fasting. Trimethylamine levels were measured using the quantitative sandwich enzyme linked immunosorbent assay method, with Human (ELISA) kit (Cat No E4733Hu). The plate has been pre-coated with human TMAO antibody. The standard curve was constructed by plotting the average optical density (OD) for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and a best fit curve was drawn through the points on the graph. These calculations were performed with computer-based curve-fitting software.

Statistical analysis

Data were coded, computed then analysed using Statistical package for social science (IBM SPSS) version 24 for Windows. Qualitative data were presented by frequency tables. For quantitative variables the normality of data was first tested with Shapiro-Wilk test and presented data by central indices and dispersion: mean ± standard deviation (SD) for normally distributed variables and median (minimum – maximum) for non-normally distributed variables. Chi-square test was used to test association between categorical variables. It was replaced by Fisher Exact Test if the expected cell count was less than 5 in four-cells tables, while it was replaced by Monte Carlo test if the expected cell count was less than 5 in more than four-cells tables. Association between normally distributed continuous variables was tested using independent sample t-test in 2 independent groups, while Mann-Whitney U test (z) was used to compare two independent nonnormally distributed continuous variables. The one-way analysis of variance (ANOVA) was used to determine whether there are any statistically significant differences between the means of two or more independent (unrelated) groups. Also, Kruskal-Wallis H test was used to compare nonparametric continuous variables in more than two different groups. The Spearman correlation coefficient (r) to assess the strength of the correlations between pairs of variables. Significant predictors in the univariate analysis were entered into regression model. For all above-mentioned statistical tests the results were considered significant when the probability of error is less than or equal 5 % ($p \leq 0.05$).

Results

The results showed no statistically significant difference in the clinical and demographic criteria between SLE with and without nephritis except for gender as all the cases in the NN group were females while males represented 10.5 % of the LN group (p = 0.047). The included patients had a mean age of 32.8 ± 12.6 and 30.7 ± 9.8 in the NN and LN groups, respectively. Comparing the clinical profile of patients in the LN and NN groups, there was no statistically significant difference in the incidence of diabetes mellitus, cardiac manifestations, respiratory manifestations, haematological manifestations, musculoskeletal manifestations, mucocutaneous manifestations and vascular manifestations (Table 1).

The prevalence of hypertension (HTN) was higher in LN patients (57.9 %) as compared with the NN group (21.4 %) (p = 0.002). The prevalence of neurological manifestations was 42.1 % in the LN patients and 21.4 % in the NN group (p = 0.05). In both LN and NN groups, male gender, patients with neurological, respiratory and vascular manifestations, as well as patients with urinary casts and protein of more than 500 mg/day had statistically significant higher SLEDAI-2K Score (Table 2). Urinary protein was significantly higher in LN patients with a median of 2.100 mg/day as compared to the NN patients (p ≤ 0.001). There was no statistically significant difference in the haemoglobin level, RBC count, total and differential WBC count, platelet count, serum Na level, ANA and complement (C3, C4 count between the two comparison groups (Table 3).

Table 1: Basic clinical data and extra renal manifestations of the studied groups (n = 80)

Parameters	Non-nephritis SLE	Nephritis SLE	Sig
Blood pressure			
Hypotensive	2 (4.8 %)	0 (0.0 %)	MC = 12.1
Normal range	31 (73.8 %)	16 (42.1 %)	p = 0.002*
Hypertensive	9 (21.4 %)	22 (57.9 %)	
Diabetes mellitus			
Yes	4 (9.5 %)	0 (0.0 %)	FET**
No	38 (90.5 %)	38 (100.0 %)	p = 0.12
Neurological manifestations			
Yes	9 (21.4 %)	16 (42.1 %)	χ^2 = 4.00***
No	33 (78.6 %)	22 (57.9 %)	p = 0.05
Cardiac manifestations			
Yes	7 (16.7 %)	6 (15.8 %)	χ^2 = 0.01
No	35 (83.3 %)	32 (84.2 %)	p = 0.91
Respiratory manifestations			
Yes	14 (33.3 %)	17 (44.7 %)	χ^2 = 1.10
No	28 (66.7 %)	21 (55.3 %)	p = 0.29
Haematological manifestations			
Yes	19 (45.2 %)	21 (55.3 %)	χ^2 = 0.80
No	23 (54.8 %)	17 (44.7 %)	p = 0.37
Musculoskeletal manifestations			
Yes	32 (76.2 %)	33 (86.8 %)	χ^2 = 1.50
No	10 (23.8 %)	5 (13.2 %)	p = 0.26
Mucocutaneous manifestations			
Yes	29 (69.0 %)	25 (65.8 %)	χ^2 = 0.09
No	13 (31.0 %)	13 (34.2 %)	p = 0.76
Vascular manifestations			
Yes	5 (11.9 %)	4 (10.5 %)	χ^2 = 0.04
No	37 (88.1 %)	34 (89.5 %)	p = 0.85
Lower limb oedema			
Yes	16 (38.1 %)	19 (50.0 %)	χ^2 = 1.10
No	26 (61.9 %)	19 (50.0 %)	p = 0.28

*Sig: test of significance; SLE: systemic lupus erythematosus; *MC: Monte Carlo test; ** FET: Fisher's Exact test; ***χ^2: Chi-square test;*

CONTENT

Table 2: Association between trimethylamine N-oxide (TMAO) and other parameters of the studied group

Parameters	TMAO	Sig
Gender		
Male	8.8 (7.3 – 32.4)	Z = 0.89
Female	8.1 (4.2 – 46.2)	p = 0.39
Blood pressure		
Hypotensive	10.7 (8.6 – 12.8)	KW = 1.6
Normal range	8.1 (5.6 – 46.2)	p = 0.44
Hypertensive	8.1 (4.2 – 32.4)	
Diabetes mellitus		
Yes	8.4 (6.3 – 39.2)	Z = 0.17
No	8.2 (4.2 – 46.2)	p = 0.89
Neurological manifestations		
Yes	8.0 (4.2 – 32.4)	Z = 1.4
No	8.3 (5.6 – 46.2)	p = 0.16
Cardiac manifestations		
Yes	8.6 (6.5 – 27.6)	Z = 0.54
No	8.1 (4.2 – 46.2)	p = 0.59
Respiratory manifestations		
Yes	8.4 (4.2 – 39.2)	Z = 0.89
No	8.0 (6.1 – 46.2)	p = 0.37
Haematological manifestations		
Yes	8.6 (6.3 – 46.2)	Z = 1.2
No	7.9 (4.2 – 39.2)	p = 0.23
Musculoskeletal manifestations		
Yes	8.3 (6.1 – 46.2)	Z = 2.1
No	7.5 (4.2 – 12.1)	p = 0.04
Mucocutaneous manifestations		
Yes	8.3 (5.6 – 46.2)	Z = 1.5
No	8.0 (4.2 – 20.4)	p = 0.14
Vascular manifestations		
Yes	7.5 (4.2 – 10.9)	Z = 1.5
No	8.2 (5.6 – 46.2)	p = 0.14
Lower limb oedema		
Yes	8.3 (4.2 – 46.2)	Z = 0.54
No	8.1 (5.6 – 27.6)	p = 0.59
Proteinuria (mg/day)		
< 500 mg/day	8.1 (5.6 – 39.2)	Z = 0.18
≥ 500 mg/day	8.3 (4.2 – 46.2)	p = 0.86
Casts in urine		
Yes	8.4 (5.6 – 46.2)	Z = 0.46
No	8.1 (4.2 – 39.2)	p = 0.64

Z: Mann Whitney test; KW: Kruskal-Wallis test; Sig: test of significance;

Patients with LN exhibited higher titres of anti-dsDNA. The median level of TMAO was 8.1 ng/mL, 8.3 ng/mL and 16.6 ng/mL in the NN, LN and control group respectively (p = 0.01) (Figure 1).

There was no significant difference in the TMAO level in male and female SLE patients. Increased TMAO levels have been only associated with musculoskeletal manifestations in SLE patients.

Table 3: Spearman's correlation between trimethylamine N-oxide (TMAO) and other parameters in LN and NN groups (n = 80)

Parameters	TMAO in LN		TMAO in NN	
	r	p	r	p
Age	-0.17	0.28	0.030	0.88
Hb (g/dL)	-0.11	0.48	0.200	0.24
RBC ($\times 10^6$/mm^3)	-0.13	0.41	-0.380	**0.02**
WBC ($\times 10^3$/mm^3)	-0.09	0.56	-0.030	0.85
Lymphocytes ($\times 10^3$/mm^3)	0.25	0.11	-0.080	0.65
Platelet count ($\times 10^3$/mm^3)	0.26	0.09	0.090	0.59
Serum creatinine level (mg/dL)	0.05	0.75	0.070	0.66
Serum Na level	0.16	0.30	-0.100	0.56
Serum K level	-0.05	0.77	0.100	0.55
ANA	0.02	0.89	-0.010	0.95
Anti-ds DNA	0.20	0.20	0.290	**0.02**
C3	0.06	0.69	0.040	0.80
C4	0.15	0.33	0.010	0.94
Urinary protein (mg/day)	-0.24	0.13	-0.007	0.97
Count of WBCs or pus cells in blood urine	-0.05	0.78	-0.060	0.74
Count of RBCs in urine	-0.26	0.09	-0.230	0.17
SLEDAI-2K score	-0.15	0.36	-0.020	0.89
Class of renal biopsy	-	-	0.120	0.48
Number of glomeruli	-	-	0.090	0.59
Activity index	-	-	-0.150	0.40
Chronicity index	-	-	-0.030	0.89

r: correlation coefficient; Group NN: included patients who were diagnosed as SLE without nephritis; Group LN: included patients who were diagnosed as lupus nephritis; Hb: haemoglobin; RBC: red blood cells; WBC: white blood cells; SLEDAI: Systemic Lupus Erythematosus Disease Activity index; ANA: antinuclear antibodies;

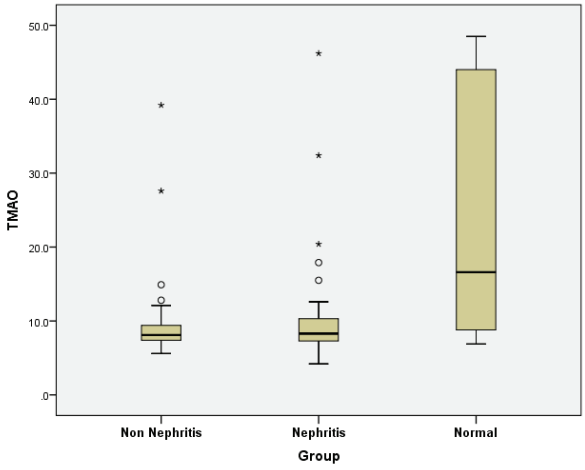

Figure 1: Serum trimethylamine N-oxide (TMAO) concentration in non-nephritis SLE, nephritis SLE and control group

TMAO positively correlated to the Anti-ds DNA titres and negatively correlated to the blood and urinary RBCs in the LN group. Nevertheless, other parameters did not reveal any significant correlation with TMAO (Table 3).

Table 4: Multiple linear regression with trimethylamine N-oxide (TMAO) as dependent variable in the studied group

Predictor (s)	β	t	p
RBC	-0.12	1.1	0.31
Count of RBCs in urine	-0.14	1.2	0.25
Anti-ds DNA	0.27	2.4	0.02*
Musculoskeletal manifestations			
Yes	-0.16	1.4	0.15
No (r)			

R^2 = 0.14; F = 3.1; Constant = 1.1; Overall p = 0.02; RBC: red blood cells;

In the Multiple linear regressions with TMAO as a dependent variable in the studied group, only anti-dsDNA revealed a statistically significant association with TMAO levels (Table 4).

Discussion

The microbiota, as an enormous human body population, plays a great role in human health and disease. This involves mastering the development of the immune system and homeostasis. Through developing a number of metabolites, microbes can support human health and prevent disease, but it can be a double-edged weapon for the host.[28] Recent advancements in metabolomics have widened understanding of the metabolites developed or metabolised by gut microbes which can function in a wide range of diseases, including autoimmune diseases, as significant immune regulators or activators.[10]

Serum TMA levels were found to be elevated in a group of patients with active rheumatoid arthritis (RA).[11] In psoriatic arthritis patients, serum TMAO correlated to disease activity in both skin and peripheral joints.[12] In humans, TMA is synthesised exclusively by gut microbiota from dietary nutrients contained in high-fat foods including choline, L-carnitine and other TMA-containing nutrients. TMAO has been recognised as a metabolite that serves as a potent RA discriminator in the urine compared to healthy subjects. Whether TMAO could help as a discriminator of SLE from healthy subjects or could discriminate LN from NN patients and whether it has an association with the disease severity including clinical, laboratory and histopathological criteria; this was the aim of this study. In the present study, TMAO levels have been evaluated in patients with SLE with and without nephritis in comparison with controls.

It has been previously found that urinary TMAO levels were higher in SLE patients than in healthy participants. This result suggests that gut microbiota and specific dietary nutrients that enhance TMAO generation are associated with disease severity.[29] This contradicts the results of the present study as it has been found that TMAO levels were significantly higher in healthy controls compared to SLE patients with and/or without nephritis. Disturbances in the composition and balance of the microflora can occur due to unhealthy lifestyle (eg unhealthy diet, low degree of physical activity and stress), consequent diseases or the use of some medications can lead to a number of disorders both locally in the gut as well as systemically.[30] This reflects an important limitation of the present study; the assessment of dietary pattern in patients and healthy controls and its effect on the gut microbiome and its derived metabolites as TMAO. As an instance, a known confounding factor is the fact that certain types of fish intrinsically contain very high amounts of TMAO[31] and thus having such a diet can affect the TMAO assessment. Another important point that should be considered the polypharmacy usually used by SLE patients and the effect of the commonly used immunosuppressive medications on the gut microbiome.

Whether certain immunosuppressive could suppress the normal gut microbiota; this raises a doubt to the largely supporting evidence to the validity of TMAO as a biomarker. Furthermore, whereas the role of bacterial-derived TMAO in cardiovascular disease pathogenesis has recently gained significant interest, it has not remained indisputable. Jia and colleagues recently used a Mendelian randomisation approach to find that genetically predicted higher TMAO was not associated with higher odds of cardiovascular disease.[32] These authors instead conclude that the observational evidence for cardiovascular diseases may be due to confounding or reverse causality.[30] The correlations between TMAO and clinical-laboratory parameters of LN and NN groups revealed only significant correlation with RBCS counts in LN patients.

Correlating the values of TMAO to the clinical-laboratory parameters of the total SLE population, a significant correlation has been found between TMAO and RBCs count, urinary RBCs and anti-dsDNA titres. The current literature suggests a regulatory effect of the gut microbiome on the haematopoietic system.[33] This might explain

the correlation between TMAO and RBCs count shown in the present study. The positive correlation between TMAO and autoantibodies such as anti-dsDNA titres has been supported by previous studies. TMAO was suggested as a diagnostic biomarker in animal models with MPO-ANCA vasculitis[34] as well as it correlated to the activity of various autoimmune disease.[35, 36] Reported that translocation of gut commensals drives IFN and anti-dsDNA in mice with lupus like disease.[36] Among the positively correlating variables to TMAO, it has been revealed in a multiple linear regression analysis with TMAO as a dependent variable that TMAO significantly predicted anti-dsDNA levels but no other parameters. Hormonal regulation of the processes of microbe control suggests that the commensal composition should vary between the two genders.[37]

In the present work, TMAO levels did not vary between male and female patients, but this can be simply attributed to the greater predominance of females in the included study sample which reflects the classic gender predominance in SLE patients. It has been previously shown that in animal SLE models, the diversity of gut microbiota differs between female and male lupus-prone mice and the female mice have more severe disease[6] and castration of male mice reversed this difference indicating a protective androgen-dependent pathway.[37]

The present study failed to report an association between TMAO and the different studied demographic and clinical parameters. An exception was the significant association between TMAO and the musculoskeletal manifestations of SLE patients. The gut derived metabolite, TMAO appears to be a major player in the various determinants of rheumatic and musculoskeletal diseases.[38] As well, raised TMAO levels have been shown to exhibit a strong negative correlation with the degree of bone mineral density (BMD) in patients with osteoporosis.[39]

In the present study, there was no significant statistical difference of TMAO levels between NN and LN patients while SLEDAI-2K score was significantly higher in LN patients. Previous studies have emphasised the prognostic value of SLEDAI-2K score particularly in LN patients.[40] TMAO has been previously identified as marker of renal medullary injury and may be an indicator of the tubulointerstitial nephritis. However, it did not show correlation to the tubulointerstitial lesions in the studied kidney biopsies. In an earlier study, TMAO has been found to be the strongest predictor of histological injury, suggesting that it may reflect the totality of renal parenchymal injury. The majority of LN patients included in this study had active proliferative lupus nephritis with class IV representing (56.8 %) and class V representing (21.6 %) of the total nephritis population. This copes with previous reports revealing a higher prevalence of proliferative class III, IV in the studied renal biopsies.[40] The exact molecular pathways linking gut microbiota to SLE and lupus nephritis are not fully elucidated. SLE patients exhibit limited gut microbiota diversity[36] however, further studies are warranted in this field.

Based on the results, the diagnostic and prognostic utility of TMAO generally in the setting of autoimmunity and specifically, in SLE faces many challenges. The reliance on TMAO as a diagnostic marker should put into consideration several dietary and comorbid conditions. This study presented two important limitations; first, the small sample size reflects the need for further studies with larger sample sizes. Second, the dietary patterns and polypharmacy might have impacted the results, however, most of the current evidence supporting the potentiality of TMAO as a diagnostic biomarker did not investigate the dietary pattern of the participants.

Conclusion

Contrary to the results of the previous studies, TMAO levels were found to be higher in healthy controls rather than SLE patients and did not discriminate between LN and NN patients as well as did not show significant correlation to the studied criteria of disease severity excluding the Anti-dsDNA titres. The possible confounding effect of the dietary pattern and ingested drugs on the gut microbiome underestimate the diagnostic and prognostic utility of TMAO as a potential marker in different diseases.

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Ethics

This study was approved by the Institutional Review Board of the Mansoura Faculty of Medicine, Mansoura University (decision No MS.18.12.388, dated 6 February 2019). Written informed consent was obtained from patients prior to their participation in the study and for publishing of the anonymised patient data. The authors have followed Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Guidelines. The study was organised and implemented based on the adherence to the Ethical Principles for Medical Research Involving Human subjects (The Declaration of Helsinki, 8th Revision, 2013).

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Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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ORIGINAL ARTICLE

Features of Metabolism in Chronic Wound Remodelling

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Abstract

Background/Aim: The treatment of chronic wounds continues to be a pressing problem throughout the world. Healing occurs through some evolutionarily conserved biochemical pathways. The mechanisms of development of disorders of reparative regeneration are not fully understood. The work aimed to study the dynamics of changes in metabolic parameters during the healing of chronic wounds.

Methods: Healthy Wistar rats were divided into two groups. The animals of the first group were intact. Chronic wounds were simulated for the animals of the second group. On days 7, 14 and 28 after wound creation, the animals were euthanised. Biochemical parameters such as glucose, total protein, albumin, cholesterol, urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were assessed in the blood serum of animals.

Results: It was found that the maximum decrease in glucose and total protein levels in the blood serum of animals in the experimental groups compared to intact animals was observed 2 weeks after surgery: the glucose concentration in rats was 1.7 times lower ($p < 0.001$). The level of albumin in the blood serum of experimental animals compared to intact animals was reduced by 1.5 times after 14 days ($p < 0.001$) and by 1.2 times after 28 days ($p < 0.01$). A week after surgery, the concentration of urea in the blood serum of experimental animals was 1.3 times higher ($p < 0.01$) than in intact rats and by day 28 after surgery, the urea level was 1.4 times higher ($p < 0.001$). The reduction in cholesterol and creatinine levels was not significant. An increase in AST, AST and ALP levels in the blood serum of experimental animals was shown. An increase in the blood serum of animals 7 days after surgery compared to the indicators of intact animals: ALP concentrations by 2.8 times ($p < 0.001$) and ALT concentrations by 1.4 times ($p < 0.001$) was established. The AST level significantly increased 14 days after surgery ($p < 0.05$).

Conclusions: The study of metabolic parameters allows monitoring of the state of the body during the healing process of wounds to correct treatment tactics.

Key words: Wound healing; Metabolism; Rats.

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Introduction

The treatment of chronic wounds continues to be a pressing problem throughout the world. About 6.7 million people worldwide suffer from chronic wounds.[1] Problems in healing chronic wounds are associated with many factors, including the elderly age of patients, concomitant diseases, treatment of wound infections, the influence of emotional and psychological stress, etc.[2] The

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degree of involvement of endocrine mechanisms regulating metabolic processes can also serve as a predictor of the chronicity of the process.[3] Metabolism plays an important role in wound healing and defects in metabolic regulation are also implicated in the pathogenesis of chronic wounds.[4] The problem is especially acute in the case of combat trauma, since in combat conditions several factors occur simultaneously that lead to an increased risk of the formation of chronic wounds.

Wound healing is a physiological response to tissue damage. Healing occurs through several evolutionarily conserved biochemical pathways regulated by various cytokines, growth factors and immune cells.[5] Wound healing is divided into three distinct phases: inflammatory, proliferative and remodelling.[6] The inflammatory phase includes haemostasis, chemotaxis of neutrophils and macrophages and increased vascular permeability, which promotes wound cleansing, cell migration, cytokines and reactive oxygen species formations. With the development of chronic inflammation, an imbalance of regulatory mechanisms occurs, characterised by the activity of proinflammatory cytokines and the insufficient effect of their inhibitors and antagonists.[7] Prolongation or delay of the inflammatory phase negatively affects the remaining stages of wound healing.[8] During the proliferative phase, granulation tissue formation, re-epithelialisation and angiogenesis occur. The new blood vessels that are formed help to better meet the metabolic needs of wound healing. During the remodelling phase, granulation tissue is replaced by a permanent scar. Changes that occur during any of these phases may contribute to the development of a chronic wound and interfere with its healing.[9, 10] The other extreme is excessive extracellular matrix formation with disrupted architecture, leading to organ fibrosis (such as hypertrophic scarring of the skin and keloid formation).[11] Skin wound healing involves metabolic reprogramming of glucose, lipids, amino acids and other nutrients that play a vital role in the proliferation, differentiation and migration of various cell types.[12]

The mechanisms of development of disorders of reparative regeneration are not fully understood. There is insufficient information about the features of changes in the basic indicators of metabolic processes during the healing of chronic wounds. The work aimed to study the dynamics of changes in metabolic parameters during the healing of chronic wounds.

Methods

Twenty-four clinically healthy Wistar rats of both sexes aged 8-9 months with a body weight of 220-250 g were used for the study. The animals were divided into two groups and kept under normal vivarium conditions. The first group (n = 6) was intact rats (Int). Eighteen animals of the second group had simulated wounds. The dorsal thoracic area was depilated and prepared for aseptic surgical procedures. Animals were placed under general anaesthesia to create wounds. Each animal was administered tiletamine hydrochloride and zolazepam hydrochloride combination *Zoletil* (Virbac, France) 10 mg/kg intramuscularly (im). Round skin wounds 2 cm in diameter were simulated. The edges of the wound were trimmed with a perpendicular loop-shaped fasciocutaneous suture. On the surface of the wound bottom, the superficial fascia was dissected with mutually perpendicular transverse and longitudinal cuts in the form of a figure measuring 5x5 mm. Then the formed areas were sutured with U-shaped sutures (Figure 1) to create a picture of trophic changes not only in the skin but also in part of the superficial fascia, as well as the underlying tissues. The wound thus obtained represented a model of a chronic wound, reproducing the conditions of local hypoxia and impaired microcirculation.

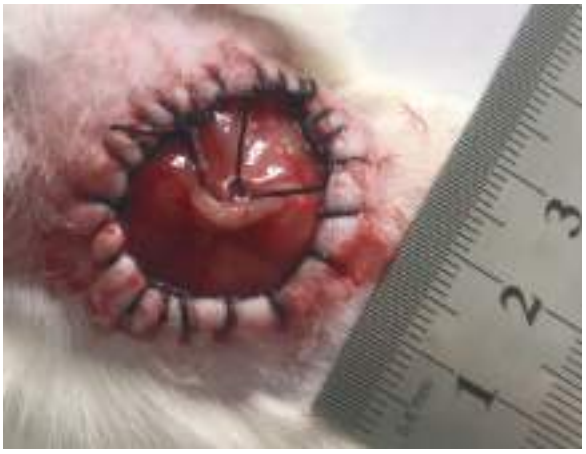

Figure 1: The induced injury

On days 7 (group Exp7, n = 6), 14 (group Exp14, n = 6) and 28 (group Exp28, n = 6) after wound creation, the animals were euthanised. Blood was

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collected from the heart by an open puncture. Biochemical parameters were assessed in animals with wounds and in intact animals. Levels of glucose, total protein, albumin, cholesterol, urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in blood serum were determined with the help of reagent kits of *DAC-SpectroMed* (Moldova).

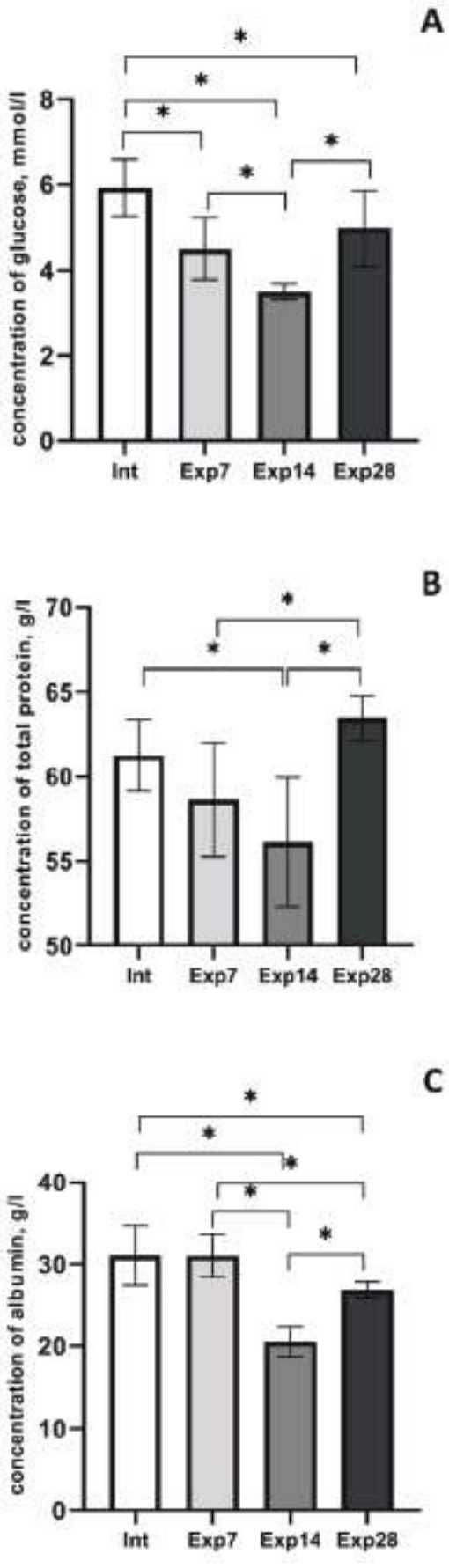
Data analysis

Statistical analysis was performed using *Statistica* 12.0 software (*StatSoft*, USA). The descriptive data were presented as M ± SE, where M is the arithmetic mean and SE is the standard error of the arithmetic mean. The significance of the differences between groups was evaluated using the non-parametric Kruskal–Wallis ANOVA test for independent samples. Differences were considered statistically significant at $p < 0.05$. The relationship between the levels of the studied metabolic parameters was evaluated according to Spearman's rank correlation coefficient (r). Histograms were plotted by *GraphPad Prism* 9 (GraphPad Software, USA).

Results

The concentrations of glucose, total protein, albumin, cholesterol, urea, creatinine, AST, ALT and ALP in the blood serum of animals are presented in Figure 2.

It was found that the level of glucose in the blood serum of animals in the experimental groups was reduced throughout the experiment compared to intact animals (Figure 2A). Thus, two weeks after surgery, the glucose concentration in rats was 1.7 times lower ($p < 0.001$) than the glucose level in intact animals. By the end of the experiment, the glucose level in animals with wounds increased but remained 1.2 times lower ($p < 0.05$) than in intact animals. The concentration of total protein in the blood serum of animals with surgery, compared with that of intact animals, decreased 7 days ($p > 0.05$) and 14 days ($p < 0.01$) after surgery (Figure 2B). However, 28 days after surgery, the total protein levels of the experimental animals returned to the values of intact rats. The level of albumin in the blood serum of experimental animals compared to



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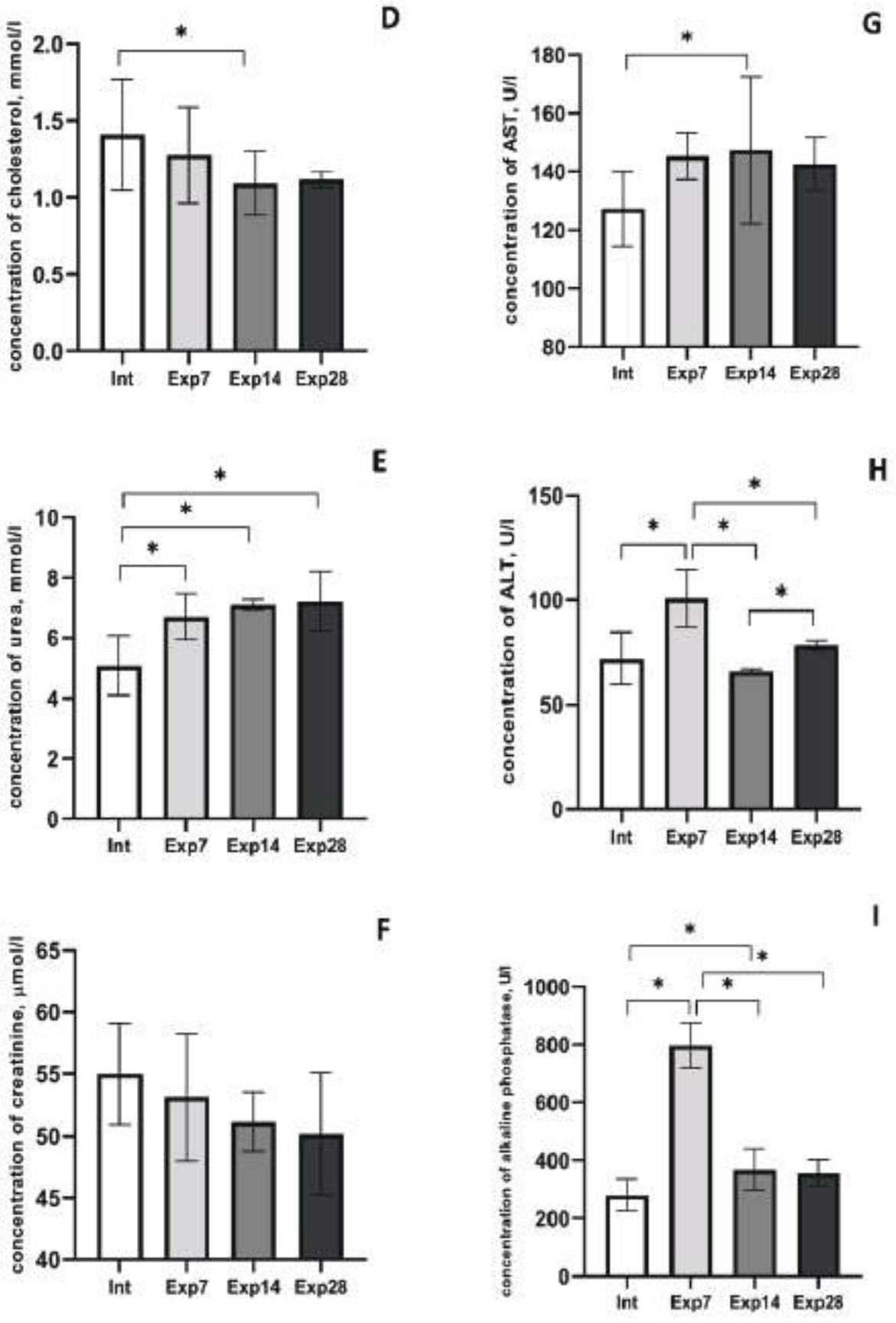

Figure 2: Levels of the studied indicators in the blood serum of animals: (A) glucose, (B) total protein, (C) albumin, (D) cholesterol, (E) urea, (F) creatinine, (G) aspartate aminotransferase (AST), (H) alanine aminotransferase (ALT) and (I) alkaline phosphatase (ALP)

**: $p < 0.05$; The error bars represent the standard error of the arithmetic mean for each indicator (n = 6). Int: intact rats. Exp: experimental groups with simulated wounds euthanised on days 7 (Exp7), 14 (Exp14) and 28 (Exp28) after wound creation.*

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intact animals was reduced by 1.5 times after 14 days ($p < 0.001$) and by 1.2 times after 28 days ($p < 0.01$) after surgery (Figure 2C). The levels of cholesterol (Figure 2D) and creatinine (Figure 2F) in the blood serum of animals after surgery, compared with the indicators of intact animals, tended to decrease at all periods of the experiment, although this difference was not statistically significant. The urea concentration in postoperative animals was increased compared with the urea level in intact animals at all experimental times (Figure 2E). Moreover, a week after the operation, the concentration of urea in the blood serum of experimental animals was 1.3 times higher ($p < 0.01$) than in intact rats. And by day 28 after surgery, the urea level was 1.4 times higher ($p < 0.001$). The level of AST (Figure 2G) in the blood serum of animals after surgery had a significant increase compared to the concentration of this enzyme in intact animals only 14 days after surgery ($p < 0.05$). During all other periods of the experiment, the increase in AST levels in experimental animals blood serum was insignificant. The concentration of ALT in the blood serum of animals with surgery, compared with that of intact animals, was increased by 1.4 times 7 days ($p < 0.001$) after surgery (Figure 2H). Fourteen and 28 days after surgery, AST values practically returned to the AST values of intact rats. The concentration of ALP in the blood serum of animals after surgery compared with the indicators of intact animals was 2.8 times increased 7 days ($p < 0.001$) after surgery (Figure 2I). Fourteen and 28 days after surgery, ALP levels decreased, while remaining higher than ALP levels in intact rats.

The relationships between the levels of metabolic parameters in animals of different groups were studied. According to the results of studies in rats of the experimental group, on day 7 after wound induction, positive correlations were noted between the levels of total protein and urea ($r = 0.959$; $p < 0.05$) and between the levels of AST and albumin ($r = 0.935$; $p < 0.05$); a negative correlation was noted between glucose and urea levels ($r = -0.813$; $p < 0.05$). In the experimental group, on the 14 day after surgery, positive correlations were noted between the levels of cholesterol and total protein ($r = 0.846$; $p < 0.05$), between the levels of AST and albumin ($r = 0,865$; $p < 0.05$), between the levels of ALT and albumin ($r = 0.933$; $p < 0.05$); negative correlations were noted between the levels of ALP and total protein ($r = -0.974$; $p < 0.05$) and between the levels of ALP and cholesterol ($r = -0.924$; $p < 0.05$). In the experimental group, on day 28 after wound creation, a negative correlation was established between urea and creatinine concentration ($r = -0.898$; $p < 0.05$).

Discussion

Currently, the research trend is to study the activity of bioactive molecules at various stages of wound healing. Given the high cost of such studies metabolic parameters in the blood serum of animals with chronic wounds were examined to monitor the state of the body, predict healing and adjust treatment tactics using simple and widely available analytes in an outpatient clinic.

Since wound healing is an energy-intensive process, the dynamics of changes in glucose levels during wound healing have been studied (Figure 2A). Glucose concentrations in animals with simulated wounds decreased significantly two weeks after surgery ($p < 0.001$). This may be due to increased glycolysis found in the late stages of acute wound healing and chronic wounds. There is evidence that differences in the increased expression of genes associated with the transport and metabolism of glucose, as well as glycolytic metabolites and enzymes, were more significant in chronic wounds compared to acute ones.[13] Presented chronic wound model replicated hypoxic conditions. The literature has shown an increase in glycolysis and a weakening of mitochondrial function during hypoxia with the development of fibrosis.[14] The hypoglycaemic state of animals, which were observed for 14 days, apparently indicates body exhaustion. Similar results were obtained on day 14 after the hip fracture simulation.[15] On the 28th day of the experiment, glucose concentrations increased, although they did not reach the level of intact animals ($p < 0.05$).

Since a significant decrease in total blood protein levels may be a risk factor for developing postoperative wound healing complications,[16] presented study examined the dynamics of total protein (Figure 2B) and albumin (Figure 2C) levels. A significant decrease in the levels of total protein and albumin ($p < 0.01$), shown in this work on the 14th day of the experiment is associated with the processes of synthesis and breakdown as a result of injury and tissue repair. Thus, the predominance of catabolic processes in

the body of animals was observed. By the end of the study period, an increase in the total protein and albumin concentrations in the blood serum of animals was observed. This can be explained by an increase in the synthesis of amino acids and proteins in the remodelling phase of the wound process and a shift in the balance towards anabolic processes. According to the literature, the total protein and albumin levels in the plasma of rabbits with wound defects did not change significantly on days 3, 7, 14 and 21.[17]

In this study, cholesterol levels were reduced at all stages of wound healing (Figure 2D). In the context of wound healing, lipids act as signalling agents in wound healing and tissue regeneration.[18] The literature has shown that lipid metabolism gene expression is significantly reduced in both acute and chronic wounds, suggesting the downregulation of lipid metabolism.[13]

It is known that the level of urea as the final product of protein metabolism and the main component of residual nitrogen depends on the intensity of its synthesis and excretion. Therefore, in presented study urea concentrations were determined both to assess the health of the liver, where it is synthesised and the kidneys, through which it is excreted (Figure 2E). Urea levels were elevated throughout the wound healing period ($p < 0.005$). It has been shown that shock from extensive trauma can have adverse effects on the harmful kidneys, with outcomes ranging from mild azotaemia to severe kidney damage requiring renal replacement therapy.[19] For example, burn rats at 11 days post-injury showed significant renal and hepatic tissue damage consistent with acute tubular necrosis and multifocal necrosis and changes in blood chemistry (eg, urea and ALT) reflecting deterioration in renal and hepatic function.[20] However, creatinine levels were not elevated. In presented study, creatinine concentrations (Figure 2F) decreased ($p > 0.05$). It can be assumed that this is due to muscle atrophy. This condition is common among patients with persistent critical illness after injury and is associated with increased urea production but decreased creatinine production.[21]

Levels of enzymes such as AST (Figure 2G) and ALT (Figure 2H) were also analysed as markers of tissue destruction.[22] This study showed an increase in the levels of AST and ALT in the blood serum of experimental animals. Seven days after surgery an increase in ALT concentrations in the blood serum of animals was shown compared to the indicators of intact animals. The AST level significantly increased 14 days after surgery.

Presented study also showed an increase in ALP levels in the blood serum of experimental animals 7 days after surgery compared to the values of intact animals (Figure 2I). ALP activity is a marker of acute inflammation because enzyme levels increase in acute wounds but not chronic inflammatory conditions. However, when chronic lesions or scars are healing, an increase in ALP activity is evident.[23]

Increased serum levels of liver biomarkers AST, ALT and ALP may appear to reflect hepatocellular damage.[24] ALT is more specific for hepatocellular injury due to its much higher concentration in liver tissue than in other tissues. In contrast, AST is widely found in a variety of cells, such as hepatocytes, cardiac and skeletal myocytes and erythrocytes.[25] Presented study shows that soft tissue injury affects liver function by causing changes in blood enzyme levels.

Analysis of the relationships between the levels of biochemical parameters showed statistically significant correlations. Regeneration is associated with metabolic processes in different phases of healing. The data obtained are associated with the activation of various signalling pathways that control the process of wound healing. Thus, the results may indicate the essential role of metabolic parameters in the regulation of reparative processes. The wounding process causes metabolic disorders, which are an important factor in the formation of a chronic wound.

Wound treatment must be carried out considering dynamic changes in metabolic parameters. For example, given that during wound healing, glucose metabolism increases and the activity of key enzymes in this process also changes, when treating diabetic wounds it is necessary, first of all, to influence glucose metabolism.[26] The administration of compound protein can accelerate wound healing and improve nutritional status. Because the compound protein accelerates the entire wound healing process, accelerating the transition of wounds from the inflammatory phase to the proliferation phase and entering the remodelling phase earlier.[27] Cholesterol level control is necessary because hypercholesterolaemia delayed wound closure

in mice for several days, likely due to decreased angiogenesis in the wound bed.[28]

Limitations

This study has several limitations. The disadvantages of the chosen model include anatomical differences in the skin of rats and humans. Rodent skin has a layer of *panniculus carnosus*, which causes rapid wound contraction after injury. In contrast, wounds in humans heal through reepithelialisation and granulation tissue formation. Further research is needed to understand whether the regulatory elements of cell metabolism identified in animal models are conserved in the human.[11]

In presented work, it is impossible to statistically reliably determine the role of each metabolic indicator in wound healing. To do this, it is necessary to significantly increase the research volume to conduct factor and cluster analysis. Taking into account the fact that there is insufficient data in the literature to adequately answer this question, there is a plan to carry out this type of research in the future.

Conclusion

The study of metabolic parameters allows monitoring of the state of the body during the healing process of wounds to correct treatment tactics. Proper systemic support with normalisation of metabolic parameters (adequate levels of protein, sugar, etc in the blood serum involved in tissue regeneration) is an important factor in optimising the reparative process. The change in correlations at different phases of the reparative process apparently reflects the activation of various regulatory mechanisms corresponding to a given stage of the reparative process.

Ethics

The experiments were carried out in compliance with the requirements of the Committee on Ethics and Bioethics of Kharkiv Medical Academy of Postgraduate Education (protocol No 2, dated 6 September 2022), consistent with the principles of the European Convention on the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986) and the Resolutions of the First National Congress on Bioethics (Kyiv, 2001).

Acknowledgement

None.

Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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ORIGINAL ARTICLE

Diuretic Activity of the Hydroalcoholic Extracts of Rhizomes and Leaves of *Artemisia Abyssinica* Sch. Bip. ex A. Rich: *In Silico* and *in Vivo* Study

Nebiyu Abdu,[1] Yonatan Alebachew[1]

Abstract

Background/Aim: The majority of communities in developing nations utilise traditional medicine as an alternative or a combination therapy with a clinically approved diuretic regimen. The present study aimed to investigate the *in vivo* and *in silico* diuretic properties of the 80 % methanol extracts of the rhizomes and leaves of *Artemisia abyssinica*, an indigenous traditional diuretic medicinal plant of Ethiopia.

Methods: Acute oral toxicity tests of 80 % methanol rhizome and leaf extracts of the plant were conducted in mice. For the diuretic test, six treatment groups were administered 100, 200 and 400 mg/kg doses of rhizome and leaf extracts of the plant. The negative and positive control groups were treated with distilled water (2 mL/100 g) and furosemide (10 mg/kg), respectively. Cumulative urine volume, diuretic action, diuretic activity and saluretic index were then determined. In addition, virtual screening and molecular docking study of the compounds of the genus *Artemisia* were done.

Results: The rhizome and leaf extracts of *A abyssinica* were found safe at a dose of 2000 mg/kg. Moreover, both extracts showed a significant diuretic action ($p < 0.05$). However, compared to the standard drug furosemide, the extracts had lower diuretic activity. The rhizome extract increased electrolyte excretion at all doses; particularly at the 200 and 400 mg/kg doses, it exhibited a profound natriuretic, chloruretic and kaliuretic effect with the concentration of 109 and 110 mmol/L for Na^+, 93 and 106 mmol/L for Cl^- and 79 and 86 mmol/L for K^+, respectively. These suggested inhibition of Na^+-K^+-$2Cl^-$ cotransporter as the potential mechanism of action of the extracts. Accordingly, virtual screening and a molecular docking analysis of the compounds of the genus *Artemisia* revealed that a few of them displayed a strong binding interaction with the cation-chloride cotransporter NKCC1 (PDB: 7S1Y), further indicating the cation-chloride cotransporter as a diuretic target of the constituents of the plant.

Conclusion: The current study supports the traditional claim of the plant for diuresis and recommends further isolation of the active constituents.

Key words: *Artemisia abyssinica*; Diuretics; *In silico*; *In vivo*; Furosemide.

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Introduction

Currently, cardiovascular diseases are the leading global cause of death. In 2021, it was estimated that hypertension affects 1.39 billion adult individuals,[1] while heart failure affects 64 million people worldwide.[2] These diseases are approximately equally prevalent in low and high income nations, 28.5 % and 31.5 %, respectively.[3] According to Kibret and Mesfin,[4] the prevalence of hypertension in Ethiopia is 19.6 %.

Diuretics are substances that increase urine and solute production by the kidneys. Most diuretics exert their action by decreasing renal tubular sodium reabsorption, thereby reducing the luminal-cellular osmotic gradient, which limits water reabsorption and results in diuresis.[5] Different types of diuretics are clinically used for the treatment of various cardiovascular and renal disorders, such as hypertension, heart failure, ascites, acute kidney injury, ect.[6-8] However, there are certain adverse effects to using a diuretic regularly. They cause electrolyte imbalances and metabolic abnormalities, including hyponatraemia, hypokalaemia or hyperkalaemia, hypomagnesemia, acid-base abnormalities, hyperuricaemia, hyperglycaemia, hyperlipidaemia and impotence.[9] They also show a variety of drug-drug interactions with other medications.[10]

In addition to their adverse effects and drug-drug interactions, the affordability and availability of diuretics are a major problem in developing countries as they are long-term prescription drugs taken on a regular basis. Consequently, the majority of communities in developing nations utilise traditional medicine as an alternative or a combination therapy with a clinically approved diuretic regimen.[11] Therefore, scientific validation and standardisation of these traditional medical practices are important. Artemisia species are among the most commonly utilised traditional diuretic therapeutic plants in this regard. However, only a few of them have been studied pharmacologically for their diuretic properties.

Hence, the aim of this study was to analyse the *in silico* and *in vivo* diuretic activity of the 80 % methanol extracts of the rhizomes and leaves of *Artemisia abyssinica*, an indigenous Ethiopian medicinal plant that has a traditional diuretic claim.

Methods

Drugs

Drug and chemicals used in this study were: distilled water, normal saline (0.9 %), absolute methanol (*Carlo Erba*, Spain) and the standard drug furosemide (*Epharm, Addis Ababa*, Ethiopia).

Plant material

A abyssinica was collected from the Wondo Genet Agricultural Research Centre found at Wondo Genet SNNRP, Ethiopia. The plant was authenticated by Mr Melaku Wondafrash, a senior botanist at the National Herbarium, College of Natural and Computational Sciences, AAU, where a voucher specimen was deposited (NA001 - *Artemisia abyssinica* Sch. Bip. ex A. Rich) for future references.

Experimental animals

Five to six-week-old healthy Swiss albino mice of either sex weighing 18–29 g were employed during the experiment. The mice were obtained from the animal house of the School of Pharmacy, Addis Ababa University and Ethiopian Public Health Institute and were kept at the School of Pharmacy animal house with a 12 h/12 h light/dark cycle. They were allowed access to water and pellets *ad libitum*. The handling of the mice and all procedures followed were in accordance with the Guide for the Care and Use of Laboratory Animals[12] and were approved by the Institutional Review Board of the School of Pharmacy, College of Health Sciences, Addis Ababa University.

Extraction

The dried powdered rhizomes (55 g) and leaves (200 g) of *A abyssinica* were separately cold macerated with 80 % methanol for 72 h. The extracts were then filtered and the methanol was removed by a rotatory evaporator (*BUCHI Rota vapor R-200*, Switzerland). The remaining water portions of the extracts were freeze-dried using a lyophiliser (OPR-FDU-5012, Korea). Finally, the resulting dried 80 % methanol extracts were weighed, labelled and filled into sample vials and their percentage yield was calculated.

Acute toxicity

Acute oral toxicity tests of the 80 % methanol extracts were carried out as per the producers of the Organisation for Economic Co-operation and Development (OECD) guideline 425 (OECD,

2001). Ten female Swiss albino mice, five for each extract, were used for the test. First, a single mouse from each group was administered 2000 mg/kg of the extracts orally. The mice were fasted for 4 h before and 2 h after the extract administration. After dose administration, the mice were observed continuously for the first 4 h with 30 min intervals and until 24 h for any behavioural changes or signs of toxicity. Since no death was observed within 24 h, an additional four mice were successively administered the same dose of the extracts for the next four days and a similar procedure was followed. Eventually, each mouse was followed for 14 consecutive days with an interval of 24 h for the general signs and symptoms of toxicity and mortality.[13]

Diuretic activity

Diuretic activity was determined following a protocol applied by Lahlou et al[14] with slight modifications. Eighty mice were randomly grouped into 10 groups: six treatment groups and two negative and two positive control groups, each with 8 mice. The mice were placed in a metabolic cage 24 h prior to the commencement of the experiment for acclimatisation and they were also fasted overnight with free access to water. The mice were then orally pretreated with normal saline (0.9 %) at a dose of 0.15 mL/10 g body weight to impose a uniform water and salt load.[15] Then, each treatment group received 100 mg/kg, 200 mg/kg and 400 mg/kg of the 80 % methanol extracts of the leaves and rhizomes of *A abyssinica.* Negative controls were orally administered with the vehicles (distilled water) and positive controls were given furosemide (10 mg/kg) orally. After administration, urine output was measured every h for 5 h. Finally, the total urine collected was stored at -20 °C for further analysis.

The parameters: urinary excretion, diuretic action and diuretic activity were calculated by the formulas given below.

$$\text{Urinary Excretion} = \frac{\text{Total urinary output}}{\text{Total liquid administered}} \times 100\ \%$$

$$\text{Diuretic Action} = \frac{\text{Urinary excretion of treatment group}}{\text{Urinary excretion of control group}} \times 100\ \%$$

$$\text{Diuretic Activity} = \frac{\text{Diuretic action of test drug}}{\text{Diuretic action of standard drug}} \times 100\ \%$$

Analytical procedures

The electrolyte content (sodium, potassium and chloride) of the urine was determined using ion-selective electrode analysis (*COBAS 6000, Roche*, USA). Ratios of electrolytes; Na^+/K^+ and Cl^-/K^+Na^+ were calculated to evaluate the saluretic activity of the extract. Moreover, the salt content of the extract had also been determined to rule out its contribution to urinary electrolyte concentration. In addition, pH was directly determined on fresh urine samples using a pH meter (*JENWAY 370*, England).

Statistical analysis

Data are presented as mean ± standard error of mean (SEM). The data were entered into Statistical Package for Social Sciences (SPSS) version 26.0 and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to compare differences in mean among the groups. A p value of less than 0.05 was considered statistically significant.

Virtual screening and molecular docking study

Virtual screening and molecular docking study were carried out on crystal structures of Cation-chloride cotransporter NKCC1 (PDB: 7S1Y) using SeeSar13.0 software (*BioSolveIT*, Sankt Augustin, Germany). The ligands bumetanide found in the crystal structure of the 7S1Y was identified, the binding sites were selected for docking and its binding modes were calculated. The 206 compounds isolated from the genus *Artemisia* were loaded as sdf files and docking was carried out on the target protein in the docking mode. The best pose solutions were identified and the physiochemical parameters were computed. The HYDE score was used to estimate the binding affinity of the molecules.[16, 17]

Results

Acute toxicity study

The acute oral toxicity test of the extracts of the leaves and rhizomes of *A abyssinica* did not show any signs of toxicity such as tremor, loss of weight, lethargy, paralysis, stress or other adverse behaviours or mortality within 14 days of follow-up. This entails that the LD_{50} value of the extracts was above 2000 mg/kg in mice.

CONTENT

Diuretic activity

As shown in Table 1, the 80 % methanol rhizome extracts of *A abyssinica* were found to have a significant diuretic action at all dose levels tested ($p < 0.05$). Particularly, following 5 h of therapy, the extracts had a significantly higher diuretic activity at 200 mg/kg and 400 mg/kg doses, 2.06 and 2.05, respectively, in comparison to the 100 mg/kg dose, 1.80. However, compared to the positive standard furosemide, the treatment groups exhibited reduced diuretic action and activity. Similarly, the 80 % methanol leaf extracts of *A abyssinica* showed significant diuretic action with values of 1.5 and 1.6 at 200 mg/kg and 400 mg/kg doses, respectively, after 5 h of treatment (Table 2). Nevertheless, the extract displayed no effect at 100 mg/kg and all the treated groups had lower activity than the positive control, the furosemide-treated group.

Table 1: Effect of 80 % methanol extracts of the rhizomes of Artemisia abyssinica on urinary volume excretion in mice

Group	Dose (mg/kg)	Urine volume (mL)					Diuretic action	Diuretic activity
		1 h	2 h	3 h	4 h	5 h		
Distilled water	0.2 mL	0.12 ± 0.04	0.37 ± 0.05	0.12 ± 0.03	0.00 ± 0.00	0.050 ± 0.02	1.00	-
AAR100	100	0.25 ± 0.06	0.00 ± 0.00	0.12 ± 0.02	0.00 ± 0.00	0.090 ± 0.03 $^{a, b, e*}$	1.80	0.27
AAR200	200	0.50 ± 0.17 ^{a**b*e*}	0.43 ± 0.12	0.00 ± 0.00	0.30 ± 0.11 $^{a, b**e*}$	0.103 ± 0.04 $^{a, e*}$	2.06	0.80
AAR400	400	0.40 ± 0.12 ^{a*b*e*}	0.37 ± 0.08	0.30 ± 0.08 ^{a*b*c*}	0.20 ± 0.05 $^{a, b*}$	0.102 ± 0.02 $^{a, b, e*}$	2.05	0.82
Furosemide	10	0.75 ± 0.17 $^{a*,b**c*, d*}$	0.40 ± 0.12	0.25 ± 0.08 ^{a*b*c**}	0.15 ± 0.04	0.120 ± 0.04 $^{a-d*}$	2.40	1.00

*Values are presented as mean ± SEM; n = 8; a = compared to negative control (Distilled water), b = compared to AAR100, c = compared AAR200, d = compared to AAR400, e = compared to furosemide; *(p < 0.05); ** (p < 0.01); AAR = 80 % methanol extract of the rhizomes of Artemisia abyssinica; numbers refer to doses in mg/kg/day.*

Table 2: Effect of 80 % methanol extracts of the leaves of Artemisia abyssinica on urinary volume excretion in mice

Group	Dose (mg/kg)	Urine volume (mL)					Diuretic action	Diuretic activity
		1 h	2 h	3 h	4 h	5 h		
Distilled water	0.2 mL	0.13 ± 0.03	0.40 ± 0.03	0.12 ± 0.05	0.00 ± 0.00	0.05 ± 0.03	1.0	-
AAL100	100	0.13 ± 0.06	0.00 ± 0.00	0.07 ± 0.03	0.00 ± 0.00	0.05 ± 0.02	1.0	0.14
AAL200	200	0.45 ± 0.11 $^{a, b*e**}$	0.40 ± 0.07^{e*}	0.00 ± 0.00	0.25 ± 0.05 $^{a, b, d, e*}$	0.75 ± 0.05 $^{a, b, d, e*}$	1.5	1.01
AAL400	400	0.50 ± 0.10 $^{a, b**e*}$	0.45 ± 0.03	0.17 ± 0.07	0.13 ± 0.04 $^{a, b, c*}$	0.08 ± 0.04 $^{a, b, c*e**}$	1.6	0.72
Furosemide	10	0.72 ± 0.17 $^{a, b, c**d*}$	0.51 ± 0.10 $^{a, b, c, *}$	0.31 ± 0.09	0.16 ± 0.04 $^{a, b, c*}$	0.13 ± 0.04 $^{a, b, d**c*}$	2.5	1.00

*Values are presented as mean ± SEM; n = 8; a = compared to negative control (Distilled water), b = compared to AAL100, c = compared AAL200, d = compared to AAL400, e = compared to Furosemide; *(p < 0.05); ** (p < 0.01); AAL = 80 % methanol extract of the leaves of Artemisia abyssinica; numbers refer to doses in mg/kg/day.*

Saluretic activity

Urine electrolyte was measured from the pooled urine sample at the end of the fifth hour and the saluretic index was measured for all test samples. Accordingly, the rhizome extracts significantly increased electrolyte (Na^+, Cl^- and K^+) excretion in a dose-dependent manner at all doses ($p < 0.05$) (Table 3). It showed a strong natriuretic and chloruretic effect at 200 and 400 mg/kg doses with concentrations of 109 and 110 mmol/L for Na^+ and 93 and 106 mmol/L for Cl^-, respectively. It also exhibited noticeably greater kaliuresis with concentrations of 79 (200 mg/kg) and 86 mmol/L (400 mg/kg). However, compared to furosemide-treated groups, it has less electrolyte excretion. Furthermore, leaf extracts displayed the unexpectedly highest electrolyte loss at 100 mg/kg dose with natriuretic, chloruretic and kaliuretic concentrations of 120, 154 and 104 mmol/L, respectively (Table 4). Yet, at higher doses, it showed only mild natriuretic and chloruretic action (63 and 66 mmol/L) at 400 mg/kg.

Table 3: Effect of 80 % methanol extracts of the rhizomes of Artemisia abyssinica on 5 h urinary electrolyte excretion in mice

Group	Dose (mg/kg)	Urinary electrolyte concentration (mmol/L)			Saluretic Index*			Na^+/K^+	$Cl^-/Na^+ K^+$
		Na^+	K^+	Cl^-	Na^+	K^+	Cl^-		
Distilled water	0.2 mL	64.0 ± 6.32	44.9 ± 0.35	74.4 ± 4.58	-	-	-	1.00	0.68
AAR100	100	97.0 ± 3.94 a, e**d*	67.1 ± 7.51 a, c, d*e**	95.0 ± 5.37 d*a, e**	1.52	1.49	1.28	1.80	0.58
AAR200	200	109.0 ± 3.83 a, e**	79.5 ± 3.48 b, c* a, e**	93.5 ± 4.29 a, c*e**	1.70	1.77	1.26	2.06	0.50
AAR400	400	110.7 ± 6.27 a, e**b*	86.0 ± 5.92 a** b, c, e *	106.7 ± 3.86 a**b, c, e*	1.73	1.92	1.43	2.05	0.54
Furosemide	10	136.1 ± 6.74 a, b, c, d**	104.4 ± 5.98 a, b**c, d**	118.7 ± 4.65 a, b**c, d*	2.13	2.32	1.60	2.40	0.49

*Values are presented as mean ± SEM; n = 8; a = compared to negative control (Distilled water), b = compared to AAR100, c = compared AAR200, d = compared to AAR400, e = compared to Furosemide; *(p < 0.05); ** (p < 0.01); AAR = 80 % methanol extract of the rhizome of Artemisia abyssinica; numbers refer to doses in mg/kg/day.*

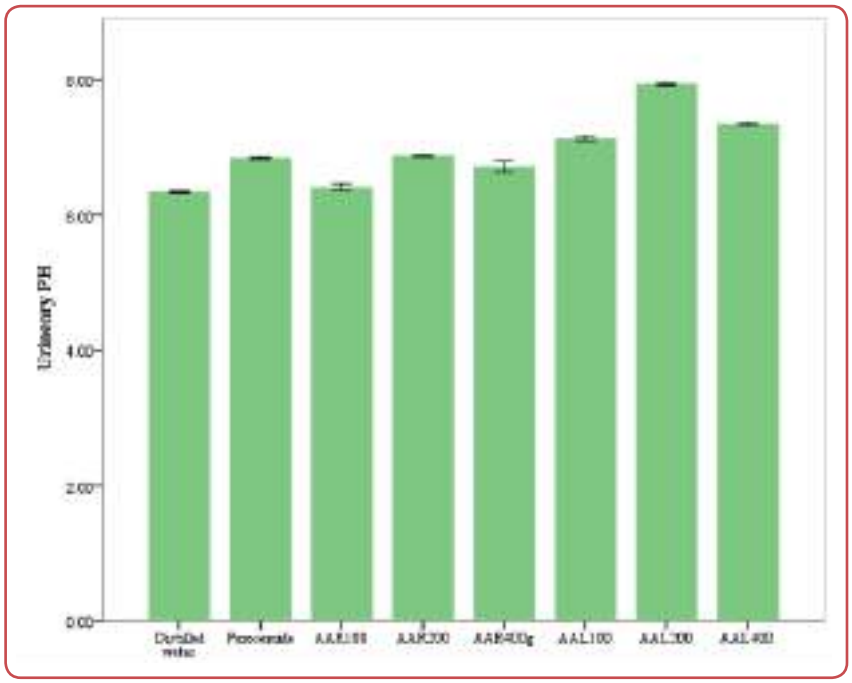
Table 4: Effect of 80 % methanol extract of the leaves of Artemisia abyssinica on 5 h urinary electrolyte excretion in mice

Group	Dose (mg/kg)	Urinary electrolyte concentration (mmol/L)			Saluretic Index*			Na^+/K^+	$Cl^-/Na^+ K^+$
		Na^+	K^+	Cl^-	Na^+	K^+	Cl^-		
Distilled water	0.2 mL	48.0 ± 4.61	46.6 ± 6.35	54.2 ± 3.85	-	-	-	1.02	0.57
AAL100	100	120.0 ± 3.94 a, c, d, **e*	104.2 ± 7.51 a, c, d, e**	154.9 ± 5.37 a, c, d**e*	2.50	2.24	2.86	1.15	0.69
AAL200	200	43.0 ± 5.72 b**d, e*	35.0 ± 4.12 b**d*	45.3 ± 4.29 b, e**d*	0.89	0.75	0.84	1.23	0.58
AAL400	400	63.7 ± 2.83 a, e*b**	48.0 ± 4.52 b** e**	66.7 ± 3.86 a, c*b, e**	1.33	1.03	1.23	1.33	0.60
Furosemide	10	88.0 ± 6.74 a, c** b d*	27.4 ± 5.98 a, c, d *b**	98.0 ± 4.65 a, c, d, **b*	1.83	0.59	1.81	3.23	0.85

*Values are presented as mean ± SEM; n = 8; a = compared to negative control (Distilled water), b = compared to AAL100, c = compared AAL200, d = compared to AAL400, e = compared to Furosemide; *(p < 0.05); ** (p < 0.01); AAL = 80 % methanol extract of the leaves of Artemisia abyssinica; numbers refer to doses in mg/kg/day.*

Urinary pH

The urinary PH was measured from the pooled sample urine. Figure 1 indicated that there was no significant urinary PH disparity among all treatment and negative and positive control groups after 5 h of treatment administration, except for the leaf extract-treated group at 200 mg/kg. It has significantly higher pH compared to just the untreated (negative control) groups.



Values are presented as mean ± SEM; n = 8; AAR and AAL refer to 80 % methanol extracts of the rhizomes and leaves of A abyssinica, respectively; numbers refer to doses in mg/kg/day.

Figure 1: Urinary pH of mice treated with the 80 % methanol extracts of the rhizomes and leaves of Artemisia abyssinica

CONTENT

Virtual screening and molecular docking study

Virtual screening of 206 compounds that were previously reported from the genus Artemisia and a loop diuretic bumetanide was carried out on the crystal structures of the target protein Na^+-K^+-$2Cl^-$ cotransporter (NKCC1) (cation-chloride cotransporter NKCC1) (PDB: 7S1Y). A few of the compounds showed strong binding interactions with the target protein. Interestingly, compounds 151 and 53 exhibited stronger affinity than even the ligand bumetanide. Moreover, they displayed key hydrogen bond interactions with amino acid residues in the bumetanide binding site with HYDE scores of -42.5 and -40.4 kJ/mol. The binding modes of compounds to NKCC1 are shown in Figure 2. The Table 5 shows docking results and *in silico* physicochemical predictions of the four top-scoring compounds. All the compounds had optimum drug-like properties, such as Log P, in the range of 1 to 5.

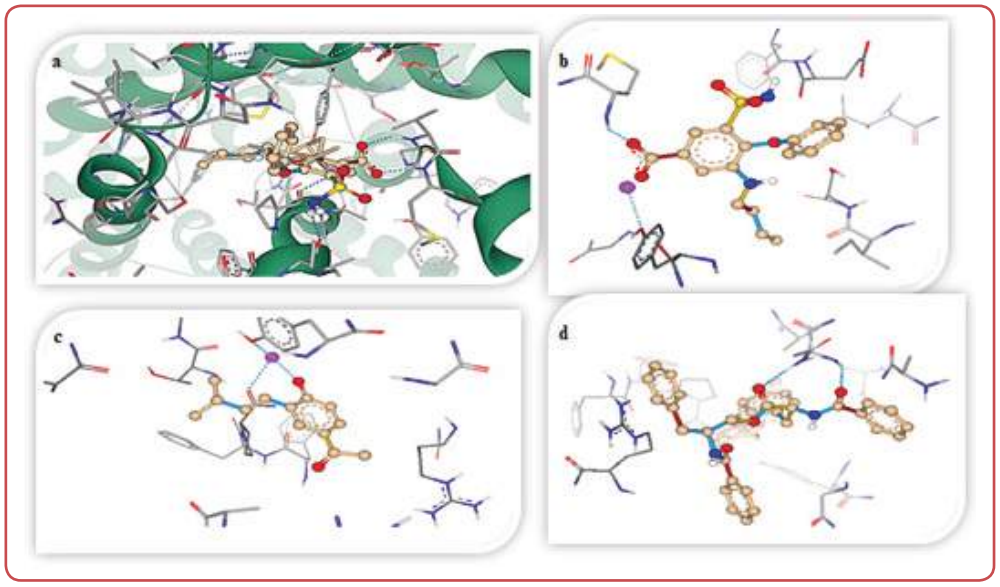
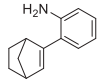
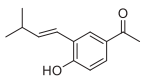
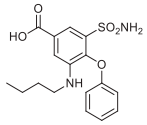
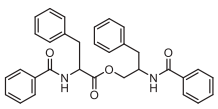


Figure 2: The binding modes of compounds to the cation-chloride cotransporter NKCC1 (PDB: 7S1Y) bumetanide binding site

a) Ribbon diagram of superimposed top-score compounds in the bumetanide binding site of NKCC1. Bumetanide, the template compound, is shown in the ball-stick model. b) Binding interaction of bumetanide with amino acid residues of NKCC1 c) Binding interaction of compound 53 with amino acid residues of NKCC1 d) Binding interaction of compound 197 with amino acid residues of NKCC1. Molecular docking was carried out using SeeSar13.0 software (BioSolveIT, Sankt Augustin, Germany).

Table 5: Docking results and prediction of partition coefficient Log P, aqueous solubility Log S and hERG toxicity of the top score compounds carried out using SeeSar13.0 software

Compounds	Structure	ΔGHYDE (kJ/mol)	Log P	Log S	Log D	hERG pIC_{50}
Compound 151		-42.5	3.320	2.630	1.534	4.9000
Compound 53		-40.4	2.955	3.272	2.955	5.0670
Bumetanide		-39.6	2.522	2.357	-0.104	4.4921
Compound 197		-37.8	4.703	-0.224	4.703	4.8640

CONTENT

Discussion

The 80 % methanol rhisomes and leaf extracts of *A abyssinica* displayed moderate diuretic activity (0.72-1.00), especially at 200 and 400 mg/kg.[18, 19] Similar urine output and electrolyte excretion results have been shown in previous *in vivo* diuretic studies on other Artemisia (L) species, such as *A annua*[20] and *A thuscula*. [21] Moreover, the rhizome extract exhibited a higher dose-dependent diuretic action than the leaf extract, indicating a relatively abundant accumulation of active secondary metabolites in the rhizomes of the plant. In addition, the increased efflux of the electrolytes, especially the sodium, in the extract-treated groups *vis-à-vis* the negative control indicated the inhibition of the Na^+-K^+-$2Cl^-$ symporter in the thick ascending loop of Henle within the kidney as the potential target for the diuretic mechanism of action of the constituents of the plant.[22, 23] The less alkalinity of the urine sample and the higher Cl^-/Na^+K^+ ratio further strengthen the suggestion of Na^+-K^+-$2Cl^-$ (NK2CC) cotransporter as a diuretic target of the extracts than carbonic anhydrase, since carbonic anhydrase inhibition requires alkalinity of the urine and a lower Cl^-/Na^+K^+ ratio. The *in silico* screening and molecular docking results also provided an additional insight into the diuretic mechanism of action of the extracts. To the best of authors' knowledge, this is the first study to examine the diuretic effects of 80 % methanol extracts of *A abyssinica*.

Conclusion

In conclusion, the rhizomes and leaves of *A abyssinica* proved to have diuretic activity. These, together with the absence of toxicity from the acute toxicity result, support their ethnobotanical claims. In addition, the virtual screening and molecular docking study of the compounds signify inhibition of Na^+-K^+-$2Cl^-$ (NK2CC) cotransporter as possible mechanism of action of the diuretic constituents of the plant.

Ethics

This experiment was approved by the Institutional Review Board of the School of Pharmacy, College of Health Sciences, Addis Ababa University, decision No ERB/SOP/361/13/2021, dated 27 December 2021. The handling of the mice and all procedures followed were in accordance with the Guide for the Care and Use of Laboratory Animals.

Acknowledgement

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Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request. The structure of compounds (1-206) isolated from the species of the genus *Artemisia* is available in the supplementary figure (Figure S1).

CONTENT

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Data curation: NA
Writing - original draft: NA
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ORIGINAL ARTICLE

Impact of COVID-19 on Mortality in the Canton of Sarajevo in Period 2020-2022

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Abstract

Background/Aim: Until March 2023, it has been reported over 676 million cases of COVID-19 globally with almost 7 million deaths caused by this disease. Aim of this study was to determine COVID-19-related deaths and to study how COVID-19 pandemic impacted mortality of residents in the Canton of Sarajevo in 2020-2022 time period. Also, aim was to analyse to what extent the number of registered non-COVID-19-related deaths have changed in the Canton of Sarajevo compared to what would have been expected in the absence of the virus to better measure the impact of COVID-19.

Methods: This study used mortality data obtained from Federal Institute for Statistics for period 2017-2022. Data was analysed and presented as raw numbers, age-, sex-, cause of death - crude death rates (CDR), excess mortality and P-score.

Results: CDR in the Canton of Sarajevo in 2020 was 1246.3 per 100.000 persons, 1488.6 in 2021 and 1153.4 in 2022, while in period from 2017-2019 CDR ranged from 1051.3 to 1057.9. Total CDR in 2020 increased by 18.3 % compared to 2017-2019 average CDR, this relative difference being even greater in 2021 (41.3 %) and lower in 2022 (9.5 %). In 2020-2022 time period, 9 of the 10 leading causes of death remained the same as in 2019. COVID-19 was the 3rd leading cause of death in 2020 and 2022, while in 2021 spiked as the leading cause of death. In 2020 there was increase of 7 % in deaths from non-COVID-19 related deaths compared to mean number of deaths for period 2017-2019. As for 2021, this number goes higher (9.5 %) and in 2022 was much lower (1.0 %).

Conclusion: In the Canton of Sarajevo, COVID-19 pandemic made a big impact on mortality in 2020-2022 years period. Data have changed in total mortality, leading causes of death and excess mortality. Deep-rooted organisational weaknesses that were exposed during pandemic that can bring harm to population from preventable chronic diseases needs to be addressed which have impact on morbidity and at the end, on mortality.

Key words: Mortality; COVID-19; Crude death rate; Excess mortality.

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Introduction

Until 10th March 2023, it has been reported over 676 million cases of COVID-19 globally with almost 7 million deaths caused by this disease.[1] In Bosnia and Herzegovina, the death count has reached more than 16,000 people, while over 400,000 people have been diagnosed with COVID-19 as of the same date.[1] It is obvious that the SARS-CoV-2 virus has claimed many lives, while there is possibility that some COVID-19 patients may have died without being diagnosed or had false negative test on COVID-19.

It is quite difficult to assess the data on deaths attributed to COVID-19. The main problem for researchers is not confirmed cases but ones whose death is caused by several pathological states interconnected with COVID-19. Besides, positive COVID-19 test is not enough to determine the cause of death, as some patients may die while infected with SARS-CoV-2 virus and not having serious clinical manifestation of disease.

On the other hand, this "once in a lifetime" situation that was last seen 100 years ago during Spanish flu, might be causing additional health problems or exacerbation of current ones. Some deaths may have occurred due to limited access to health care during the pandemic because main part of healthcare system was redirected to diagnose and care for COVID-19 patients. Hospital capacities were stretched to their limits and many non-life-threatening conditions weren't treated. There was a lack of maintenance of chronic illnesses like diabetes and chronic heart diseases because primary healthcare facilities were overburdened with COVID-19 patients, while chronic patients were fearful to visit their primary physician or to call 911 during an emergency, such as a heart attack or stroke.[2] Of course, there is also reasonable possibility that some of the COVID-19 deaths could be due to other causes and not COVID-19.[3]

There are reports of increase of death rates in many countries during the COVID-19 pandemic as well as shortening of life expectancy at birth.[4, 5] USA study reported that the death rate in USA in 2020 jumped nearly 17 % compared to 2019 and this is the biggest increase in more than a century since the Centers for Disease Control and Prevention has been tracking this data.[4] Also, it was found that COVID-19 mortality figures in USA underestimate the actual death toll and that COVID-19 mortality is likely to be twice as high as reported.[5]

As the health care system in Bosnia and Herzegovina is characterised by extreme fragmentation considering the fact that the system is organised in various ways in the Federation of B&H, the Republic of Srpska and Brčko District, the response to COVID-19 pandemic was lacking in many ways. In terms of the organisational structure and management, this system generally operates through 13 completely different sub-systems at the level of entities, cantons (one of them being the Canton of Sarajevo) and Brčko District, which significantly complicates the way health care services are provided, increases management and coordination costs and adversely affects the rationality of management of healthcare institutions. During pandemic these problems were heighten as there was no national response to COVID-19 and every previously mentioned fragmented part of health care system had its own risk management plan, own guidelines and own crisis response team, which in summary could worsen the outcomes for rescuing lives.

The aim of this paper was to determine COVID-19-related deaths and to study how COVID-19 pandemic impacted mortality of residents in the Canton of Sarajevo in period 2020-2022. Also, aim was to analyse to what extent the number of registered non-COVID-19-related deaths have changed in the Canton of Sarajevo compared to what would have been expected in the absence of the virus to better measure the impact of COVID-19.

Methods

Type of study and data collection

This descriptive retrospective study used mortality data obtained from Federal Institute for Statistics for years 2017-2022. Data was extracted on 5 June 2023 with accordance to Federal laws and in cooperation with Federal Institute for Statistics as main public institution of Federation of Bosnia and Herzegovina for mortality data collection and distribution. Data used in this study was obtained as electronical databases and values included in these datasets were final and could not be changed in later times. Data were reported by gender, age group, regions/cantons and main cause of death.

Age groups used in this study were: 0–14, 15–64, ≥ 65 as for males and females. As aim of this study was analysis of deaths of residents of the Canton of Sarajevo, only region used from these datasets was the Canton of Sarajevo as integral part of Federation of Bosnia and Herzegovina.

Federal Institute for Statistics Mortality Database included data from Statistical reports of death that were filled for every deceased person in Federation of Bosnia and Herzegovina by doctor coroner, specially educated to recognise main cause

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of death. All coroners in Federation of Bosnia and Herzegovina were working in concurrence with international guidelines for certification and classification (coding) of COVID-19 as cause of death formulated by World Health Organization (WHO) as well as others causes of death defined by WHO.[6] All causes of death were reported as International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) codes.

Statistical analysis

Excess mortality was estimated by subtracting the number of deaths in 2020, 2021, 2022 and average number of deaths from pre-COVID-19 period 2017-2019. A similar approach has been used in studies of Swedish and German researches.[7,8]

This approach was also strengthened as crude death rates (CDR) during period 2017-2019 were stable and there was no significant difference in CDR during this period. Exact and the average number of deaths were used for the three previous years as a control group, which also helped smooth out any short-term spikes. As raw number of excess deaths did not provide a sense of scale and it was difficult to compare with other regions and countries, for better comparisons across regions and countries, excess mortality was measured as the percentage difference between the reported and projected number of deaths (P-score) as used by other researchers.[9] P-score was calculated as:

$$\text{P-score} = [(\text{Reported deaths} - \text{Projected deaths}) / \text{Projected deaths}] \times 100$$

The total number of deaths (of any cause) was used as well as the number of COVID-19-related deaths, in order to calculate the number of deaths that were not officially associated to COVID-19 in excess death count. To asses statistical significance Pearson Chi square test was used. The accepted statistical significance was at $p < 0.05$. All calculations were made in software IBM SPSS 21 (IBM Corp, Armonk, NY, USA).

Results

In three-year period 2020-2022, there were 16,334 deaths in the Canton of Sarajevo (5,254 in 2020, 6,241 in 2021 and 4,839 in 2022). Number of deaths of female residents was 8,097 and male residents 8,237. During 3-year period 2016-2019 there were total of 13,262 deaths of residents of the Canton of Sarajevo (4,400 in 2017, 4,437 in 2018, 4,425 in 2019). Average number of deaths in the Canton of Sarajevo during period 2017-2019 was 4,421 ± 19 while in period 2020-2022 was 5,445 ± 720. There was 25.4 % increase in number of deaths in 2020-2022 period compared to average number of deaths in 2017-2019. Detailed view of number of deaths in the Canton of Sarajevo is shown in Figure 1.

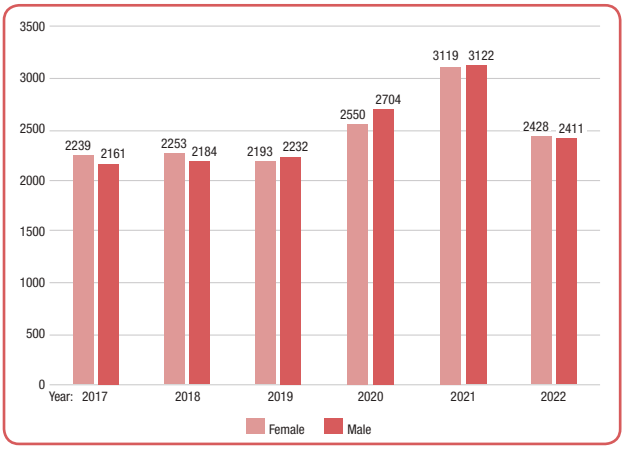

Figure 1: Number of deaths in the Canton of Sarajevo in period 2017-2022, by sex

Impact of COVID-19 on CDR, total and by sex

Figure 2 presents the relative changes in CDR when comparing COVID-19 pandemic period with the years prior to COVID-19 in the Canton of Sarajevo. CDR in the Canton of Sarajevo during COVID-19 pandemic was 1246.3, 1488.6 and 1153.4 per 100.000 persons in 2020, 2021 and in 2022, respectively, while in period from 2016-2019 CDR ranged from 1051.3 (2017) to 1057.9 (2018).

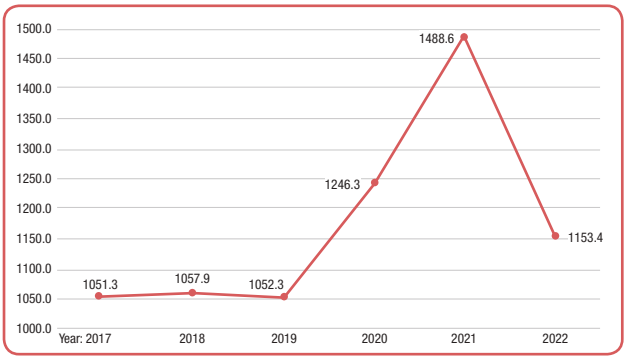

Figure 2: Crude death rates (CDR) per 100,000 people in the Canton of Sarajevo, 2017-2022

CONTENT

The observed CDR in 2020 increased by 18.3 % compared to 2017-2019 average CDR (1053.8 ± 3.6); this relative difference was greater for women (23.8 %) than for men (22.7 %) but with no significant difference ($p > 0.05$). In 2021, this increase had spiked due to large number of registered deaths. CDR increased in 2021 compared to 2017-2019 was 41.3 %, 47.8 % for women and 42.4 % for men. As seen from Table 1, CDR increased in 2022 compared to 2017-2019 average CDR was much lower and it was 9.5 % overall (10.1 % for men and 14.5 % for women).

Table 1: Summary of changes in crude death rates (CDR) comparing 2020-2022 to average CDR in 2017-2019, total and by sex

Year	CDR (%)		
	Total	Men	Women
2017-2019 (mean ± SD)	1053.8 ± 3.6	1106.2 ± 18.3	1007.0 ± 14.2
2020	18.3	22.7	23.8
2021	41.3	42.4	47.8
2022	9.5	10.1	14.5

%: CDR change compared to 2017-2019 average;

Age-specific CDR in period 2020-2022 compared to 2017-2019 average

Comparing 2020-2022 period with 2017-2019 average, CDR increased in every year for age groups 15-64 and ≥ 65 (Table 2). One exception was reported in 2022 for age group 15-64 with CDR decline of -3.2 %. Biggest reported CDR increase was 35.5 %, reported in 2021 for age group ≥ 65. Smallest reported CDR increase was 3.7 %, reported in 2022 for age group ≥ 65. As seen from Figure 3, every year in 2022-2022 period had CDR decline for age group 0-14, as in these years occurred smaller number of infant deaths compared to 2017-2019 period in the Canton of Sarajevo, as infant deaths make the majority in number of deaths in age group 0-14.

Table 2: Summary of changes in crude death rates (CDR) comparing period 2020-2022 to 2017-2019 average, by age groups

Year	CDR (%)			
	Total	0-14 years	15-64 years	≥ 65 years
2017-2019 (mean ± SD)	1053.8 ± 3.6	68.2 ± 9.4	333.6 ± 10.5	5046.8 ± 116.6
2020	18.3	-18.2	12.6	12.9
2021	41.3	-49.1	29.0	35.5
2022	9.5	-20.0	-3.2	3.7

%: CDR change compared to 2017-2019 average;

Age-specific CDR for age groups 15-64 and ≥ 65 had changed significantly in 2020 and 2021 compared to 2017-2019 average ($p < 0.05$). As for 2022, there was no significant change in CDR comparing it to 2017-2019 average ($p > 0.05$).

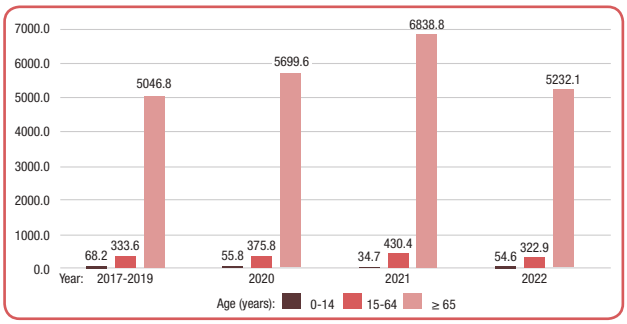

Figure 3: Crude death rates (CDR) for period 2017-2019, 2020, 2021 and 2022, by age groups

Changes in CDR for the 10 leading causes of death

For the analysis of leading causes of death, comparison was made only with 2019, as comparation with other years would be too excessive. Other reasoning for this was that 10 leading causes of death in period 2017-2019 were almost the same with some causes switching positions on the chart.

In years 2020-2022, 9 of the 10 leading causes of death remained the same as in 2019 (Figure 4). As seen from the Figure 4, most causes of death were diseases of the circulatory system. COVID-19 was newly emerged cause of death in 2020-2022, becoming the 3rd leading cause of death in 2020 and 2022 and spiking as number one cause of death in 2021 with more than doubled CDR in comparison to number two cause of death in 2021 (CDR 333.9 for COVID-19 compared to CDR 156.5 for chronic ischaemic heart disease). Of the remaining 10 leading causes of death in 2022, 2021, 2020 and 2019, all were the same, some swapping places on the chart. Excluding COVID-19, in these four years, first five places as causes of death dominantly held these five diseases: chronic ischaemic heart disease, cerebral infarction, myocardial infarction, diabetes mellitus and malignant neoplasm of bronchus and lung. Hypertension, atherosclerosis, cardiomyopathy and chronic obstructive pulmonary disease (COPD) hold, in all these analysed years, bottom four leading causes on the charts of 10 leading causes of death in the Canton of Sarajevo. Analysing death rates for the 10 leading causes of death while comparing pandemic years to 2019, can be seen that there was significant correlation between increase of total CDR and COVID-19 deaths ($p < 0.05$), as COVID-19 being main reason for this increase.

Excess mortality in 2020-2022

The differences in number of deaths between

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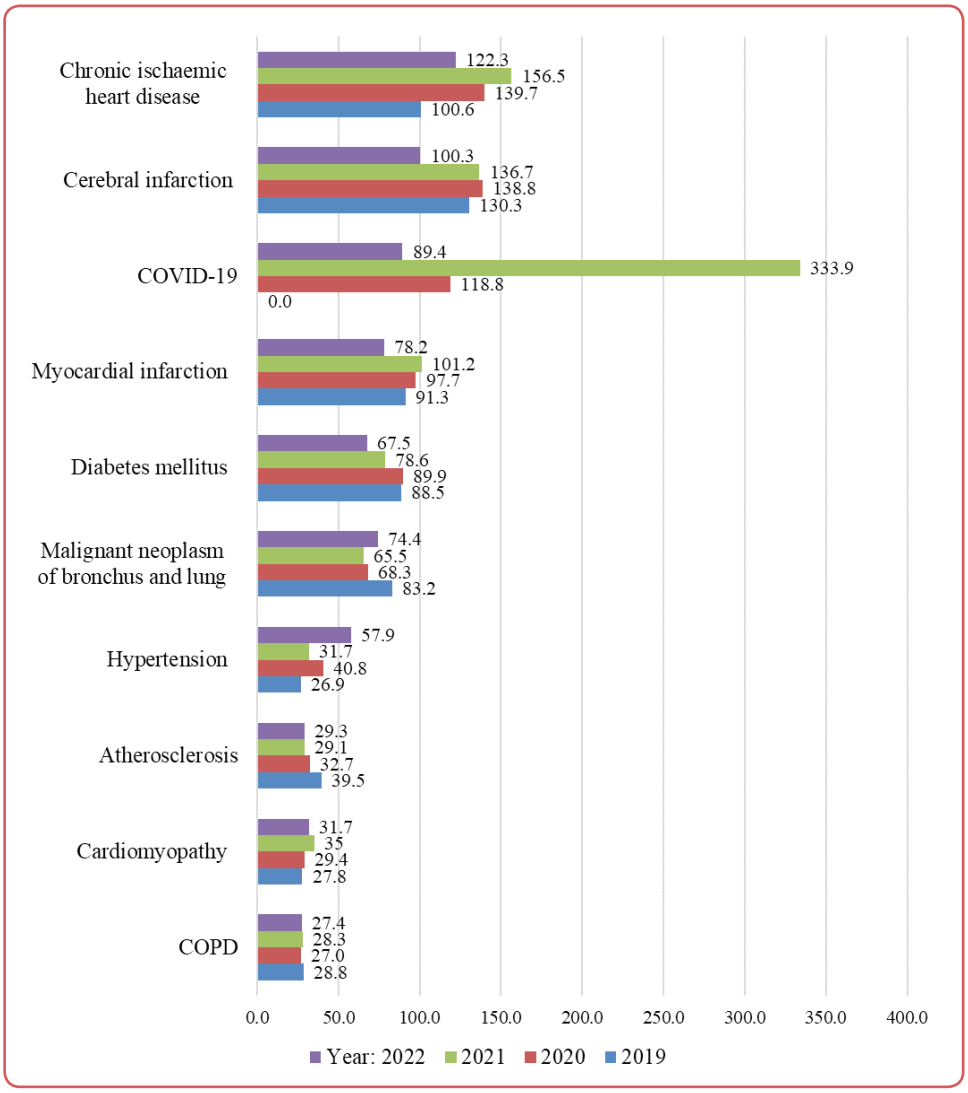

Figure 4: Death rates for the 10 leading causes of death in the Canton of Sarajevo, 2019 - 2022

COPD: Chronic obstructive pulmonary disease;

Table 3: P-score values, number of deaths and difference in number of deaths

Year	N	P-score	Difference	N (COVID-19)
2020	5,245	18.8	+824	+824
2021	6,241	41.2	+1,820	+1,820
2022	4,839	9.5	+418	+418

N: number of deaths; N (COVID-19): number of registered deaths caused by COVID-19; Difference: Difference in number of deaths compared to 2017-2019 mean;

2022 (4,839), 2021 (6,241), 2020 (5,245) and previous three-years mean (4,421) for the Canton of Sarajevo were 418, 1820 and 824, respectively. P-score in 2020 was 18.8 indicating that mortality in 2020 was almost 19 % higher than the mean value for the previous 3 years in the Canton of Sarajevo. Analysing 2021, P-score gets staggeringly high with 41.2 % higher mortality compared to 2017-2019. P-score in 2022 was much lower, with 9.5 % higher mortality compared to 2017-2019 (Table 3).

There were more non-COVID-19 related deaths in 2020-2022 compared to mean number of deaths in 2017-2019, so excess death count cannot be contributed only to COVID-19. There were more than 7 % more deaths from non-COVID-19 related deaths in 2020 compared to mean number of deaths for period 2017-2019. This percentage was even higher for 2021 (9.5 %), while for 2022 was much lower (1.0 %) and it can be stated that there were no non-COVID-19 related excess deaths in 2022.

*Table 4: Death rates and number of deaths for 5 leading causes of death by ICD-10 chapters in 2022, 2021, 2020 and 2019**

	2022		2021		2020		2019	
ICD-10 chapter	**N**	**CDR**	**N**	**CDR**	**N**	**CDR**	**N**	**CDR**
Diseases of the circulatory system	2,071	493.6	2,331	555.1	2,281	541.1	2,000	475.6
Neoplasms	1,141	272.0	1,181	281.2	1,157	274.5	1,195	284.2
Endocrine, nutritional and metabolic diseases	321	76.5	347	82.6	379	89.9	374	88.9
Diseases of the respiratory system	179	42.7	201	47.9	221	52.4	204	48.5
Diseases of the digestive system	109	26.0	121	28.8	127	30.1	111	26.4

** Only excluded chapter was codes for special purpose (U00-U99) as leading cause of death by ICD-10 chapters which was only composed of COVID-19 deaths. N: Number of deaths; CDR: crude death rates.*

As seen from the Table 4, the biggest increase of CDR by chapters in ICD-10 in 2020 and 2021 compared to 2019 was in chapter "diseases of circulatory system". There were 2,281 deaths caused by circulatory diseases in 2020 and 2,331 in 2021 compared to 2,000 in 2019. Increase of CDR for this group of diseases was 13.8 % in 2020 and 16.6 % in 2021 compared to 2019 and the difference was statistically significant ($p < 0.05$). There was no statistically significant change of CDR for other chapters including neoplasms, endocrine, nutritional and metabolic diseases, diseases of the respiratory system and diseases of the digestive system in 2020 and 2021 compared to 2019.

Discussion

The definite consequences that COVID-19 pandemic had on mortality, not only in 2020-2022 years period but years that follow and its impact on other aspects of life will be known after many studies and research. However, by analysing age and sex-specific death rates for all-cause mortality during 2020-2022 and previous years, a perception can be made of the mortality burden. This analysis showed that in 2020-2022 period total mortality in the Canton of Sarajevo increased, as for male and female citizens. There was increase in total CDR ranged from 9.5 – 41.3 % compared to years 2017–2019. In 2020 in USA was registered 16.8 % CDR increase compared to 2019.[4] In Switzerland CDR was 8.8 % higher in 2020 than in 2019.[10] This increase was larger for women than for men in the Canton of Sarajevo.

It has been argued that the pandemic affected the frailest and the oldest the most and in such case it would be expected that the biggest increase in CDR would be in age group ≥ 65. This was the case in the Canton of Sarajevo, where this happened in all three analysed years. If absolute numbers were analysed, most deaths occurred in age group ≥ 65, from all causes, as well as from COVID-19. But most indicative for this assumption is CDR, where biggest CDR increase was reported in age group ≥ 65. Comparing 2020-2022 period with previous years, for age group 15-64 CDR increase ranged from -3.2 to +29.0 %, while these numbers ranged from +3.7 to 35.5 % for age group ≥ 65. These results correspond with results found in analysis of USA mortality in 2020 where biggest increases in CDR were reported in older age groups.[4]

In 2020-2022, 9 of the 10 leading causes of death remained the same as in 2019. Excluding COVID-19, in these four years, first five places as causes of death dominantly hold same five diseases: chronic ischaemic heart disease, cerebral infarction, myocardial infarction, diabetes mellitus and malignant neoplasm of bronchus and lung. There was CDR increase of diseases of the circulatory system in 2020-2022. Similar changes reported Murphy et al in their study in USA.[4] It must be added that COVID-19 infection provokes elevated troponin values in 20–30 % of hospitalised COVID-19 patients that may aggravate clinical outcomes of patients with preexisting diseases of the circulatory system.[11] Both direct and indirect effects of infection with SARS-CoV-2 have been proposed to underlie these adverse cardiovascular effects and significantly increase the burden of morbidity and mortality related to COVID-19.[12] These could be reasons why an in-

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crease of CDR of diseases of the circulatory system during pandemics was noted. COVID-19 became the 3rd leading cause of death, newly added as a cause of death in 2020 and 2022, while in 2021 was leading cause of death, spiking in death chats with CDR of 333.9. It was expected that this number would be this staggering in 2021 as during third COVID-19 pandemic wave in February, March and April of 2021 was noticed the biggest spike in COVID-19 death count.

Analysing excess mortality, P-score in 2020-2022 ranged from 9.5 to 41.2 indicating that mortality in 2020 and 2021 was much higher than the mean value of the previous three years in the Canton of Sarajevo. This score was by far the highest in Latin America: Peru (153 %), Bolivia (68 %) and Mexico (61 %), while in Europe these numbers were lower.[13]

Confirmed COVID-19 deaths accounted for about 60.8 % of the excess mortality in 2020 observed. These numbers went higher for 2021 and 2022 (77.3 % and 89.7 %, respectively). The remaining percentage represented excess mortality from non-COVID-19-related causes of death. This data might represent an increase of deaths from other causes as an indirect consequence of the pandemic, eg due to restriction of healthcare resources, treatments and/or surgeries, or a changed behaviour of seeking medical care for severe conditions. But some of these death cases could have been people with false negative COVID-19 tests or people dying from COVID-19 and not being previously tested. Unfortunately, during 2020, especially in first half of the year, in Bosnia and Herzegovina testing was scarce as there were lack of access to large number of tests. On the other hand, some of the COVID-19 deaths could be due to other causes and not COVID-19.[3]

Official data in this study reported increase of 7 % in deaths from non-COVID-19 related deaths in 2020 compared to mean number of deaths for period 2017-2019. As for 2021 this number was higher (9.5 %) and in 2022 was much lower (1.0 %). Main part of this increase can be associated with increase of number of deaths caused by circulatory and hearth diseases. Considering pathophysiology of COVID-19 and its effects on cardiovascular system that have been shown in studies of Cheng et al as well as Mitrani et al, this increase could be expected.[11, 12] On the other hand, in 2020-2022 CDR for neoplasms decreased compared to 2019. This kind of effect on mortality caused by COVID-19 was also reported in study of USA mortality in 2020.[4] The reasoning behind this could be because the mortality rate of COVID-19 was high in cancer patients and cause of death for these patients was mainly reported as COVID-19 infection and not cancer, consequently decreasing numbers for neoplasm mortality.[14, 15] In non-pandemic circumstances deaths of these patients would be associated to cancers and neoplasm mortality rates would be higher.

Conclusion

In the Canton of Sarajevo, COVID-19 pandemic made a big impact on mortality in 2020-2022 years period. Data has changed dramatically in total mortality, leading causes of death and excess mortality. In 2023, we are comprehending how big of an impact COVID-19 pandemic had on population of the Canton of Sarajevo in 2020-2022 and the consequences the pandemic will eventually have later on demographics. Predicting long-term impacts requires caution, but the abrupt and extensive reprioritisation of healthcare services, that happened during last three years, might have consequences on health status of general population. Deep-rooted organisational weaknesses that were exposed during pandemic needs to be addressed that can bring harm to population from preventable chronic diseases which will have impact on morbidity and at the end, on mortality. Similar estimates of age-, sex- and cause of death- specific death rates as well as excess mortality data from other countries are necessary to be able to do properly compare the impact of COVID-19 on mortality in the Canton of Sarajevo.

Ethics

This study was a secondary analysis based on the currently existing database from the Federal Institute for Statistics and did not directly involve human participants or experimental animals. Therefore, the ethics approval was not required for this paper.

Acknowledgement

None.

CONTENT

Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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Data curation: AŽ
Writing - original draft: DA, AŽ
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Visualisation: DA
Project administration: DA

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CONTENT

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ORIGINAL ARTICLE

Effect of Proteolytic Enzymes and Insulin Sensitiser in Treatment of Joint Osteoarthritis in Diabetic Patients

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Abstract

Background/Aim: Knee osteoarthritis is a frequently crippling chronic condition. Numerous pharmacological medications have been successfully utilised to treat knee osteoarthritis. This research aimed to compare the efficiency of metformin and serratiopeptidase in treating and preventing osteoarthritis development *via* distinct mechanisms.
Methods: Between 1 January and 30 May 2019, a randomised-clinical-trial was done at Al-Kindy Hospital on 80 osteoarthritis patients, divided in two groups. Group I was given metformin 850 mg orally, whereas Group II was given serratiopeptidase 20 mg and metformin 850 mg orally. Parameters in these groups were compared with forty healthy normal controls.
Results: Following treatment, patients in Group II have shown a significant decrease in pain levels ($p = 0.001$). Interleukin 8 (IL-8), tumour necrosis factor-alpha (TNF-α) and interleukin 1 beta (IL-1ß) levels were significantly decreased in Group II ($p = 0.001$).
Conclusion: The combination of serratiopeptidase and metformin was effective and safe in treating knee osteoarthritis.

Key words: Metformin; Inflammatory parameters; Knee osteoarthritis; Serratiopeptidase.

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Introduction

Osteoarthritis (OA) of knee is a prevalent global chronic debilitating disease.[1] It is a synovial joint condition that is pathologically defined by cartilage damage, increased load, capsule thickness, osteophytosis and subchondral bone alterations.[2] Obesity is a global epidemic strongly associated with OA and has a multifactorial effect on OA.[3] Similarly, the influence of metabolic factors such as humoral and lipids mediators, obesity initiates the mechanical effects that result in knee joint injury due to excessive load, muscle weakness and biomechanical alterations.[4, 5]

Knee OA therapy aims to alleviate pain, improve the overall quality of life, limit joint cartilage degradation and maintain movement. The disease is managed in three distinct ways: non-pharmacologically, pharmacologically and surgically. Non-pharmacological approaches frequently entail lifestyle modifications, for instance, increased physical activity, weight loss and programmed dieting.[6]

Weight loss reduction has been shown to improve movement in people with knee OA. Nu-

merous pharmaceutical treatments, including systemic non-steroidal-anti-inflammatory drugs (NSAIDs), metformin, platelet-rich plasma, diacerein, glucosamine, topical creams and serratiopeptidase, have been utilised successfully in the treatment of knee OA.[7,8]

Metformin (biguanide) is a medication that is commonly used to treat type II diabetes mellitus (T2DM). Metformin affects glucose production and may influence insulin production in people with diabetes. Additionally, it aided in weight loss.[9] Along with its anti-diabetic impact, metformin has been proven to alleviate the inflammation and pain intensity associated with OA with no documented adverse effects, making it a viable therapy option for individuals with OA of knee and a prospective medication for inflammation-related problems. Although, the mechanism of metformin anti-inflammatory effect is uncertain, numerous researches have demonstrated that it affects oxidative stress and reduces inflammatory markers.[10]

Serratiopeptidase, is proteolytic enzyme that exhibits anti-inflammatory properties. Nowadays, serratiopeptidase enzymes are widely employed as the preferred inflammatory and anti-pain treatment in Japan and Europe.[11]

The prevalence of obesity and overweight has increased significantly over the last two decades in Iraq, resulting in improved knee OA cases, particularly between the elderly, poor life quality, resulting in impaired function and significant burden on the health services. Several medications were introduced for the treatment of knee OA, showing little evidence of efficacy and numerous bad effects. Aim of this study was to determine the efficacy of metformin and serratiopeptidase in treating and reversing OA development *via* various mechanisms.

Methods

The research was a randomised clinical trial undertaken at Al-Kindy Teaching Hospital, Baghdad, Iraq from 1 January to 30 May 2019. The study population consisted of individuals with knee OA who were offered to the Al-Kindy Teaching Hospital's Consultation Clinic. Adulthood, overweight or obesity and knee OA were all considered inclusion criteria. Pregnancy, coagulation, bleeding disorders, systemic conditions such as diabetes mellitus, hypertension and existing knee treatment OA with other prescription regimens were an non-inclusion/exclusion criteria.

Patients

Eighty patients with OA of the knee were divided into two groups using digital randomisation and non-randomisation: Group I was given metformin 850 mg orally, whereas Group II was given serratiopeptidase 20 mg and metformin 850 mg orally.

The rheumatologists of Al-Kindy Teaching Hospital have performed clinical and radiological diagnosis of patients with knee OA. A control group of 40 healthy volunteers were selected. Before inclusion in the trial, the height and weight of all research participants was analysed (n = 120). To determine their body mass index (BMI), they were measured on a calibrated scale. The BMI of all research patients was matched.

Patients were instructed to discontinue use of the medicine and to report to the researchers any instances of bruising or bleeding that occurred while they were taking it.

Pain scores (ranging from 1-10) were obtained from patients after being labelled and were input into specifically created questionnaire for each trial participant. Five mL blood sample was collected from each participant to evaluate their inflammatory markers serum level [tumour necrosis factor-α (TNF-α), resistin, interleukin 8 (IL-8), IL-1ß and adiponectin] using ELISA. The kits were as follows: *Human Tumour Necrosis Factor α (TNF-α) ELISA Kit*, product number: RAB1089, *Sigma-Aldrich*, Germany; *IL-1 beta Human ELISA Kit*, P01584, *Invitrogen,* MA, USA; *IL-8 Human ELISA Kit*, P10145, *Invitrogen,* MA, USA; *Resistin Human ELISA Kit,* Q9HD89, *Invitrogen,* MA, USA; *Adiponectin Human ELISA Kit*, Q15848, *Invitrogen,* MA, USA.

Rheumatologists divided the two treatment regimens into two research groups after collecting baseline data from patients and patients were monitored for 12 weeks. At the second visit, each patient's pain and BMI were recorded in a questionnaire and anti-inflammatory parameters were determined in the hospital's laboratory for three groups of research participants. Pain

scores, levels of inflammatory and BMI markers in knee joint OA patients were compared to those in the healthy group to determine the effectiveness of treatments. Throughout 12 weeks, the negative consequences of both research groups were observed.

Statistical analysis

Analysis was conducted using an SPSS application. Outcomes were classified using a contingency table. To compare two means before and after therapy, a paired t-test was performed. ANOVA was implemented to compare the means, while the Fisher exact test was utilised to examine categorical data.

Results

A total of 80 patients with knee OA were included in this study. Between study groups of patients, there were no significant variations in age or gender. In two study groups, female OA patients were substantially more common than male OA patients (Table 1).

BMI of OA patients for both study groups were slightly and non-significantly reduced (p = 0.10). Pain scores have shown significant decrease (p = 0.001) in Group II, but no significant change (p = 0.07) in Group I. Regarding IL-1ß and IL-8 levels they have shown significant decline in Group II (p = 0.001), but no significant change in Group I. Resistin serum didn't show significant changes after treatment of OA patients. TNF-α was significantly lowered in both research groups of OA patients following treatment (p = 0.001). Adiponectin serum didn't show significant changes after treatment for OA patients (Table 2).

Levels of IL-1ß, IL-8, TNF-α and adiponectin were significantly different between the study group and the healthy controls when inflammatory markers from OA patients in both study groups were compared to controls. There was a difference (p = 0.001), with Group II patients having lower parameter levels than Group I patients and healthy controls having lower levels. There was no significant difference in resistin levels (p = 0.2) (Table 3).

Table 1: Demographic characteristics of obese patients with osteoarthritis

Parameters	Group I N (%)	Group II N (%)	p-value
Age (years)			
< 40	4 (10.0 %)	4 (10.0 %)	0.8*
40-49	10 (25.0 %)	11 (27.5 %)	
50-59	9 (22.5 %)	6 (15.0 %)	
≥ 60	17 (42.5 %)	19 (47.5 %)	
Gender			
Male	12 (30.0 %)	11 (27.5 %)	0.6**
Female	28 (70.0 %)	29 (72.5 %)	

**Fishers exact test; **Chi-square test; Group I was given metformin 850 mg orally; Group II was given serratiopeptidase 20 mg and metformin 850 mg orally;*

Table 2: Body mass index (BMI) and inflammatory markers levels for two groups of treated knee osteoarthritis patients pre- and post-treatment

Parameters	Group I			Group II		
	Pre-treatment	Post-treatment	p-value	Pre-treatment	Post-treatment	p-value
BMI	34 ± 5.1	34.0 ± 4.6	0.70	33.7 ± 5.0	32.0 ± 4.2	0.10
Pain score	7.9 ± 2.0	6.9 ± 2.0	0.07	8.0 ± 2.0	4.0 ± 2.5	**< 0.01**
IL-1ß [pg/mL]	425.0 ± 22.1	419.0 ± 20.9	0.20	427.0 ± 20.3	412.0 ± 17.5	**< 0.01**
IL-8 [pg/mL]	370.0 ± 25.0	366.0 ± 28.5	0.50	368.0 ± 30.3	228.0 ± 21.4	**< 0.01**
Resistin [µg/mL]	0.018 ± 0.010	0.016 ± 0.008	0.20	0.024 ± 0.001	0.022 ± 0.009	0.30
TNF-α [pg/mL]	65.0 ± 1.5	60.0 ± 0.7	**< 0.01**	70.3 ± 1.7	58.4 ± 0.7	**< 0.001**
Adiponectin [µg/mL]	31.0 ± 4.2	29.0 ± 4.5	0.10	30.4 ± 5.0	29.4 ± 4.9	0.30

**Paired t-test; BMI: body mass index; IL-1ß: interleukin 1 beta; IL-8: Interleukin 8; TNF-α: tumour necrosis factor-α; Group I was given metformin 850 mg orally; Group II was given serratiopeptidase 20 mg and metformin 850 mg orally;*

Table 3: Inflammatory markers expression in study groups and healthy controls

Parameters	Group I	Group II	Control	p-value
IL-1ß [pg/mL]	419.0 ± 20.9	412.0 ± 17.5	3.0 ± 0.8	< 0.01
IL-8 [pg/mL]	366.0 ± 28.5	228.0 ± 21.4	33.0 ± 12.4	< 0.01
Resistin [µg/mL]	0.016 ± 0.007	0.020 ± 0.009	0.020 ± 0.010	0.20
TNF-α [pg/mL]	60.0 ± 0.7	58.0 ± 0.7	38.0 ± 2.9	< 0.01
Adiponectin [µg/mL]	29.0 ± 4.5	29.0 ± 4.9	17.0 ± 0.0	< 0.01

**One-way ANOVA analysis; BMI: body mass index; IL-1ß: interleukin 1 beta; IL-8: interleukin 8; TNF-α: tumour necrosis factor-α; Group I was given metformin 850 mg orally; Group II was given serratiopeptidase 20 mg and metformin 850 mg orally;*

Table 4: Adverse effects reported in patients treated for knee osteoarthritis

Adverse effect	Group I	Group II	p-value
Nausea and vomiting	4 (10.0 %)	1 (2.5 %)	0.30
Diarrhoea	1 (2.5 %)	0 (0.0 %)	1.00
Vertigo	2 (5.0 %)	1 (2.5 %)	0.60
Headache	2 (5.0 %)	0 (0.0 %)	0.20
Bleeding/bruising	0 (0.0 %)	0 (0.0 %)	-
Muscle weakness	1 (2.5 %)	0 (0.0 %)	1.00

** Fisher Exact test; Group I was given metformin 850 mg orally; Group II was given serratiopeptidase 20 mg and metformin 850 mg orally;*

As demonstrated in Table 4, no significant changes in side effects were detected between the two study groups three months after medication. Nausea and vomiting (10 %), vertigo (5 %) and headache (5 %) were the most frequent adverse reactions associated with Group I regimen. Nausea and vomiting (2.5 %) and vertigo (2.5 %) were the most commonly reported adverse effects associated with Group II regimen.

Discussion

Numerous medication formulations, such as NSAIDs, have been utilised to treat knee OA and these drugs can disrupt extracellular matrix metabolism, particularly proteoglycan production.[12] Moreover, these pharmacological treatments were associated with several side events, including gastrointestinal ulcerations. As a result, novel medication formulations are required to alleviate symptoms safely and be long-lasting.[12] These data demonstrated the metformin and serratiopeptidase regimen's superior symptomatic efficacy and laboratory efficiency in the treatment of knee OA.

Metformin has been shown to have a synergistic impact with various medications and in a variety of disorders.[13] Mohammed et al[14] have shown that combining metformin with anti-inflammatory drugs like NSAIDs for knee OA has been demonstrated to improve outcome scores for knee OA. Metformin was reported to lower inflammatory indicators and contribute to reducing oxidative stress *via* an unknown mechanism.[15] These inflammatory indicators, such as cytokines and chemokines are increased following knee joint damage, indicating a significant role for oxidative stress in knee OA pathophysiology.[16]

Several authors validated metformin's osteogenic effect *in vitro*.[17] Study in China reported the beneficial effect of metformin in management of intervertebral disc herniation.[18] Although there was no significant association, after three months of treatment, patients BMI of both trial groups decreased. This outcome is consistent with the findings of study conducted in the United States of America, which concluded that there is insufficient evidence to support the use of metformin to treat overweight and obesity.[19]

In a study in India, both patients groups with knee OA used serratiopeptidase combined with various drugs and both modalities, including serratiopeptidase, had a better effect on knee treatment with mild side effects. The underlying mechanism of serratiopeptidase in knee OA is not fully understood but demonstrated to lyse dead, damaged tissue while sparing living tissue.[20] Indian studies have previously established that serratiopeptidase has anti-inflammatory properties after surgery.[21] When inflammatory indicators from both trial groups were compared to those from healthy controls, serum levels of IL-8, IL-1ß, adiponectin and TNF-α, were considerably lower in patients treated with serratiopeptidase and metformin than in patients treated with metformin alone. Such finding further established anti-inflammatory synergy between serratiopeptidase and metformin.

Furthermore, presented findings corroborate those of Bhagatet et al[22] and Nair et al,[8] both of which demonstrated the anti-inflammatory impact of serratiopeptidase enzyme and metformin. There was limited adverse effects in both groups, particularly for group II (serratiopeptidase and metformin), with no significant differences between groups. These results points to regimen's safety, proven in several prior studies.[14, 20]

Limitations of this trial were lack of follow-up, a single-centre design and a limited period for evaluating side effects; therefore, additional long-term follow-up studies are required to verify long-term effect of serratiopeptidase and metformin medication combination.

Conclusion

Serratiopeptidase and metformin in knee OA treatment are safe and effective. This regimen effectively reduces pain and inflammatory markers in people with knee OA.

Ethics

The study was approved by the Al-Kindy College of Medicine, University of Baghdad's Ethics and Scientific Committee, which gave it the registration No EAC-125603, dated November 2018. Written informed consent was obtained from patients prior to their participation in the study and for publishing of the anonymised patient data. The study was organised and implemented based on the adherence to the Ethical Principles for Medical Research Involving Human subjects (The Declaration of Helsinki, 8th Revision, 2013).

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Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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REVIEW ARTICLE

Complementary Therapies and Factors Related to *Dysmenorrhoea* in Adolescents: A Bibliometric Analysis

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Abstract

Complementary therapies for decreasing *dysmenorrhoea* and factors behind *dysmenorrhoea* in adolescents is not clear enough. Researchers need information about trends and novelties for the future research topic of complementary therapies and factors related to *dysmenorrhoea* in adolescents. Thus, the purpose of this study was to identify the complementary therapies for decreasing *dysmenorrhoea* and factors related to *dysmenorrhoea* in adolescents by analysing network visualisation, overlay visualisation and density visualisation on the topic through bibliometric analysis. The data sources used in this study were based on online searches *via https://app.dimensions.ai/*. The literature search used the stages following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flowchart. Paper restricted in publications years 2014-2023, focus in the field of health sciences, nursing, public health, clinical sciences and publication type were included as criteria for inclusion in the study. The data were analysed using *VOSviewer*. After identifying the clusters, the types of complementary therapies that were able to reduce *dysmenorrhoea* were acupressure, acupuncture, calcium sufficiency, curcumin, yoga, aromatherapy, massage and physical activity. Moreover, factors related to *dysmenorrhoea* were HIV, reproductive coercion, reproductive health, oxytocin, prolactin, trauma, metformin, anxiety and breast cancer. Moreover, the newest topics that were being widely researched related to adolescent *dysmenorrhoea* were education, non-suicidal self-injury (NSSI), prolactin and physical activity. On the other hand, topics that were rarely researched related to the topic of adolescent *dysmenorrhoea* were topics about sexual health, reproductive coercion, inflammation, curcumin and physical activity. Complementary therapies and factors that are still rarely studied offer potential novelty in results in future studies.

Key words: *Dysmenorrhoea*; Factors; Complementary therapy; Adolescent.

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Introduction

Female adolescents are populations that expe-rience various changes in hormonal, physical, cognitive and emotional maturity as well as social status in society.[1–4] This change can be identified from the responsibilities they will face in society and their new roles.[5] Physical changes are another issue that occurs in the adolescent population. These physical changes include enlarged hips and breasts, increased height and weight, hair beginning to grow in the armpit and pubic area and menstruation.[6]

Menstruation is a hallmark of puberty signs in adolescents. Generally, menstruation occurs regularly every month.[1, 7] Most prominent complaint during menstruation or even before menstruation is pain.[8, 9] World Health Organization found that 1,769,425 (90 %) of women experience *dysmenorrhoea* with 10-15 % experiencing heavy *dysmenorrhoea*.[10] The pain they experience can occur in 3-7 days, depending on personal conditions. If this problem is not controlled, discomfort and anxiety will happen.[11, 12]

The studies showed that complementary therapy such as progressive muscle relaxation therapy (PMRT) can reduce *dysmenorrhoea*.[2, 13, 14] In another study endorphin massage therapy (EMT) was also able to reduce *dysmenorrhoea*.[13, 15, 16] Researchers who focused on complementary therapies with natural ingredients state that various natural ingredients such as ginger, turmeric, cinnamon and bay leaves can reduce *dysmenorrhoea* if processed way appropriately.[17–19]

Apart from being a therapy, the factors related to *dysmenorrhoea* are essential to identify the impact of interventions in therapy. From the data, the factor that can influence the occurrence of *dysmenorrhoea* is anxiety.[11, 12, 20] Moreover, other studies mention that *dysmenorrhoea* is influenced by reproductive health and hormonal status.[21, 22] The hormone that has the most influence on *dysmenorrhoea* is the prolactin.[23–25]

Interest in research on the topic of complementary therapies for decreasing *dysmenorrhoea* and the factor behind *dysmenorrhoea* in adolescents is not clear enough. In future studies, researchers need information about trends and novelties for the future research topic of complementary therapies and factors related to *dysmenorrhoea* in adolescents. This is a problem that arises among researchers currently. The results of bibliometric analyses may guide future studies by determining the quality and main research areas of existing publications in specific fields. Moreover, the bibliometric analysis enables researchers to easily obtain information about subjects of interest from among numerous and increasing number of published articles.

There are five types of study metrics for data analysis, namely: scientometrics, bibliometrics, cybermetrics, informetrics and altmetrics.[26] Bibliometric analysis is more suitable for quantitatively analysing the distribution of research papers, terms and keywords in determining research trends.[27] In addition, bibliometric analysis is a research method used in library and information science to evaluate research performance.[28] Bibliometric analysis is essential in assessing research impact where studies are ranked based on the citations received.[29]

There is no bibliometric analysis on the publication of research topics on complementary therapies for decreasing *dysmenorrhoea* in adolescents. Thus, the purpose of the study was to identify the complementary therapies for decreasing *dysmenorrhoea* and factors related to *dysmenorrhoea* in adolescents by analysing network visualisation, overlay visualisation and density visualisation on the topic through bibliometric analysis.

Methods

Data sources

The data sources used in this study were based on online searches *via https://app.dimensions.ai/.* The literature search used the stages following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flowchart.[30] Paper were restricted in publications years 2014-2023, focus was in the field of health sciences, nursing, public health, clinical sciences and publication type of article.

Selecting data

The stages in PRISMA included identification, screening and including as shown in Figure 1. Stage 1 (identification) detected 49,652 records from dimensions.ai, taking into account, each of the main search terms (complementary therapy AND decreasing *dysmenorrhoea* AND adolescent), "article and proceeding document type" and all published data in the data range from 2014 to 2023. In stage 2 (screening), the option "article title, abstract" was selected in the field of each search term, resulting in biomedical sciences, clinical sciences, health sciences, reproductive medicine, neurosciences, health service and systems, public health articles being excluded. In phase 3 (included), the final paper yielded 1,877 articles.

Data analysis

Data were analysed using *VOSviewer*. *VOSviewer* is a computer program for creating and viewing bibliometric maps.[31] The type of analysis was

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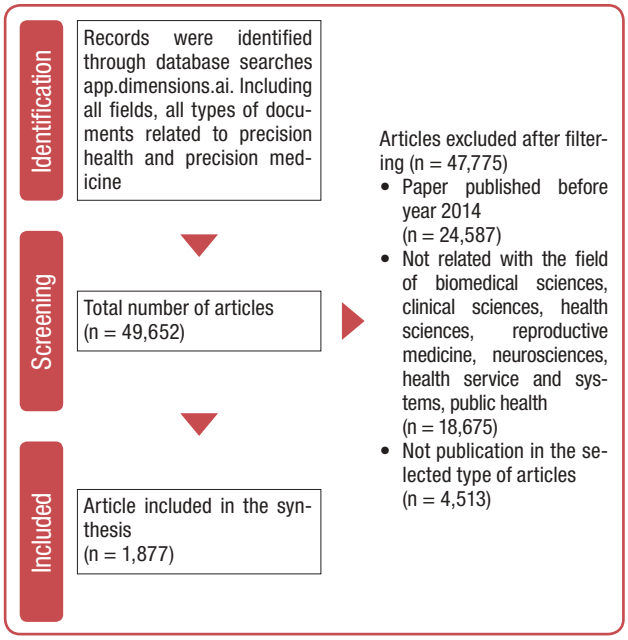

Figure 1: PRISMA Flowchart

selected to create a map based on text data. In this study, the analysis was reviewed by co-occurrence and co-authors.

The procedure for co-occurrence analysis went through the following stages: the data source was selected, data were read from references manager files. Fields were chosen, fields were selected from which title and abstract was extracted. The counting method selected was full counting. The minimum number of occurrences of a term 10 was selected as a threshold. The number of selected terms was 133.

The procedure for co-author analysis went through the following stages: the type of data was chosen, a map based on bibliographic data was created. An option to create a co-authorship map based on bibliographic data was chosen. Data source was chosen, data from reference manager files were read. Supported file type was: ris. The type of analysis and counting method was chosen: the type of analysis was co-authorship and the counting method was full counting. Threshold was chosen: the maximum number of documents of an author was 2. Out of the 2,624 authors, 49 met the threshold. The author was chosen: for each of the 49 authors, the total strength of the co-authorship links with other authors was calculated. The authors with the greatest total link strength were selected. The number of authors selected was 49.

Results

From the network visualisation (Figure 2), it was identified that there were 151 items divided into 7 clusters with several links 3,740 with a total link strength of 28,710. After identifying the clusters, the types of complementary therapies that were able to reduce *dysmenorrhoea* were: acupressure, acupuncture, calcium, curcumin, yoga, aromatherapy, massage and physical activity.

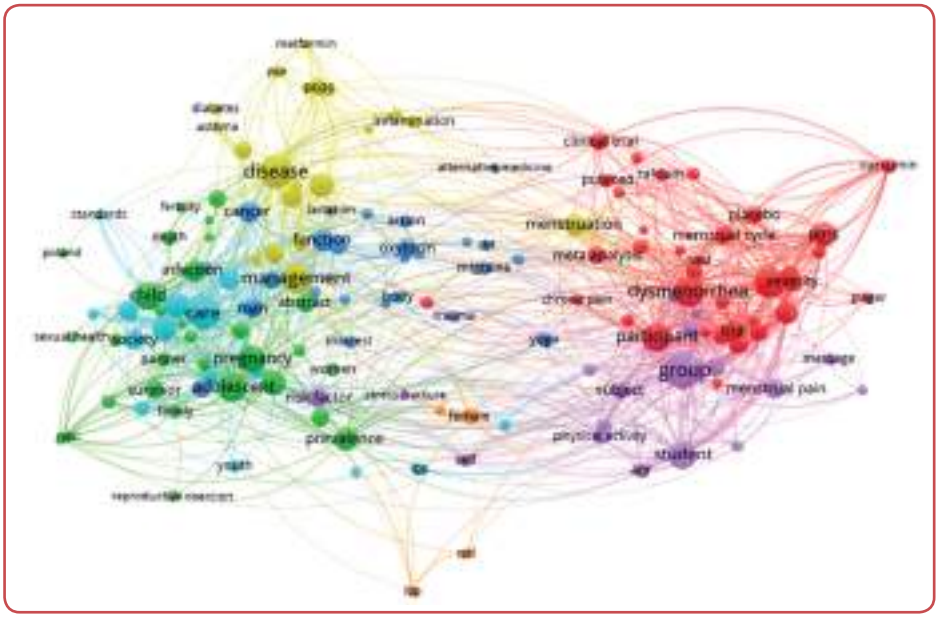

Figure 2: Network visualisation

Moreover, factors related to *dysmenorrhoea* were: HIV, reproductive coercion, reproductive health, oxytocin, prolactin, trauma, metformin, anxiety and breast cancer.

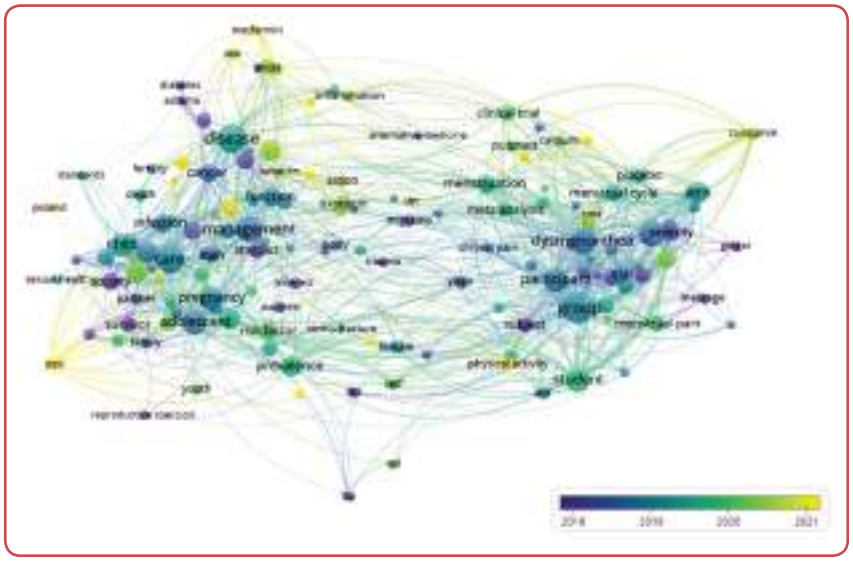

Figure 3: *Overlay visualisation*

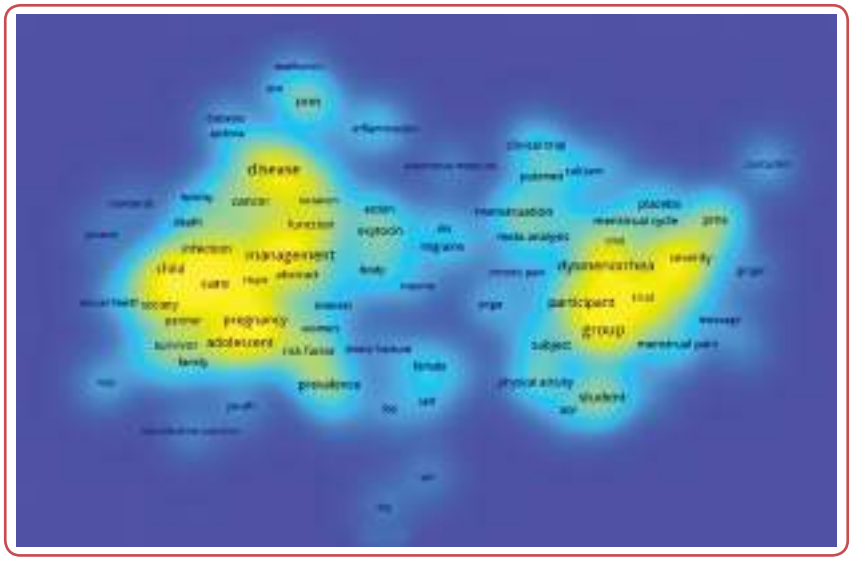

Figure 4: *Density visualisation*

From the overlay visualisation (Figure 3), it was indicated that the newest topics that were being widely researched related to adolescent *dysmenorrhoea* were education, non-suicidal self-injury (NSSI), prolactin and physical activity.

From density visualisation (Figure 4), it was indicated that topics that were rarely researched related to the topic of adolescent *dysmenorrhoea* were topics about sexual health, reproductive coercion, inflammation, curcumin and physical activity.

Discussion

This study focused on identifying complementary therapies that can reduce *dysmenorrhoea* and factors related to *dysmenorrhoea*. The types of complementary therapies that were able to reduce *dysmenorrhoea* were acupressure, acupuncture, calcium sufficiency, curcumin, alternative medicine, yoga, aromatherapy, massage and physical activity.

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Complementary therapies

Acupressure

Acupressure is a traditional complementary therapy originating in China. One of the benefits can be used to minimise *dysmenorrhoea* using the fingers by pressing on certain meridian points (acupuncture points). Emphasis on acupressure meridian points can produce endorphins in the body, emphasis is placed on the Hequ/LI4 (located between the base of the thumb and forefinger) directly increasing endorphins so that it will accelerate pain release.[32] Endorphins are hormones that reduce pain and also act as a sedative. When the body feels pain and then acupressure therapy is carried out at a certain point, the body will release endorphins which make the body feel calmer.[33]

Acupuncture

Acupuncture therapy has been widely used in many countries to treat primary *dysmenorrhoea* (PD).[34] Evidence shows that acupuncture can reduce *dysmenorrhoea* and accompanying symptoms by reducing prostaglandin F2α which is specific for *dysmenorrhoea*, relaxing the uterine muscles, increasing blood flow in the uterus, balancing hormones and psychological symptoms. In PD most patients are symptomatic and tend to use drugs such as analgesics, anti-inflammatory steroids, contraceptive drugs and others and current clinical guidelines for treating PD recommend using NSAIDs and stimulation of acupuncture points as therapy complementary alternatives to medical therapy. The use of NSAIDs has side effects, especially on the digestive side while acupuncture therapy is preferred.[34]

Calcium supplement

Vitamin D has an important role in female reproduction, vitamin D receptors are expressed in ovarian, endometrial, decidual, placental and fallopian tube epithelial cells.[35] Based on several studies that have been conducted, it is known that calcium levels are known to decrease in the luteal phase in response to changes in oestradiol levels. Women who consumed three to four servings of dairy products a day were found to have a lower risk of developing *dysmenorrhoea* compared to those who did not consume dairy products.[36] To reduce pain in PD, calcium consumption is needed around 800 - 1,000 mg. The percentage of absorption at the time of calcium consumption depends on the total amount of elemental calcium consumed at one time and the absorption of the highest amount of calcium consumption at a dose of about 500 mg. With an increase in the amount of calcium intake consumed, the effectiveness of calcium absorption will decrease. So, if someone is taking 1,000 mg/day of calcium from a supplement, they can split the dose and take 500 mg in two separate doses over time, by taking 250–500 mg every 4 h in pain.[10]

Curcumin

Curcumin can work as an analgesic which could inhibit excessive prostaglandin release through the uterine epithelial tissue and inhibit uterine contractions so that it could reduce pain during menstruation.[37] The mechanism of inhibition of uterine contractions through curcumin is by reducing the influx of calcium ions (Ca^{2+}) into the calcium channels of the uterine epithelial cells. Curcumenol as an analgesic agent will inhibit the excessive release of prostaglandins through the uterine epithelial tissue and will inhibit uterine contractions thereby reducing the occurrence of *dysmenorrhoea*. It was found that the *dysmenorrhoea* pain scale after being given turmeric water (*Curcuma longa*) was increased with an average score of 1.93.[38]

Yoga

One of the non-pharmacological therapies to reduce *dysmenorrhoea* was yoga. Yoga is a relaxation technique that teaches a set of techniques such as breathing, meditation and body positions to increase strength and balance. Relaxation techniques in yoga can stimulate the body to release endogenous opioids, namely endorphins and enkephalins (compounds that function to inhibit pain). Endorphins are produced in the brain and spinal cord. This hormone can function as a natural sedative that is produced by the brain, causing a feeling of comfort.[39] There have been many studies on the effectiveness of yoga in reducing *dysmenorrhoea* and it was found that yoga can be used as an effective complementary therapies in reducing *dysmenorrhoea* in women with PD.[40]

Aromatherapy

The principal effect of aromatherapy is to stimulate the *hypothalamus*. The *hypothalamus* is a gland in the brain that controls the hormone system and regulates important bodily functions such as sleep and emotional response. After reaching the *hypothalamus*, the scent stimulus travels through the limbic system and into the *hippocampus*, which is a part of the brain important

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for memory. This not only helps the individual associate certain scents with certain memories, but this is also what allows the body to react to the aromas from the aromatherapy. When the aroma is inhaled, the molecules of the aromatherapy oil are carried to the receptor cells in the nose, when these molecules attach to the fine hairs of the nose, an electrochemical message occurs which is transmitted through the olfactory tract to the brain and continues to the olfactory system. The electrochemical messages will stimulate the *hypothalamus* to release the hormone serotonin and endorphin hormones, where the function of the serotonin hormone is to improve mood while the endorphin hormone is a natural pain reliever and produces feelings of relaxation, calm and joy.[41] Aromatherapy that is commonly used to reduce *dysmenorrhoea* is the aroma of shallots, olive oil, *Citrus Hystrix*, *Jasminum officinale*, *Mentha Piperita*, orange, rose, *Nigella Sativa* and ginger.[42]

Massage

Massage can be a choice in the medical world that has a positive effect in reducing the level of muscle fatigue and pain relief and also as a tool to improve the level of human health both physically and psychologically.[43] Through massage, the process of expelling burnt residue (lactic acid) into the bloodstream is accelerated, so recovery is also faster.[44]

Physical activity

Studies on the relationship of physical activity with a decrease in pain, especially *dysmenorrhoea* began to be done a lot.[45] The results of the study show that physically active individuals have a higher tolerance for pain and do not experience as much chronic pain as those who are sedentary. Individuals who engage in physical activity are associated with releasing endorphins, having better blood flow, lower inflammation and better heart health. This can result in the ability to endure more pain.[46]

Factors related to *dysmenorrhoea*

HIV

Adolescents experience menstrual cycle irre-gularities at certain times. Complaints such as irregular menstruation or changes in menstrual flow and worsening of premenstrual symptoms, sometimes indicate an underlying health problem. Most of the menstrual changes reported by women living with HIV do not appear to be directly related to the virus.[47] There is evidence to suggest that women living with HIV are more likely to experience the absence of menstruation (*amenorrhoea*). Further analysis of international studies conducted from 1996 to 2010 in nearly 9,000 women found that there is a significant association between HIV status and *amenorrhoea*. Women living with HIV have a 70 % chance of having *amenorrhoea* for more than three months.[48]

The association of *amenorrhoea* with HIV was first detected in 1988 when it was noted as an initial complaint in 26 % of newly diagnosed seropositive Ugandan women. Since then many reports of this problem have been reported which may be due to low BMI, chronic stress state, immune dysfunction or from comorbidities. Besides *amenorrhoea*, other mechanisms such as hyperprolactinaemia, polycystic ovary syndrome and premature ovarian failure have also been suggested as a cause of some of the menstrual disorders seen in HIV.[48]

The cause is still being debated. It remains unclear whether *amenorrhoea* is a complication of HIV infection itself or due to other risk factors that are more common in women with HIV, such as low body weight, immunosuppression or a combination of risk factors.

Reproductive coercion

Puberty is marked by the first discharge of menstrual blood (*menarche*) experienced by women, which is a characteristic of the maturity of a healthy, non-pregnant woman. *Menarche* occurs due to increased follicle stimulating hormone (FSH) and luteinising hormone (LH) which can stimulate ovarian target cells. FSH and LH combine with FSH and LH receptors which will further increase the rate of cell secretion, growth and proliferation.[49] Oestrogen and progesterone hormones will stimulate the uterus and breast glands to be competent to allow ovulation to occur.[47] *Dysmenorrhoea* was found in 58.8 % of all women. The mean age and age at *menarche* were younger in women with *dysmenorrhoea*. In that study, it was found that the risk of *dysmenorrhoea* occurred 0.97 times lower with age, meaning that the risk of *dysmenorrhoea* was higher in women with a younger age of *menarche* and age of *menarche* affected the incidence of *dysmenorrhoea*.[50]

Reproduction health

Reproductive health is a healthy condition psychologically, socially and physically related to the functions and processes of reproduction. Every

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individual must be responsible for maintaining and protecting their reproductive organs, both men and women, especially women who experience menstrual periods because the intimate organs are very susceptible to exposure to bacteria. One of the reproductive health problems in women, especially teenagers during menstruation is *dysmenorrhoea*, the sensation of pain due to contractions of the uterine muscles in every woman is different, this *dysmenorrhoea* condition sometimes disturbs adolescents psychologically due to the uncomfortable sensation of pain felt so that it disrupts activities and quality of life in adolescents.[50]

Oxytocin

Oxytocin is a natural hormone that acts as a neurotransmitter in the brain which is produced by the *hypothalamus* and secreted through the pituitary gland. The hormone oxytocin plays an important role in the male and female reproductive systems. In women, one of the functions of the hormone oxytocin is to stimulate uterine contractions during childbirth and stimulate contractions of breast tissue during the lactation process. During menstruation, increased pre-ovulatory oxytocin secretion in the *hypothalamus* and ovaries can increase gonadotropin-releasing hormone secretion and facilitate ovulation.[51]

One of the causes of PD is an increase in the hormone prostaglandin produced in the lining of the uterus. During the menstrual phase, prostaglandins increase the myometrial response which stimulates the hormone oxytocin, where the hormone oxytocin also has the property of increasing uterine contractions. These uterine contractions can cause complaints of pain. In women who are not pregnant or breastfeeding and have high levels of the hormone oxytocin, it will make uterine contractions more active and cause an increase in uterine muscle mass (hypertrophy), so women with high levels of oxytocin experience *dysmenorrhoea* compared to women who have low oxytocin.[52]

Prolactin

Prolactin hormone is a hormone produced in the pituitary gland, uterus, brain, breast, skin, fat layer and immune cells. Prolactin hormone is owned by men and women. The hormone prolactin is controlled by the main hormones, namely dopamine and oestrogen in the pro-duction process. Both of these hormones will send messages to the pituitary gland to produce or stop them.[53]

Dysmenorrhoea usually occurs during the premenstrual phase. This phase is marked by an increase in the hormones prolactin and oestrogen, one of the signs of an increase in the hormone prolactin in the premenstrual phase up to the first day of the menstrual cycle, namely breasts that feel sore and tight. Conditions where prolactin levels are above normal (*hyperprolactinaemia*) cause irregular menstruation and even *amenorrhoea* so the disruption of the hormone prolactin affects the menstrual cycle.[54]

Psychological trauma

PD often occurs in more than 50 % of women and 15 % of them experience severe pain. PD is experienced by 60-75 % of young women. Three-quarters of these suffer *dysmenorrhoea* with mild and moderate intensity, while another quarter experience *dysmenorrhoea* with severe levels.[55] *Dysmenorrhoea* results in psychological disorders where one of the psychological factors is stress which is a physiological, psychological response from humans who try to adapt and regulate both internal and external pressures. Sustained stress can lead to depression. The stress response of each person is different, namely due to health conditions, personality, first experience with *dysmenorrhoea*, knowledge, coping mechanisms, level of education, age and emotional management abilities of each individual. Stress can have both positive and negative effects. Positive influence, namely encourage individuals to raise awareness and produce new experiences. The negative effects, cause feelings of insecurity, anger, depression, headaches, stomach aches and insomnia.[55]

Metformin

One of the disorders in the reproductive system is irregular menstrual cycles and not even getting menstruation (*amenorrhoea*). Pelvic inflammatory disease, uterine polyps, cysts, uterine tumours, endometriosis and so on. At this time, a new medical therapy emerged in the treatment of endometriosis, namely by administering the drug metformin.[56]

Research on the effectiveness of metformin in patients with endometriosis, polycystic ovary syndrome (PCOS) and other reproductive disorders has started to be carried out a lot. Until now, the exact cause of endometriosis is not known, so definitive treatment has not been found. Many experts suspect that this condition arises due to abnormal proliferation of endometrial cells caused by reverse menstrual blood flow, mutations in

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peritoneal cells or embryonic cells, immune system disorders and hormonal disorders. According to various studies, administering several types of hormonal drugs (including metformin) to patients with endometriosis can suppress the growth of their cysts, minimise the clinical symptoms that appear, relieve the inflammation they cause, as well as increase the chances of pregnancy.[56]

Anxiety

Experiencing menstrual pain recurring every month can increase the risk of experiencing depression, anxiety or stress. In some cases, this psychological disorder exacerbates the severity of menstrual pain so that it has a two-way relationship. Even having depression and *dysmenorrhoea* together can increase the perception of pain severity and reduce response to treatment.[55] In a critical review, patients with severe *dysmenorrhoea* experienced increased pain sensitivity which could not only be explained by increased prostaglandin factors alone, but there were alternative explanations, which is possibly due to central sensitivity to pain, the hypothesis is that *dysmenorrhoea* may be caused by increased sensitivity to pain, so *dysmenorrhoea* does not only have physical aspects but can have psychological aspects that affect the condition.[55]

Breast cancer

Cancer is a disease that is feared by all indi-viduals, both men and women. In women, one of the most common cancers is breast cancer, according to several studies it was found that women who experience *menarche* at a very young age (< 12 years of age) can increase the risk of breast cancer caused by high levels of oestrogen in the body. There is still no definite causal factor for the occurrence of breast cancer, including the very young age of *menarche*. Other factors that are suspected include the length of time menstruation, menopause, obesity, hormone therapy, use of oral contraceptive therapy, preeclampsia, height, physical activity, radiation exposure and others that need to be studied more deeply.[57]

Education

Health education is an activity to provide and improve knowledge, attitudes and good behaviour of individuals, families and communities to maintain and improve health. Health education is very necessary as an effort to prevent disease, as well as for the problem of *dysmenorrhoea*. *Dysmenorrhoea* has negative effects that cannot be taken for granted, the uncomfortable sensation of pain causes individuals to feel anxious, stressed, depressed, decreased activity and decreased quality of life so education needs to be given on how to deal with *dysmenorrhoea*. The studies that have been conducted regarding education on how to treat *dysmenorrhoea* have on average obtained good results in increasing knowledge, attitudes and ways of handling *dysmenorrhoea*. The provision of health education is an effective method for increasing knowledge, attitudes and skills.[58]

NSSI

NSSI is a symptom of depression by self-harm. Experiencing menstrual pain repeatedly makes individuals feel anxious when approaching menstruation, if this happens continuously without proper treatment, education and support systems from family and environment, the risk will increase towards depression. *Dysmenorrhoea* is not only physically disturbing to sufferers, from a psychological aspect it can be disturbing to sufferers.[55]

Conclusion

The types of complementary therapies that were able to reduce *dysmenorrhoea* were: acupressure, acupuncture, calcium sufficiency, curcumin, yoga, aromatherapy, massage and physical activity. Moreover, factors related to *dysmenorrhoea* were: HIV, reproductive coercion, reproductive health, oxytocin, prolactin, trauma, metformin, anxiety and breast cancer.

Ethics

This study was a secondary analysis based on the currently existing dataset from the *Dimensions* and did not directly involve with human participants or experimental animals. Therefore, the ethics approval was not required in this paper.

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CONTENT

Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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REVIEW ARTICLE

Association Between Null Genotypes of Glutathione S-Transferase M1 and T1 and Susceptibility to Systemic Lupus Erythematosus: A Meta-Analysis

Mostafa Saadat[1]

Abstract

Oxidative stress is involved in the development of systemic lupus erythematosus (SLE). It is well known that activity of the glutathione S-transferase superfamily has a protective effect against oxidative stress. Several studies have investigated the association between the *GSTT1/GSTM1* polymorphisms and the risk of SLE with inconsistent results. The present meta-analysis was performed to investigate the association between susceptibility to SLE and the null genotypes of *GSTT1* and *GSTM1*. Eligible publications were identified by searching several databases, 18 case-control studies with 2483 cases and 3643 controls met the inclusion criteria. The raw data of three reports have internal inconsistencies, therefore these studies were excluded from the final analysis. The results showed that the *GSTM1* null genotype significantly increased the risk of SLE (OR = 1.17, 95 % CI: 1.03-1.32, p = 0.012) with no evidence of significant heterogeneity (Q = 14.53, df = 14, p = 0.411; I^2 = 3.4 %). The *GSTT1* null genotype was not associated with the risk of SLE (OR = 0.94, 95 % CI: 0.80-1.10, p = 0.447). There was no evidence of heterogeneity between studies. The present study showed that the null genotype of *GSTM1* was weakly associated with the risk of SLE.

Key words: Glutathione S-transferases; *GSTT1*; *GSTM1*; Meta-analysis; SLE.

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Introduction

Systemic lupus erythematosus (SLE) is one of the best studied autoimmune diseases. Although the aetiology of SLE is not fully understood, oxidative stress (an imbalance between free radical production and cellular antioxidant capacity) has been implicated in its pathogenesis. Oxidative stress has been positively correlated with the risk of SLE.[1, 2] The enzyme activity of some antioxidant enzymes, such as the activity of paraoxonase 1 (PON1)[3] and catalase (CAT),[4] is decreased in SLE patients compared to controls. Familial aggregation and twin studies have shown a high degree of heritability.[5-7]

Glutathione S-transferases (GSTs, EC 2.5.1.18) are a superfamily of detoxification enzymes that catalyse the conjugation of numerous xenobiotics with glutathione. GSTs are classified into several classes, including alpha, mu, theta, etc. The mu and theta classes have 5 and 2

members, respectively. *GSTM1* (MIM: 138350) and *GSTT1* (MIM: 600436) genes belong to the mu and theta classes, respectively. Deletion of the entire *GSTT1* and *GSTM1* genes is a rare genetic variation that produces *GSTT1* and *GSTM1* null alleles, respectively. Homozygous individuals for each null allele are referred to as null genotypes. The *GSTT1* (and *GSTM1*) null genotype results in the absence of the gene, protein and enzyme activity.[8, 9] The *GSTT1* and *GSTM1* null genotypes are important genetic markers for studying the role of these genes. There are many association studies investigating the relationship between these genetic variations and susceptibility to many multifactorial diseases.[10-19]

It is well known that GST enzyme activity has protective effect against oxidative stress.[8, 9] Alteration of GST enzyme activity due to the above-mentioned gene deletion, reduces cellular detoxification capacity. Considering that oxidative stress has been associated with SLE, it is reasonable to assume that the null genotypes of *GSTT1* and *GSTM1* may have a significant contribution to the pathological process and risk of SLE.

From 1999 to date, many studies have investigated the association between the *GSTT1/GSTM1* polymorphisms and the risk of SLE, with inconsistent results.[20-32] Because many association studies are conducted with limited numbers of participants (patients and controls), they usually fail to detect weak associations. Meta-analysis of published data can increase the sample size and statistical power to overcome the weakness of small studies and provide more precise estimates of the association between a given polymorphism and susceptibility to a multifactorial disease. Two meta-analyses have investigated the association between *GSTT1/GSTM1* polymorphisms and susceptibility to SLE. The first meta-analysis, published in 2015, was based on 9 original articles,[33] and surprisingly, the second, published in 2016, was based on 4 original articles.[34] Unfortunately, both meta-analyses suffer from the authors' inaccuracy in finding relevant articles and the authors did not include some of the studies published at the time. Therefore, their results are not reliable and a new meta-analysis is needed.

The present meta-analysis was performed to investigate the association between susceptibility to SLE and the null genotypes of *GSTT1* and *GSTM1*.

Methods

The current meta-analysis was conducted according to the recommendations of the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statement. Eligible publications were identified by searching multiple databases, including PubMed, Europe PMC, Web of Science, Scopus, Directory open access journals (DOAJ), ProQuest, African journals online (AJOL) and Islamic science citation (ISC). Last search updated on 15 July 2023. The following keywords were used to search the literature search: (*GSTT1* OR *GSTM1*) AND ("systemic lupus erythematosus" OR SLE). The search was not limited by language. References of eligible studies were also reviewed to identify additional relevant studies.

Inclusion and exclusion criteria

Inclusion criteria were as follows: studies comparing SLE patients and healthy controls; articles with sufficient genotype data to calculate odds ratios (ORs) and corresponding 95 % confidence intervals (CIs); studies written in English. Exclusion criteria were as follows: case studies, meta-analyses, reviews, letters to the editor, poster presentations, overlapping data, studies of family members based on linkage analysis, articles that did not report raw data, duplicate studies and articles written in languages other than English.

Data extraction

A customised and standardised form was used for data extraction. The researcher extracted the required information twice with an interval of 2 weeks. There was no discrepancy between two extractions. For each study, the following information was extracted: first author's surname, year of publication, country in which the study was conducted, number of cases and controls, ethnicity, genotyping method, source of controls, mean age of participants (in cases and controls), percentage of female participants (in cases and controls) and number of cases and controls with respect to the *GSTT1/GSTM1* genotypes. Ethnicity was categorised as African, Asian, Caucasian and mixed. Source of controls was categorised as population-based and hospital-based studies. For studies investigating on more than one ethnic group, data were extracted separately as independent studies.

Statistical analysis

Associations were expressed as OR with 95 % CIs. Heterogeneity between studies was measured using Cochran's Q statistic and inconsistency was quantified using I^2 statistic. The Q statistic was considered statistically significant if the p-value was less than 0.10 ($p < 0.10$). I^2 values of 0-25 %, 26-50 %, 51-75 % and > 0.75 % represent no, low, moderate and high heterogeneity, respectively. If heterogeneity was present, a random-effects model was used according to the DerSimonian and Laird method,[35] otherwise, a fixed-effects model was used for comparisons according to the Mantel-Haenszel method.[36]

Subgroup meta-analyses were performed according to ethnicity, source of control and sample size. Sensitivity analyses were performed to assess the robustness and stability of the results. To perform sensitivity analyses, a single study was serially removed from the studies included in the analysis and the effect of removal on the level of heterogeneity and the strength of the association was measured. The potential publication bias of the eligible studies was assessed using the Begg[37] and Egger[38] regression tests. Statistical analysis and generation of plots were performed using the "Comparative Meta-Analysis" software (version 2.2.064, USA).

In addition, to assess whether statistically significant associations detected in this meta-analysis were "noteworthy", the false positive report probability (FPRP) value was calculated with a prior probability level (π) of 0.001 for significant associations. A FPRP cut-off value of 0.20 was used, as previously suggested.[39] Therefore, associations with the FPRP values less than 0.20 were considered as "noteworthy" associations.

Results

A flowchart of the study selection process is shown in Figure 1. At the end of the search process, a total of 121 original articles were reviewed. Duplicate articles (n = 65) between databases were excluded. Further screening resulted in the exclusion of 43 articles due to study design, article type (review, abstract, meta-analysis, etc) and not related to the *GSTT1/GSTM1* polymorphisms or research topic. Finally, 13 articles were selected for analysis.

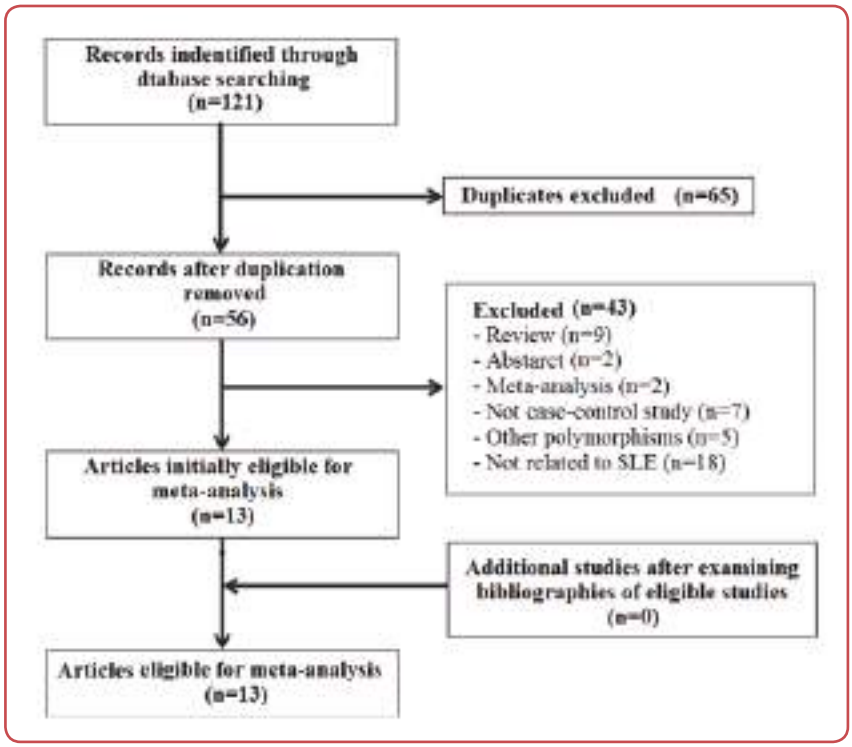

Figure 1: Flow diagram for identifying and including studies in the current meta-analysis

CONTENT

Table 1: Characteristics of included studies in this meta-analysis

Authors	Year	Country	Ethnicity	Source of controls	Cases			Controls		
					n*	Age	Female proportion (%)	n	Age	Female proportion (%)
Ollier et al[28]	1996	UK	Caucasian	NA	90	-	90.0	569	-	90.0
Tew et al (1)[31]	2001	USA	African	NA	105	-	-	56	-	-
Tew et al (2)[31]	2001	USA	Caucasian	NA	76	-	-	78	-	-
Tew et al (3)[31]	2001	USA	Hispanic	NA	71	-	-	42	-	-
Fraser et al (1)[27]	2003	USA	Caucasian	PB	85	-	-	202	-	-
Fraser et al (2)[27]	2003	USA	African	PB	144	-	-	72	-	-
Fraser et al (3)[27]	2003	USA	Mixed	PB	14	-	-	44	-	-
Kang et al[26]	2005	Korea	Asian	NA	330	33.3 (11.8)	96.6	270	34.9 (9.9)	94.5
Horiuchi et al[32]	2009	Japan	Asian	PB	152	-	100.0	427	-	100.0
Zhang et al[25]	2010	China	Asian	NA	298	-	-	284	-	-
Kiyohara et al[29]	2012	Japan	Asian	PB	151	41.2 (13.0)	100.0	421	31.7 (14.1)	100.0
Rupasaree et al[24]	2013	India	Caucasoid	HB	194	28.4 (9.8)	98.0	445	29.9 (9.9)	98.0
Glesse et al (1)[23]	2014	Brazil	Caucasian	PB	282	49.3 (15.0)	91.5	241	-	-
Glesse et al (2)[23]	2014	Brazil	African	PB	87	48.0 (13.6)	93.2	88	-	-
Salimi et al[22]	2015	Iran	Caucasian	PB	163	32.6 (8.6)	93.0	179	32.1 (11.7)	93.0
Nasr et al[30]	2017	Egypt	African	PB	40	28.1 (4.5)	100.0	40	27.3	100.0
Jevtovic-Stoimenov et al[21]	2017	Serbia	Caucasian	PB	88	52.0 median	-	88	-	-
de Oliveora et al[20]	2021	Brazil	Mixed	HB	144	33.7 (9.8)	100.0	145	32.7 (6.8)	100.0

n: number of participants; NA: data not available; PB: population based controls; HB: hospital based controls; age values are presented as mean (standard deviation);

Reports by Tew,[31] Fraser[27] and Glesse[23] that included participants from different ethnic groups were considered as three, three and two studies, respectively. The application of these criteria resulted in 18 case-control studies eligible for meta-analysis.

Table 1 shows the characteristics of the 18 eligible studies included in the meta-analysis. Of these 7, 4 and 4 studies were conducted in Caucasians, Asians, and Africans, respectively. Controls were population-based (PB) and hospital-based (HB) in 10 and 2 studies, respectively. Some studies (n = 6) did not report the source of controls. In all studies, the polymorphism was determined by PCR assays. The sample size ranged from 38 to 659 participants (patients and controls).

Table 2: Associations between GSTM1 null genotype and systemic lupus erythematosus risk

Authors	Year	Ethnicity	Cases		Controls		OR	95 % CI	P-value
			Present	Null	Present	Null			
Ollier et al[28]	1996	Caucasian	35	55	253	316	1.25	0.79-1.98	0.322
Tew et al (1)[31]	2001	African	84	21	43	13	0.82	0.37-1.81	0.634
Tew et al (2)[31]	2001	Caucasian	37	39	47	31	1.59	0.84-3.02	0.150
Tew et al (3)[31]	2001	Hispanic	40	31	25	17	1.14	0.52-2.47	0.741
Fraser et al (1)[27]	2003	Caucasian	48	37	110	92	0.92	0.55-1.53	0.754
Fraser et al (2)[27]	2003	African	111	33	54	18	0.89	0.46-1.72	0.734
Fraser et al (3)[27]	2003	Mixed	9	5	15	9	0.92	0.23-3.64	0.912
Kang et al[26]	2005	Asian	144	186	129	141	1.18	0.85-1.63	0.311
Horiuchi et al[32]	2009	Asian	75	77	231	196	1.21	0.83-1.75	0.313
Zhang et al[25]	2010	Asian	108	190	138	146	1.66	1.19-2.31	0.003
Kiyohara et al[29]	2012	Asian	75	76	227	194	1.18	0.81-1.72	0.370
Rupasaree et al[24]	2013	Caucasoid	143	51	289	154	0.66	0.46-0.97	0.036
Glesse et al (1)[23]	2014	Caucasian	149	133	112	129	0.77	0.54-1.09	0.147
Glesse et al (2)[23]	2014	African	54	33	63	25	1.54	0.81-2.90	0.182
Salimi et al[22]	2015	Caucasian	76	87	100	79	1.44	0.94-2.21	0.088
Nasr et al[30]	2017	African	18	22	23	17	1.65	0.68-4.00	0.265
Jevtovic-Stoimenov et al[21]	2017	Caucasian	34	54	37	51	1.15	0.63-2.10	0.645
de Oliveora et al[20]	2021	Mixed	98	15	101	18	0.85	0.41-1.79	0.687

OR: odds ratio; CI: confidence interval;

For the *GSTM1* polymorphism, a total of 13 articles (including 18 case-control studies) with 2483 cases and 3643 controls met the inclusion criteria (Table 2). The results showed that the *GSTM1* null genotype significantly increased the risk of SLE (OR = 1.12, 95 % CI: 1.004-1.25, p = 0.042) with no evidence of significant heterogeneity (Q = 24.19, df = 17, p = 0.114; I^2 = 29.7 %).

The raw data presented in three reports by Ollier,[32] Rupasree[24] and Salimi[22] have internal inconsistencies. Therefore, these studies were excluded from the meta-analysis. Further analysis showed that the level of heterogeneity decreased and there was no evidence of heterogeneity between studies (Q = 14.53, df = 14, p = 0.411; I^2 = 3.4 %). The association between the null genotype of *GSTM1* and the risk of SLE increased

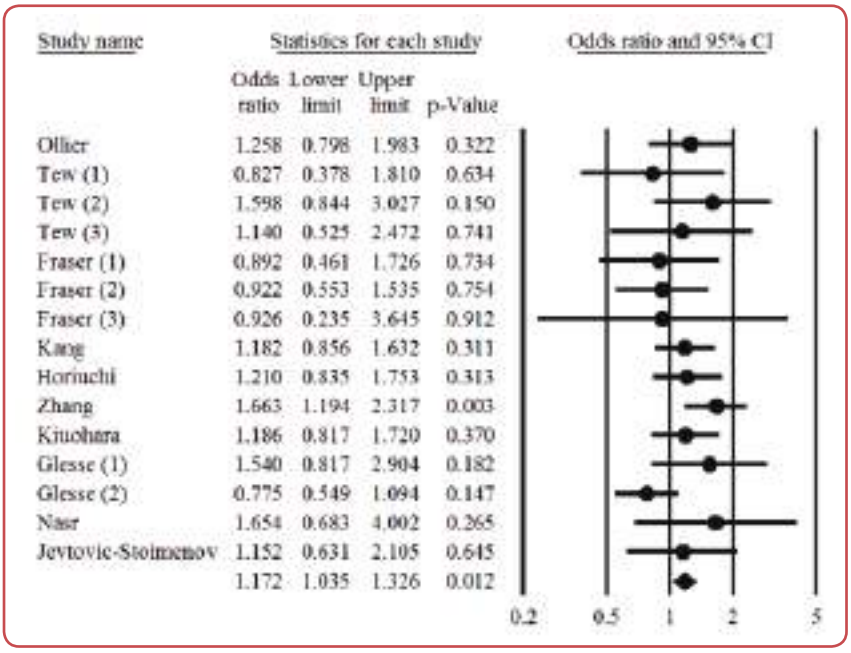

Figure 2: Forest plot of the association between GSTM1 null genotype and systemic lupus erythematosus risk

Table 3: Associations between GSTT1 null genotype and systemic lupus erythematosus risk

Authors	Year	Ethnicity	Cases		Controls		OR	95 % CI	P-value
			Present	Null	Present	Null			
Ollier et al[28]	1996	Caucasian	71	18	368	86	1.08	0.61-1.91	0.779
Tew et al (1)[31]	2001	African	78	27	38	18	0.73	0.35-1.48	0.387
Tew et al (2)[31]	2001	Caucasian	62	14	61	17	0.81	0.36-1.78	0.602
Tew et al (3)[31]	2001	Hispanic	61	10	35	7	0.82	0.28-2.34	0.711
Fraser et al (1)[27]	2003	Caucasian	73	12	181	21	1.41	0.66-3.02	0.369
Fraser et al (2)[27]	2003	African	114	30	59	13	1.17	0.57-2.42	0.669
Fraser et al (3)[27]	2003	Mixed	12	2	22	1	3.83	0.31-46.6	0.292
Kang et al[26]	2005	Asian	160	170	121	149	0.86	0.62-1.19	0.370
Horiuchi et al[32]	2009	Asian	-	-	-	-	-	-	-
Zhang et al[25]	2010	Asian	163	135	137	147	0.77	0.55-1.06	0.119
Kiyohara et al[29]	2012	Asian	-	-	-	-	-	-	-
Rupasaree et al[24]	2013	Caucasoid	131	63	360	85	2.03	1.39-2.98	< 0.001
Glesse et al (1)[23]	2014	Caucasian	226	56	197	44	1.10	0.71-1.72	0.643
Glesse et al (2)[23]	2014	African	73	14	69	18	0.73	0.34-1.59	0.435
Salimi et al[22]	2015	Caucasian	122	41	152	27	1.89	1.10-3.25	0.021
Nasr et al[30]	2017	African	30	10	35	5	2.33	0.71-7.58	0.159
Jevtovic-Stoimenov et al[21]	2017	Caucasian	71	17	75	13	1.46	0.66-3.20	0.342
de Oliveora et al[20]	2021	Mixed	98	46	101	44	1.07	0.65-1.77	0.769

OR: odds ratio; CI: confidence interval;

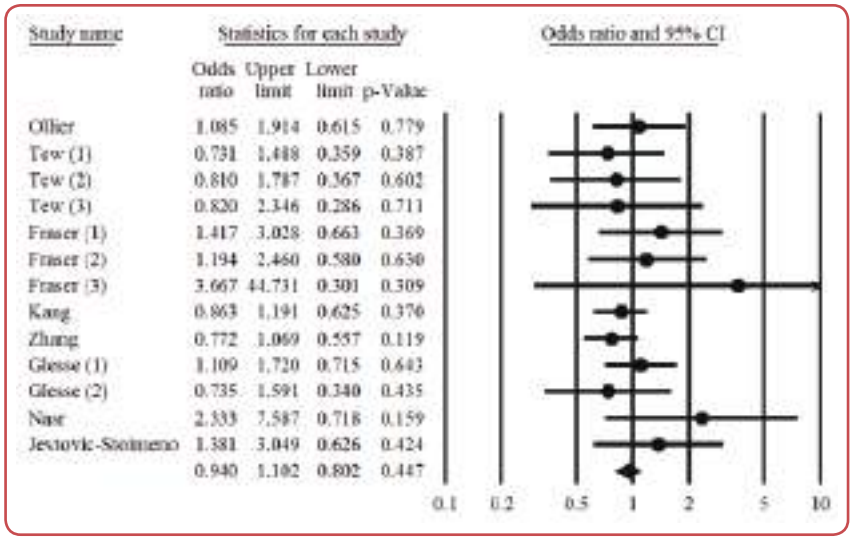

Figure 3: Forest plot of the association between GSTT1 null genotype and systemic lupus erythematosus risk

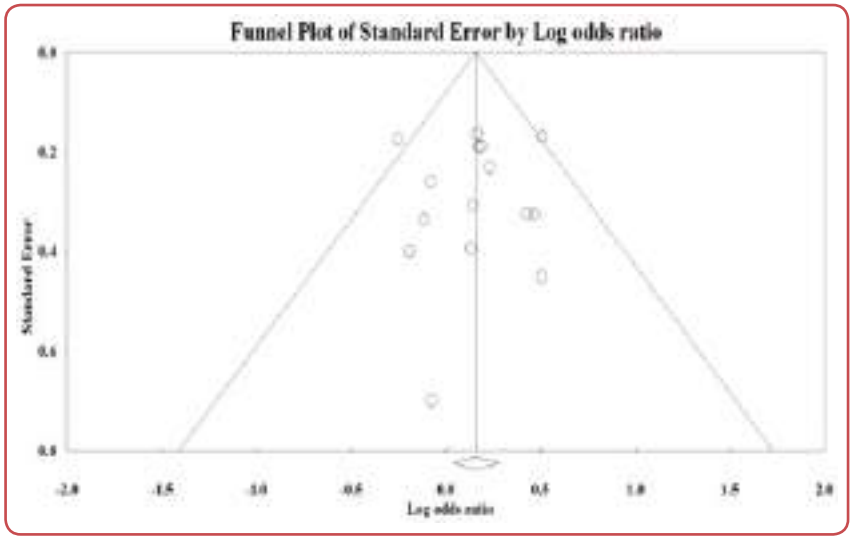

Figure 4: Funnel plot of the GSTM1 null genotype and systemic lupus erythematosus risk

slightly (OR = 1.17, 95 % CI: 1.03-1.32, p = 0.012). The forest plot for the association between the *GSTM1* null genotype and the risk of SLE is shown in Figure 2.

For the *GSTT1* polymorphism, a total of 16 case-control studies with 2211 SLE patients and 2706 controls were selected for the present analysis (Table 3). There was moderate heterogeneity among the studies (Q = 26.99, df = 15, p = 0.029; I^2 = 44.4 %). The *GSTT1* null genotype was not associated with the risk of SLE (OR = 1.12, 95 % CI: 0.92-1.37, p = 0.250).

After excluding studies due to internal inconsistency, the heterogeneity decreased remarkably (Q = 9.41, df = 12, p = 0.667; I^2 = 0.0 %), but the association was still not significant (OR = 0.94, 95 % CI: 0.80-1.10, p = 0.447). The forest plot for the association between the *GSTT1* null genotype and the risk of SLE is shown in Figure 3.

Some investigators[23, 25, 27] reported the combination genotypes in cases and controls. These reports were used to investigate the risk of SLE based on the combination of *GSTM1* and *GSTT1* genotypes. There was no association between the genotype combination and the risk of SLE (data not shown).

In meta-analyses with high heterogeneity between studies, researchers should identify the source(s) of heterogeneity. In such cases, studies are usually stratified based on some aspect (such as ethnicity, source of controls, etc) to reduce heterogeneity. In the present study, where there was no heterogeneity between studies, further analysis did not seem necessary.

Sensitivity analysis was performed to assess the influence of each study and showed that almost none of the studies significantly the results, indicating that the present findings are robust.

Finally, it should be noted that there was no evidence of publication bias for the studies used in *GSTM1* and risk of SLE (Figure 4; p-values for Begg and Egger tests were 0.656 and 0.896, respectively).

As shown in Figure 2, there was a weak association between the null genotype of *GSTM1* and the risk of SLE (OR = 1.17, 95 % CI: 1.03-1.32, p = 0.012). With a statistical power of 0.998, the FPRP value was estimated under two prior probability assumptions. With prior probabilities of 0.001 and 0.010, the FPRP value was estimated to be 0.923 and 0.545, respectively.

Discussion

There are several original articles reporting the association between *GSTT1/GSTM1* polymorphism and susceptibility to SLE, with inconsistent results.[20-32] As mentioned in the Introduction section, although there are two other meta-analyses investigating the relationship between *GSTT1/GSTM1* polymorphisms and the risk of SLE[32, 33] unfortunately, the authors of the meta-analyses did not include some of the studies published at that time, so both analyses suffer from the authors' inaccuracy in finding relevant articles. Therefore, the present study was performed. This is the first meta-analysis to comprehensively investigate the association between null genotypes of *GSTT1/GSTM1* and SLE. A weak association (OR = 1.17) was found between the *GSTM1* null genotype and susceptibility to SLE.

It is well known that SLE is a clinically heterogeneous disease and this may reflect heterogeneity in its genetic component. Therefore, the present finding of no evidence of heterogeneity between studies is unexpected. Most likely, the low strength of the association is a reason for the observed homogeneity between studies.

Some limitations of the present meta-analysis should be acknowledged. First, the uneven geographical distribution of the original articles used in the study is a very important limitation. There was only one report from Eastern Europe and one report from Northern Europe, but no report from Western and Southern Europe and Australia. Second, a high proportion of the studies used in the meta-analysis did not report the source of the control groups.[25, 26, 28, 31] Third, the false positive report probability (FPRP) value of the association between the null genotype of the *GSTM1* and the risk of SLE under two assumptions for prior probabilities of 0.001 and 0.010 was 0.923 and 0.545, respectively. These values are much higher than the FPRP cut-off value of 0.20,[39] indicating that the association was not noteworthy (true positive). Further well-designed large studies are needed to investigate the relationship between gene polymorphisms and risk of SLE.

Conclusion

In conclusion the current meta-analysis suggests that the null genotype of the *GSTM1* (but not *GSTT1*) polymorphism is associated with increased susceptibility to SLE. It should be noted that the FPRP value for this association is much higher than the previously proposed FPRP cut-off value of 0.2. Further case-control studies with larger sample sizes are needed to confirm the present findings.

Ethics

This study was a secondary analysis based on the currently existing multiple databases, including PubMed, Europe PMC, Web of Science, Scopus, Directory open access journals (DOAJ), ProQuest, African journals online (AJOL) and Islamic science citation (ISC) and did not directly involve with human participants or experimental animals. Therefore, the ethics approval was not required in this paper.

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None.

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Conflicts of interest

The author declares that there is no conflict of interest.

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Data access

All data were recorded in Tables 1 and 2.

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Author contributions

Contributed to the conceptualisation, methodology, validation, formal analysis, data curation, writing - original draft, writing - review and editing: MS

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HISTORY OF MEDICINE

Ioannis Chrysospathis, Theodoros Garofalidis and Georgios Hartofilakidis: The Pioneers of Orthopaedics in Greece

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Abstract

The aim of the present paper was to highlight the work of Chrysospathis, Garofalidis and Chartofylakidis, who dedicated themselves to the emergence of orthopaedics as an independent branch of medical science in modern Greece. The ancient Greek physicians Hippocrates, Galen and Paulus of Aegina laid the foundations for the emergence of orthopaedics and ancient Greek civilisation undoubtedly played a significant role in the evolution of this medical art throughout the following centuries. In modern Greece, Ioannis Chrysospathis fought for orthopaedics to be a separate medical branch rather than a practice within the context of general surgery. Theodoros Garofalidis also contributed to the evolution of orthopaedics in Greece, while Georgios Hartofilakidis brought pioneer knowledge to the Greek area, contributed to the creation of specialised orthopaedic departments and internationally contributed to the study of hip diseases and the art of total hip arthroplasty.

Key words: Orthopaedist; Surgery; Orthopaedic Department; University of Athens; History of Medicine.

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The evolution of orthopaedics in Greece

Greek mythology provides the first accounts of the origins of medical science in ancient Greece. According to the mythology, Apollo, the offspring of Zeus and Leto, was the god of science and medicine. He taught humans about a plethora of medicines made from different herbs, in addition to various therapies, such as thermal or herbal baths. He was also believed to have demonstrated the method of making a diagnosis based on observation and a focus on symptoms. Cheiron, a centaur, was taught the art of medicine by Apollo and was said to have been the first to perform the practice of surgery on wounds and fractures. He later passed on the knowledge he gained to Asclepius.[2]

Asclepius, the offspring of Apollo and Coronis, was recognised as a great healer with extraordinary therapeutical skills and he was worshipped as a demi-god at the Asklepieia, healing temples located throughout Greece (ca 5th-4th century BC). Asclepiads, the healer priests of the Asklepieia, had a profound knowledge of the earth and the properties of herbs and poisons and they used this knowledge in accordance with their religious perception in surgical procedures. Inscriptions

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Figure 1: The "Crooked tree" by Nicolas Andry de Bois-Regard
Source: Private collection.

about wound care, reduction of dislocations and immobilisation of fractures have been found in various Asklepieia, demonstrating the development of surgical as well as orthopaedic practice in ancient Greece.[2]

It should also be highlighted that bone fractures, reconstruction and dislocation were well-known medical subjects among physicians even in Homer's era (8th century BC). In the epic poem, "The Iliad", Homer included more than one hundred battle injuries, including bone and joint trauma. All this knowledge was collected and categorised (c 460-370 BC) inside *Corpus Hippocraticum*, which contains various chapters on orthopaedics, such as "About fractures and dislocations" [in Greek: *Περὶ ἀγμῶν* (*Peri Agmon*)], "On joints" [in Greek: *Περὶ ἄρθρων* (*Peri Arthron*)] and "On the bone restoration" (in Greek: *Μοχλικός* (*Mochlikos*)].[1, 2]

Galen (AD 129-200) from Pergamon, Asia Minor is considered the successor of Hippocrates. He studied in Alexandria and his work on kyphosis, lordosis, scoliosis, vertebral tuberculosis and spinal injuries was inspired by the Hippocratic account. His service as a gladiatorial surgeon during the Roman period allowed him to study the anatomy of the human body by examining the gladiators' wounds; however, his anatomical and physiological theories were mainly based on animal dissection because dissection of human bodies was forbidden. Unavoidably, he made errors in his attempts to extrapolate his conclusions from animal experiments to humans. Nevertheless, he had an indisputable impact on the study of spinal anatomy: he specified twenty-nine pairs of spinal nerves, he described the cervical and brachial plexus and he knew a lot of details about neural anastomoses (*epimixies*).[2, 3]

In the Byzantine times, Paulus of Aegina (ca AD 625-690) was a distinguished surgeon who made great contributions to the orthopaedic art. He wrote the *Epitomae Medicae Libri Septem* (Medical Compendium in Seven Books), a guidebook for surgery and medicine that was used until the 17th century. In his work, he extended the accumulated knowledge about the locomotor system. Furthermore, he identified bone fractures in full detail, giving emphasis to congenital bone dislocations and he recommended the use of antiseptics, painkillers and anti-inflammatories.[1-3]

During the Ottoman occupancy (c 1453-19th century), the Hellenic medical tradition was limited. This tradition was revived with the establishment of the Greek state (1830), particularly when the Practical School of Medicine and Surgery was founded (1835) and the Othonian University was established (1837). The University was later known as the National and Kapodistrian University of Athens (1932). In 1856, the Athens University Clinic, Astykliniki, was established to provide clinical training for students and medical care for paupers. In Astykliniki, people with orthopaedic problems, such as gangrene, periostitis, rickets, bone fractures and joint diseases, were treated in the context of general surgery.[2]

Notably, until the end of the 19th century, orthopaedic surgery was not defined as a separate medical field in Greece. However, owing to continuing study and practice by certain general surgeons who treated people with orthopaedic problems, orthopaedics began to develop as a distinct specialty in medicine. In 1917, an orthopaedic de-

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partment was instituted in Astykliniki, with Ioannis Chrysospathis and Athanasios Contargyris appointed as directors.[2]

Ioannis Chrysospathis (1873-1938)

Figure 2: Ioannis G Chrysospathis
Source: National and Kapodistrian University of Athens.

Ioannis G Chrysospathis (1873-1938) was born in Kalamata, Greece (Figure 2). His enthusiasm to pursue a more sophisticated education than was available to him in Greece led him to study medicine in Leipzig, Germany and he ultimately obtained his doctorate *cum laude superato* in Freiburg in 1895. In 1899, he served as an assistant in the First Surgical Department of the New General Hospital of Eppendorf, in Hamburg, under Professor Hermann Kümmell's supervision. Chrysospathis specialised in orthopaedics, after having been trained in German, Austrian and French hospitals (1895-1900). He apprenticed under the German surgeon and orthopaedist Albert Hoffa (1859-1907) in his private clinic in Würzburg and he also attended the in-depth lectures of the Austrian surgeon Lorenz Böhler (1885-1973) in Vienna.[1,2,4-6]

Chrysospathis subsequently made major contributions to the art of orthopaedics in Greece. In 1901, he returned to his native country to establish the first orthopaedic practice in Athens. During this time period, numerous pioneering physicians in Greece were practicing orthopaedics and various institutions began to include it as well. Chrysospathis's initiatives in the country's first orthopaedic clinic included a laboratory in which customised orthopaedic devices were built under his own instruction for people with scoliosis and other deformities and an X-ray diagnostic laboratory that provided radiation therapy. He was the first Greek surgeon to carry out orthopaedic operations, such as transferring healthy tendons to paralysed tendons, reducing the congenital dislocation of the hip joint and correcting orthopaedic distortions.[1,2,4,5,7]

In 1905, during the 5th Panhellenic Medical Congress in Athens, Chrysospathis displayed the first radiographic images of various bone disorders. Three years later, in 1908, the orthopaedic discipline was established in the Medical School of Athens. Chrysospathis presented his thesis on ankylosing spondylitis and he was appointed Associate Professor of Orthopaedics and Chairman of the Orthopaedic Department at Astykliniki. He continuously worked to promote this newly formed branch of medicine and in 1925 he was elected the first Professor of Orthopaedics and Head Physician of the Hippocrateion Hospital (1926-1938).[1,2,4,6,8]

Chrysospathis was married to Marika Patsidi and they had a daughter, Anna, who married an Irish physician, Professor Charis Toole (1897-1980), who was later a distinguished surgeon. Chrysospathis was fluent in three languages, Greek, German and French. Among his writings, his treatise titled "Orthopaedics" (1932) stands out—it was the first orthopaedic textbook in Greek for the students of the School of Medicine. He also wrote many manuscripts, such as his thesis "Chronic ankylosing spondylitis" (1907), "Bone tuberculosis of the vertebral spine" (1910) and "Kyrtopodie" (1936). Apart from the art of orthopaedics, Chrysospathis was interested in the history of medicine. For this reason, he published "Orthotics from antiquity until nowadays" (1909), "Hippocratic orthopaedics" (1910) and "The evolution of orthopaedics during the last years" (1917). It is also worth mentioning that around 1901, he took the initiative to publish an advertising text within the public press to further promote orthopaedics. The text implies that he was an expert in

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topics such as deformities of the vertebral spine and those caused by chronic arthritis, deformities due to rickets, congenital lameness, fractures of the extremities, lumbar radiculopathy, rheumatism and paralysis caused by central nervous system impairment.[1-4]

Ioannis Chrysospathis died in 1938 and the full extent of his legacy is still buried within the archives of the Athenian University. He fought for separating orthopaedics from general surgery, but he did not live long enough to see orthopaedics recognised as a medical specialty, which only occurred in 1947.[1, 9]

Theodoros Garofalidis (1898-1978)

Figure 3: Theodoros Garofalidis
Source: National and Kapodistrian University of Athens.

Theodoros Garofalidis (1898-1978) was born in Athens where he studied medicine, but his studies were interrupted for the five-year period when he served in the Greco-Turkish War (1919-1922) (Figure 3). In 1924, he continued his education at Asklepieion Voulas, the first sanatorium founded by the Hellenic Red Cross in Athens for children with bone tuberculosis and rickets. Recognising his contribution to the sanatorium's work, the Hellenic Red Cross designated Garofalidis as an internal director. During his training at Asklepieion Voulas, he performed spinal fusion surgery for the rehabilitation of tuberculous spondylitis. He also studied osteomyelitis, poliomyelitis, arthritis, scoliosis and congenital hip dislocation.[2, 8, 10, 11] In 1926, Garofalidis was awarded a doctorate. From 1926 to 1929, he worked at Aretaieion Hospital and then continued his studies in Paris, with a scholarship from the French Government, working next to the paediatric and plastic surgeon Louis Ombrédanne (1871-1956). He returned to Aretaieion Hospital in 1931 and five years later, in 1936, he was appointed Assistant Professor of Orthopaedics at the University of Athens. In 1938, he was appointed Chairman of the Second Orthopaedic Department of Asklepieion Voulas Hospital. In 1944, toward the end of World War II, a Department of Paediatric Surgery and Orthopaedics was established in "Evangelismos General Hospital" in Athens, with Athanasios Contargyris (1892-1954) as the chair. In 1947, this department was renamed as the Department of Orthopaedic Surgery of Athens University Medical School, where Contargyris was appointed as the second Professor of Orthopaedics in Greece. Four years later, the department was relocated to Laikon King's Paul Hospital and in 1954, after Contargyris' death, Garofalidis was elected Professor of Orthopaedics at the University of Athens as well as the chairman of the Orthopaedic Department, from 1956 until 1967.[2, 8, 10]

Garofalidis was married to Aristotle Onassis' sister, Artemis. He was the chair of the administrative council of Olympic Airlines and a board member of the Ministry of Social Welfare. He was also the first Greek orthopaedist to become a corresponding member of the American Orthopaedic Association (1953). In addition, he was the author of more than two hundred scientific papers, including ones of great significance such as "The tuberculosis of the bones and joints" (1945) and "Modern Orthopaedics" (1964) in collaboration with Georgios Hartofilakidis and Christos Rigopoulos. In 1966, Garofalidis was elected Dean of the School of Medicine at the University of Athens. He was also president of the Hellenic Association of Orthopaedic Surgery and Traumatology (HAOST), in 1957 and in 1962, chair of the Higher School of Physiotherapy, the Hellenic Society for the Protection and Rehabilitation of Disabled Children, the Hellenic Red Cross and the Patriotic Foundation for Social Protection and Awareness. In honour of his contributions to the development

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of orthopaedics in Greece, the Orthopaedic Department of University of Athens in the "Apostolos Pavlos" Accident Hospital today known as KAT Hospital named its research centre "Th. Garofalidis". He died on 19 August 1978, in Athens.[10, 11]

Georgios Hartofilakidis (1927-2022)

Figure 4: Georgios Hartofylakidis
Source: Private collection.

Georgios Hartofilakidis (1927-2022) was born in Athens and he graduated from the School of Medicine (University of Athens) in 1955 (Figure 4). He undertook postgraduate studies at the Orthopaedic Department at the Laikon King's Paul Hospital, supervised by Garofalidis and later at the Columbia University Medical Centre in New York. In 1960, he was awarded a doctorate in medicine.[10, 11] By the end of 1947, twenty-two pioneering orthopaedists—including the first Greek woman orthopaedic surgeon, Marika Daniilidou—had already founded the HAOST in Greece. The first president of the association was Richardos Livathinopoulos (1868-1954) while Athanasios Contargyris was responsible for editing the first issue of the "Bulletin of the HAOST" (later *Acta Orthopaedica et Traumatologica Hellenica*), which was published in 1948. Years later in 1969, when Hartofilakidis served as a President of HAOST arranged its first scientific meeting in Thessaloniki.[2]

On his own recommendation, which was accepted by the School of Medicine (1969), the Orthopaedic Department of University of Athens was transferred from the Laikon Hospital to the KAT Hospital in 1970. In 1969, Hartofilakidis was elected Professor of Orthopaedics and Chairman of the Orthopaedic Department, a position he held for twenty-five consecutive years while undertaking important, pioneering and enviable work, until his retirement, in 1994. He organised the Department according to international standards and promoted cooperation with specialised departments all over the world in various fields of orthopaedics, bringing pioneer knowledge to the Greek area. In the academic year 1974-1975, he was elected Dean of the School of Medicine at the University of Athens and in 1976-1977, member of the Senate. In 1978, he founded the Laboratory for Research of the Musculoskeletal System "Theodoros Garofalidis" (LRMS) while he contributed to the creation of specialised departments of KAT Hospital such as the Hand, Upper Limb and Microsurgery Department, the Department of Sports Injuries and the Scoliosis and Spine Department. In 1994, he was also awarded the title of Emeritus Professor of Orthopaedics.[2, 10, 11]

Hartofilakidis was the first President of the Hellenic College of Orthopaedic Surgeons (1983-1988) and President of the Higher School of Physiotherapy (1969-1982), as well as Vice President of the Hellenic Society for the Protection and Rehabilitation of Disabled Children in Greece. He was also Vice President of the Education and Post-Education Committee of the Central Board of Health. Furthermore, he held high administrative positions in many hospitals and institutions, such as the KAT Hospital, offering important scientific and administrative work. At the same time, he was a member of various committees on health education issues and of many scientific associations and societies, including the HAOST, the Hellenic Surgical Society, the American Orthopaedic Association, the British Orthopaedic Association and the American College of Surgeons. In addition, he was an active volunteer and scientific director of the Polyclinic of the Olympic Village, thus contributing to the field of Health of Athletes and Volunteers during the 2004 Olympic Games in Athens.[10, 11]

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Hartofilakidis devoted his career to establishing and systematising the teaching of the art of orthopaedics to the next generation of orthopaedists. He offered pioneering research work focusing on osteoarthritis of the hip, congenital hip disease and total hip arthroplasty. His authorship includes scientific papers and books both in Greek and English, with many being published after his retirement from the University (within the period from 1994 to 2022). These papers demonstrate his international contribution to the study of hip diseases. He contributed to the establishment of terminology, classification, description of the natural history and surgical treatment of congenital hip disease. All data derived from his invaluable registry established in the early 1970s. This registry includes approximately 1000 total hip arthroplasties, except from other interesting cases, performed by him from 1973 to 1994 and then followed continuously by him during lifetime. It should also be mentioned that the "Hartofilakidis classification of congenital hip disease in adults" is currently used in top-tier orthopaedic centres worldwide.[12]

He has been recognised as the father of modern orthopaedics in Greece.[2] In his honour, among others, the auditorium of KAT Hospital was named "Professor's G Hartofilakidis auditorium" in 2005, an honorary event for his 90 years was held in the Great Hall of the National and Kapodistrian University of Athens in 2017 and Professorship Hall of LRMS was named "Professor's G Hartofilakidis Hall" in 2020. In 2019, he was honoured with the EFORT Recognition Award by the EFORT which recognises his outstanding contribution to worldwide orthopaedic surgery as a lifetime achievement.[13]

Hartofilakidis was married to Anna Gorga, with whom he had two children, Maria and Konstantinos. He passed away in 2022. His excellent ability to deal with difficulties and avoid conflicts will always be remembered as a catalyst for the re-development of orthopaedics in Greece.[11, 13]

What is already known on this topic: the contribution of ancient Greek physicians, such as Hippocrates, to the foundation of practical surgery and orthopaedic art is already known through the study of written and non-written testimonies.

What this study adds: the present study delves into the contribution of innovative doctors such as Chrysospathis, Garofalidis and Chartofylakidis, who dedicated themselves to the emergence of orthopaedics as an independent branch of medical science in Modern Greece.

Conclusion

The field of orthopaedics in Greece gradually evolved over centuries. With the establishment of the Greek State in 1830, several general surgeons focused on diseases of the musculoskeletal system. The innovative achievements of the pioneering doctors profiled in this paper contributed significantly to the development and establishment of orthopaedics as an independent branch of medicine.

Ethics

Our institution does not require ethics approval for articles reporting the history of medicine.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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CONTENT

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PROFESSIONAL ARTICLE

Evaluation of Quality of Life for Women With Breast Cancer

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Abstract

Background/Aim: One of the therapeutic outcome metrics for women with breast cancer is quality of life (QoL). The purpose of the following study was to evaluate the QoL of women with breast cancer who were getting therapy or on follow-up at the Oncology Teaching Hospital in Medical City, Baghdad, Iraq.

Methods: A convenient sample of 100 women was selected from the Teaching Oncology Hospital at Medical City in Baghdad City. For the purposes of the study, a questionnaire was constructed. Physical, role, emotional, cognitive and social functioning were its five functional domains. Each study participant received a questionnaire explaining how to complete it, along with a form requesting their approval to remain anonymous. They also got the opportunity to leave the study at any time without being provided an explanation. A panel of 10 experts determined the questionnaire's content validity and its internal consistency reliability was calculated using the split-half method and the Cronbach α correlation coefficient of r = 0.92. Data were collected using the study questionnaire and analysed using a descriptive statistical data analysis approach based on frequency and percent.

Results: The study findings depict that most of the women with breast cancer were 31-49 year old and they accounted for 43 % of the studied sample. Most of these women had problems with their emotional, social and role functioning.

Conclusion: Breast cancer affects many elements of life, but it has the largest influence in modern culture on social and emotional functioning, as well as role functioning. A multidisciplinary team working with affected women would be advantageous in enhancing the QoL of breast cancer patients, particularly in the most compromised aspects of their health.

Key words: Evaluation; Quality of life; Women; Breast cancer.

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Introduction

Breast cancer is contributing for 12.5 % of all newly diagnosed cases of cancer every year. In many areas of the globe, breast cancer is the most common cancer in women and one of the major causes of cancer-related mortality.[1] A woman's risk nearly doubles if she has a first-degree family with breast cancer (mother, sister or daughter). In 15 % of affected women a family member had a breast cancer.[2] Approximately 85 % of all incidences of breast cancer are caused by women who have no family history of the disease. They are produced by genetic alterations resulting

from aging and life in general, rather than inherited mutations.[1]

One of the therapeutic outcome metrics for women with breast cancer is quality of life (QoL). QoL is influenced by a variety of factors in breast cancer patients. These variables include socioeconomic position, level of education, job status, mental issues and financial considerations.[2] The impact of the diagnosis, the impact of disease management and the development of the condition on everyday activities and recovery are all factors in the complicated and multifaceted assessment known as QoL. QoL is currently considered as a measure of the effectiveness of cancer care and treatment. QoL when used to assess psychological, physical and social health, gives insight into everyday life for patients receiving breast cancer therapy.[3]

Financial troubles that may have an impact on savings and property may be experienced by breast cancer patients. Income loss, increased health-care costs and reductions in paid and unpaid job are all major causes of financial burden for breast cancer patients, who could find it difficult to pay for needs like food and clothes.[4] Good health is one of, if not the most significant element for excellent QoL.[5, 6]

Physical symptoms and psychological distress have a detrimental impact on breast cancer patients' QoL. Some of the components that comprise QoL in general include physical functioning, mental well-being (such as anxiety and depression symptoms) and social support.[7]

The purpose of the following study was to evaluate the QoL of women with breast cancer who were getting therapy or were on follow-up at the Oncology Teaching Hospital in Medical City, Baghdad, Iraq.

Methods

The present study used the descriptive design with application of evaluation approach. A convenient sample of 100 women, with breast cancer was selected from the Teaching Oncology Hospital at Medical City in Baghdad City, Iraq. Study was carried out from January 2023 to June 2023.

For the purposes of the study, a questionnaire was constructed. Physical, role, emotional, cognitive and social functioning were its five functional domains. These domains were evaluated as low, moderate and high. Inclusion criteria included women with breast cancer, aged 18 years and older. Exclusion criteria included women aged 75 years and older, because these ages do not have stability in physical, role, emotional, cognitive and social functioning.

Each study participant received a questionnaire explaining how to complete it, along with a form requesting their approval to remain anonymous. They also got the opportunity to leave the study at any time without being provided an explanation. A panel of 10 experts determined the questionnaire's content validity and its internal consistency reliability was calculated by using the split-half method and the Cronbach α correlation coefficient of r = 0.92. Data were collected using the study questionnaire and analysed using a descriptive statistical data analysis approach based on frequency and percent.

Results

Out of 100 women, most of the women with breast cancer were 31-49 year old and they accounted for 43 % of studied sample, followed by 32 % of women aged 50-59 (Table 1).

Table 1: Distribution of the sample by age

Age (years)	Women with breast cancer	
	N	%
≤ 30	4	4
31 – 49	43	43
50 – 59	32	32
60 – 69	13	13
≥ 70	8	8
Total	**100**	**100**

N: number of patients; %: percentage;

Table 2: Evaluation of quality of life (QoL) in women with breast cancer

Domains	Low	Moderate	High
Physical functioning	20 (20 %)	30 (30 %)	50 (50 %)
Role functioning	56 (56 %)	24 (20 %)	20 (20 %)
Emotional functioning	90 (90 %)	10 (10 %)	0 (0 %)
Cognitive functioning	10 (10 %)	30 (30 %)	60 (60 %)
Social functioning	60 (60 %)	20 (20 %)	20 (20 %)

Most of the women with breast cancer had problems with their emotional, social and role functioning (Table 2).

Discussion

Throughout the course of data analysis, the study findings depict that women with breast cancer were mostly early to middle age. This may be due to genetic risk factors or may be due to infertility and the use of contraceptives. Breast cancer has a one in 69 probability of occurring in women between the age 40-60. That risk rises to one in 43 in age 50-60 and in 60-70 age group, the possibility is one in 29. Additionally, in women aged 70 and older, there is a one in 26 is at risk of contracting the disease.[8]

A cross-sectional research of 96 female breast cancer patients was carried out to examine the QoL of breast cancer patients who were sent to the Surgical Department of King Salman Armed Forces Hospital in Saudi Arabia for therapy and follow-up. According to the study, one-third of the patients (31.3 %) were over 48 years of age and 29.2 % were in the aged 18-27 years.[4] Another cross-sectional research was performed on 100 breast cancer patients, the study depicts that the mean age was 60 year for these women.[9]

The study's results in terms of their QoL confirm that the domains of emotional, social and role functioning of such quality were affected due to emerging breast cancer. Such effect is well-noticed in the low levels of these domains. A comprehensive systematic review was conducted and meta-analysis has examined a total of 9012 patients with breast cancer. The results revealed that the domain of social functioning of the QoL is found to be highly influenced by breast cancer. Those who had finished the therapy scored better on QoL than those who were still undergoing treatment.[10]

A case-control study, on a total of 356 breast cancer survivors was conducted and breast cancer survivors reported poor QoL in the domain of cognitive performance.[11] A cross-sectional study of 140 patients with breast cancer was performed and the findings showed that breast cancer has a massive influence on the physical and role functioning components of these women's QoL.[12] Another cross-sectional research of 96 women with breast cancer revealed that cancer stage is significantly influenced the physical and social functioning.[4]

A cross-sectional study was carried out on 100 woman with breast cancer. The study depict that breast cancer has been identified as having negative impact upon the domains of QoL.[9] Another cross-sectional study was steered on 112 woman with breast cancer. The findings show that the domains of physical and role functioning of these women's QoL are greatly affected by breast cancer.[13] Another cross-sectional study was done in Saudi Arabia to measure the QoL in breast cancer patients. The study findings present that patients scored higher on the emotional functioning as the domain of such quality.[14]

Conclusion

Breast cancer affects many elements of life, but it has the largest influence in modern culture on social and emotional functioning, as well as role functioning. A multidisciplinary team working with affected women would be advantageous in enhancing the QoL of breast cancer patients, particularly in the most compromised aspects of their health.

Ethics

Written permission has not been sought from the local ethics committee, since it was a non-invasive and non-interventional study. This publication contains no data that could reveal the identity of the participating patients.

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Conflicts of interest

The authors declare no conflict of interest.

CONTENT

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Data access

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CASE REPORT

Metastatic Cryptococcosis as a Manifestation of Immune Reconstitution Inflammatory Syndrome in a Patient With COVID-19 Infection

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Abstract

Disseminated cryptococcosis is an infrequent fungal illness primarily observed in immunocompromised individuals, particularly among those with human immunodeficiency virus (HIV). In this report, a case where the initiation of antiretroviral therapy revealed a previously hidden *Cryptococcus* infection in an HIV-positive male who also had COVID-19 is presented. A 30-year-old male with a medical history of HIV sought medical attention at the Emergency Department due to the presence of a widespread, non-itchy skin rash along with severe difficulty breathing. Diagnosis of unmasking immune reconstitution inflammatory syndrome (IRIS) associated with disseminated cryptococcosis, all while testing positive for COVID-19 was made based on clinical presentation and performed analyses. COVID-19 management guidelines were strictly adhered to and treatment included the administration of steroids, amphotericin B and fluconazole. Additionally, empirical coverage for *Pneumocystis carinii* pneumonia (PCP) was initiated. Regrettably, the patient's clinical condition deteriorated in the following days, ultimately resulting in his passing. The ongoing pandemic has understandably prioritised the diagnosis of COVID-19 by healthcare providers, sometimes overshadowing the exploration of alternative diagnoses. It is crucial to maintain a heightened clinical suspicion for opportunistic infections, especially among immunocompromised individuals, particularly those with HIV.

Key words: COVID-19; HIV; Cryptococcosis.

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Introduction

Disseminated cryptococcosis is an infrequent fungal illness primarily observed in immunocompromised individuals, particularly among those with human immunodeficiency virus (HIV). In this report, a case where the initiation of antiretroviral therapy revealed a previously hidden *Cryptococcus* infection in an HIV-positive male who also had COVID-19 is presented.

Case history

A 30-year-old African male, with a prior medical history of HIV, presented to the Emergency Department at Nelson Mandela Academic Hospital in South Africa. He displayed a widespread, non-pruritic skin rash and severe shortness of breath. His HIV diagnosis was established two weeks prior by his general practitioner (GP), who had initiated him on first-line antiretroviral ther-

apy using a fixed-dose combination. In South Africa, GPs have the authority to commence antiretroviral therapy. His baseline CD_4^+ cell count was measured at 2 cells/mm^3.

Upon examination, the patient was tachypnoeic and dehydrated, with an elevated heart rate and his oxygen saturation level was 84 % while breathing room air. He presented with oral thrush and a diffuse, nodular skin rash that covered his entire body (Figure 1). His Glasgow coma scale (GCS) score was 15/15 and there were no signs of neck stiffness or neurological deficits. Chest auscultation revealed clear lung fields and normal heart sounds.

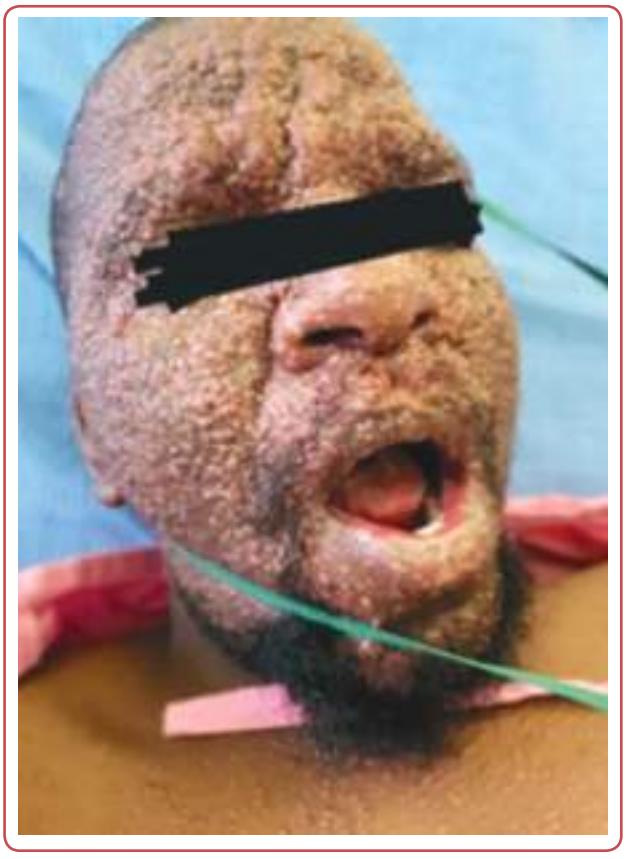

Figure 1: Oral thrush and a diffuse, nodular skin rash

Relevant diagnostic investigations were performed. Chest X-ray was within normal limits. Computed tomography (CT) and CT pulmonary angiogram (CTPA) scans showed no radiological indications of pneumonia or pulmonary embolism. Skin biopsy revealed evidence of cutaneous cryptococcosis. Lumbar puncture confirmed concomitant cryptococcal meningitis. Results of arterial blood gas analysis, serum venous sample, cerebrospinal fluid analysis and nasopharyngeal swab is shown in Table 1.

Table 1: Results of performed analyses

Analysis	Result
Arterial blood gas analysis	
pH	7.48
pCO_2	30 mmHg
O_2 saturation	87 %
pO_2	51 mmHg
HCO_3	24 mEq/L
Serum venous sample	
ALT	61 U/L
AST	66 U/L
ALP	90 U/L
GGT	361 U/L
Total bilirubin	23 µmol/L
Creatinine	206 µmol/L
Urea	17.7 mmol/L
Na^+	1387 mmol/L
K^+	5.17 mmol/L
WBC	4.35 cells × 10^9/L
Hb	14.7 g/dL
Platelets	179 cells × 10^9/L
D-dimer	1.09 mg/L
Cryptococcal Ag	+
HBV profile	-
HCV profile	-
VDRL	-
Cerebrospinal fluid analysis	
Glucose	4.2 mmol/L
Protein	0.25 g/L
ADA	0.6 U/L
Polymorphs	0
Erythrocytes	0
Lymphocytes	0
Gram stain	Moderate yeasts (+2)
RPR	-
Cryptococcal Ag	+
Culture	*Cryptococcus Neoformans*
CSF pressure	NM
Nasopharyngeal swab	
RT-PCR	+

pCO_2: partial pressure of carbon dioxide; O_2: oxygen; pO_2: partial pressure of oxygen; HCO_3: bicarbonate; ALT: alanine transaminase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; GGT: gamma glutamyl-transferase; Na^+: sodium ion; K^+: potassium ion; WBC: white blood cells; Hb: haemoglobin; Ag: Latex antigen; HBV: hepatitis B virus; HCV: hepatitis C virus; VDRL: venereal disease research laboratory (syphilis test); ADA: adenosine deaminase; RPR: rapid plasma reagin; CSF: cerebrospinal fluid pressure; RT-PCR: COVID-19 reverse transcriptase polymerase chain reaction; +: positive test; -: negative test; NM: opening pressure was not measured due to the unavailability of CSF manometer;

Diagnosis of unmasking immune reconstitution inflammatory syndrome (IRIS) associated with disseminated cryptococcosis, all while testing positive for COVID-19 was made based on clinical presentation and performed analyses. No other opportunistic infections were detected. COVID-19

management guidelines were strictly adhered to and treatment included the administration of steroids, amphotericin B and fluconazole. Additionally, empirical coverage for *Pneumocystis carinii* pneumonia (PCP) was initiated. Regrettably, despite these interventions, the patient's clinical condition deteriorated in the following days, ultimately resulting in his passing.

Discussion

IRIS is an exaggerated inflammatory response that arises when the immune system undergoes recovery. This phenomenon predominantly affects individuals with compromised immune systems, especially those with HIV and typically manifests shortly after initiating antiretroviral therapy (ART). IRIS can manifest in two distinct forms: "unmasking IRIS", which involves the re-emergence of an underlying, previously undiagnosed infection and "paradoxical IRIS" characterised by the exacerbation of a previously treated infection.[1, 2] It is important to note that IRIS can range in severity from mild to life-threatening, with an overall reported mortality rate of approximately 4.5 %.[3]

Cryptococcosis is among the opportunistic infections frequently observed in individuals with HIV and it has the potential to present as IRIS, exhibiting various clinical manifestations that can encompass cutaneous lesions and affect various organ systems. Among the manifestations, cryptococcal meningitis stands out as the most extensively documented form of the disease.

Presented patient was promptly initiated on ART by his general practitioner, even though his pre-HIV therapy screening tests were still pending. Fourteen days later, he returned with symptoms of shortness of breath (SOB) and a diffuse skin rash, which raised suspicion of a fungal infection. Unfortunately, the initial medical assessment by the hospital on-call team focused solely on the symptoms of COVID-19 pneumonia due to his respiratory distress and a positive COVID-19 polymerase chain reaction (PCR) test, however perhaps not realising the medical history and clinical signs.

During the hospitalisation, a review of his pre-screening tests revealed a positive Cryptococcal antigen latex agglutination test (CLAT). Prompt investigations were conducted, encompassing a normal chest imaging study, a skin biopsy that confirmed the presence of cryptococcosis and a lumbar puncture (LP) which definitively established the diagnosis of cryptococcal meningitis.

CT and CTPA scans conclusively ruled out the presence of concurrent pneumonia or pulmonary embolism. In the author's opinion, the apparent lack of abnormalities in the chest imaging was likely due to the significantly low CD_4^+ count, which hindered the immune response's ability to provoke and incite an inflammatory response within the lungs, rendering it undetectable on the imaging studies.

Up to the time of this case report, the literature has documented three instances of co-occurrence involving COVID-19 and cryptococcosis. One case involved cryptococcosis in a kidney transplant recipient with decompensated alcoholic liver disease and COVID-19.[4] Another case involved a patient who underwent tocilizumab treatment for a COVID-19-induced cytokine storm and subsequently developed cryptococcosis.[5] The third case described a scenario of cryptococcal meningoencephalitis in an elderly patient with SARS-COV-2 infection who was being treated with dexamethasone.[6]

Conclusion

This case underscores the challenges and complexities of dealing with multiple pathologies, particularly during the COVID-19 pandemic. It demonstrates how focusing solely on COVID-19 can sometimes overshadow other serious conditions and may diminish the significance of clinical signs pointing to other medical conditions and underlying opportunistic pathologies. A notable example is presented case of IRIS, where the presence of COVID-19 was likely incidental. In the early stages, the patient's COVID-19 status might have hindered a precise diagnosis. However, in the author's view, the patient's skin rash, medical history and a positive CLAT test should have raised suspicion of a concurrent opportunistic infection rather than COVID-19 alone.

CONTENT

Ethics

Our institution does not require ethics approval for reporting individual cases or case series. A written informed consent for anonymised patient's information to be published in this article was obtained from the patient's next of kin.

Acknowledgement

We extend our gratitude to the patient and his family, as well as to the entire medical team involved in providing care.

Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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Sarma et al. Scr Med. 2024 Jan-Feb;55(1):123-5. DOI:10.5937/scriptamed55-46001

CASE REPORT

Gangrene and Perforation of the Gallbladder as a Complication of Typhoid Fever – A Case Report

Arnabjyoti Deva Sarma,[1] Chinmoy Das,[1] Pratap Chandra Sarma[2]

Abstract

A rare consequence of typhoid illness is gall bladder gangrene with perforation. The gut and more rarely, the gall bladder are involved in surgical complications of typhoid fever. The morbidity and fatality rates are high, especially if they are not discovered and treated in time. Case report of a male adolescent patient with gall bladder gangrene with perforation is presented. Despite the difficult care, cholecystectomy and early intervention had positive outcome.

Key words: Surgery; Gallbladder; Typhoid; Gangrene; Radiology.

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Introduction

A rare complication of typhoid illness is gall bladder gangrene with perforation. Although rare and much more so in the absence of gallstones, spontaneous perforation in calculus cholecystitis can occasionally accompany typhoid fever.[1] Mortality rates are very high if such illnesses are not addressed in a timely manner. Gangrene refers to the full necrosis of one or more areas of the gallbladder wall. Acute cholecystitis can progress to gangrene of an area of the gallbladder wall, which is followed by perforation. The word "gangrene" does not indicate that the gallbladder is gangrenous completely. Here is presented a case of a male adolescent who developed gall bladder gangrene with perforation as a complication of typhoid illness.

Case history

A 14-year-old child was seen with a 10-day fever and a 1-day history of abdominal pain. Diffuse, continuing and severe pain that hampered with daily activities. The child had never vomited, had no jaundice and had not experienced burning urine in three days. Physician recommended him to hospital for additional treatment. During examination, he was alert, oriented, febrile and his tongue had an even pallor. He had a normal heartbeat and tachycardia. There was some slight abdominal distention and the entire abdomen was tender and hard. Bowel sounds were absent, there was no palpable bulk in the belly and other systems remain normal.

Complete blood count (CBC) test revealed: haemoglobin: 11.5 g/dL, platelets count: 10,530/m^3, neutrophils count: 44,000/m^3. The peripheral smear showed normal leukocytes with elevated neutrophils and normocytic, normochromic erythrocytes, reduced quantity of platelets, no signs of immature cells or parasites. Impression of neutrophilia with thrombocytopenia was seen.

Multiple fluid levels were seen on the central part of the abdomen on an erect abdomen X-ray

and there was no free air below the diaphragm, indicating a small bowel obstruction. A mildly enlarged liver with normal echo texture, a gall bladder that was suitably enlarged and had normal walls, no calculi, a small volume of free fluid present in the peritoneum and an impression of mild hepatomegaly and mild ascites had been observed on an abdomen ultrasonography. Widal test revealed high titre of: *Salmonella typhi* O titre 1:640, *S typhi* H titre 1:640; *S paratyphi* A (H) negative and *S paratyphi* B (H) had negative results, indicating a positive Widal test meaning that there was a presence of *S typhi* in the blood.

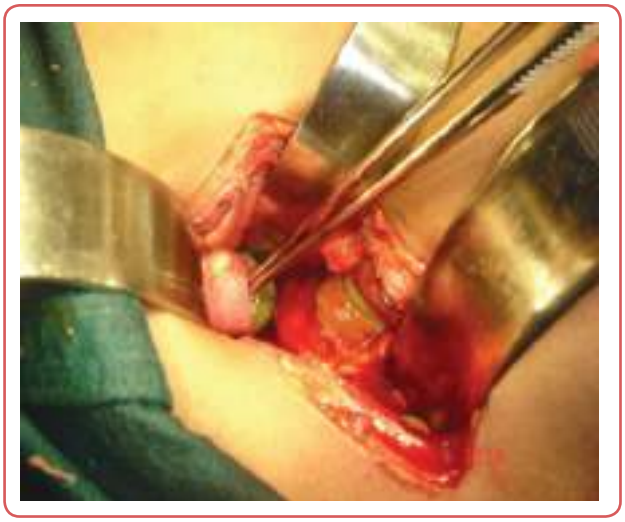

Figure 1: Gall bladder showing perforation at fundus

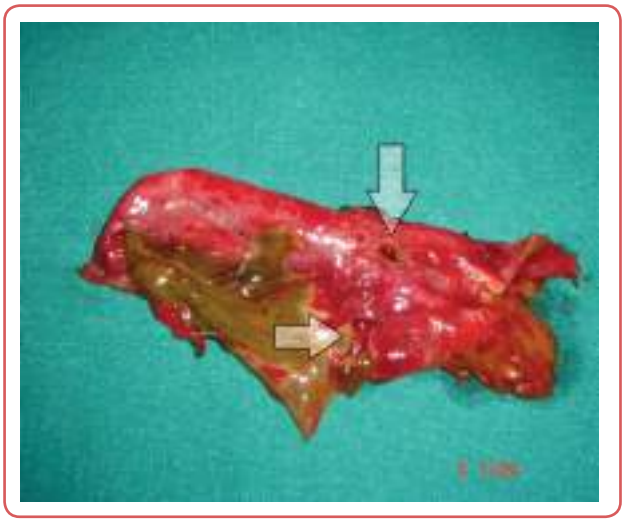

Figure 2: Gall bladder showing perforation and gangrene

Due to the high endemicity of typhoid fever and the absence of free gas under the diaphragm on the patient's X-ray, he was diagnosed with typhoid fever with paralytic ileus. Patient was treated conservatively, but did not improve.

Distention and pain in the abdomen persisted with bilious aspirate, so it was decided to perform an exploratory laparotomy on the third day of admission, which revealed a small fundus perforation (Figure 1) and a 50 % gangrenous gall bladder (Figure 2). There were large, thick pus flakes inside the peritoneal cavity. The appendix, cecum and terminal ileum were all inflamed and oedematous. Cholecystectomy was carried out and the abdomen was closed in layers and the peritoneal cavity was thoroughly cleaned with normal saline. Post-operative discomfort and distention decreased and the child recovered without any complications.

Discussion

Typhoid gall bladder perforation (GBP) is a rare surgical complication of typhoid illness and is virtually ever identified prior to surgery, even in regions where typhoid ileal perforation is widespread.[2-4] This is because generalised peritonitis is a common symptom of typhoid infection. Acalculous typhoid GBP is thought to be caused by the simultaneous existence of severe inflammation, immunosuppression and extremely virulent organisms.[5] Routine plain radiographs of the abdomen and chest to check for air under the diaphragm may have postponed intervention without significantly advancing diagnosis and care. Although Sood[6] stated that ultrasonography had a high degree of accuracy in detecting GBP, in presented case ultrasonography gallbladder imaging showed distention with normal walls and no calculi. This patient's gangrenous gallbladder, which affected 50 % of it, had a perforation at its fundus, making cholecystectomy impossible. Cholecystectomy was performed after it was discovered that the inflammatory tissues close to the gallbladder neck may be easily separated with blunt dissection. Numerous series have documented increased fatality rates in GBP patients.[7]

Conclusion

To avoid the potentially fatal consequences of perforation and gangrene, acute cholecystitis in children with typhoid fever demands a high index of suspicion when diagnosing.

CONTENT

Ethics

Our institution does not require ethics approval for reporting individual cases or case series. Since the patient described in this report was a minor, the written informed consent for anonymised patient information to be published in this article was obtained from his parents.

Acknowledgement

We acknowledge Assam Down Town University for giving opportunity to conduct this study.

Conflicts of interest

The authors declare that there is no conflict of interest.

Funding

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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Software: CS
Validation: PCS
Formal analysis: PCS
Investigation: ADS
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Data curation: ADS, CS
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Instructions to authors

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1. Manuscripts should be submitted in the *doc or *docx format (Microsoft Word), using the Times New Roman font. The text should be single spaced in 12 point. The main heading should be 14 point **bold**. Subheadings should be 12 point **bold**. Tables must be in 10 point, single spaced; headings within tables should be in 10 point **bold**; the main table heading should be in 12 point **bold**; legends should be single spaced in 10 point. Figures should be made using standard graphic formats (*pdf, *ai, *cdr). Photographs should be delivered in *jpg format, 1:1, 300 dpi or higher resolution, as separate files.

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4. Numbers in text and tables should be provided if expressed as %; means should be accompanied by standard deviations (SDs) and medians by interquartile range (IQR). In text, use the following rule: spell out numbers up to nine and then use numerical designation for 10 and above.

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6. References should be indicated in the text sequentially in the Vancouver numbering style, as superscripted numbers after any punctuation mark.

7. Units of measurement, length, height, weight and volume are to be expressed in metric units (eg, metre - m, kilogram - kg, litre -L) or subunits. Temperature should be in degrees Celsius (°C); quantities of substances are given in moles (mol) and blood pressure is expressed as millimetres of mercury (mm Hg). All values of haematological, clinical and biochemical measurements use the metric system according to the International System of Units (SI units).

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10. Ethics. Consent statement and permission obtained by the institutional ethics committee (IEC). A cover letter should state that written informed consent was obtained from all subjects (patients and volunteers) included in the study and that the study was approved by the IEC. The name of the issuing ethics committee should be clearly disclosed, as well as its decision number and date of its issuing.

11. Acknowledgement. A statement listing all persons and institutions that has assisted the authors in performing the research in question.

12. Conflict of interest. A statement listing all cases of non-financial involvement of any author that could be perceived to be potentially damaging to the objectivity of the research project.

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ment declaring any potential conflict of interest must be signed by each author. (See the policy statement on conflict of interest issued by the World Association of Medical Editors, WAME, www.wame.org or ICMJE uniform disclosure form for potential conflicts of interest, www.icmje.org.) This disclosure includes all affiliations or financial involvement (eg, employment, consulting fee or honorarium, gifts, stock ownership or options, travel/accommodations expenses, grants or patents received or pending and royalties) with any organisation having a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This information will be held in confidence while the paper is under review. If the manuscript is accepted for publication, the editors will discuss with the author how such information is communicated to the reader in the section "Conflicts of interest."

14. Data access. Statement on where and how the original data used to prepare the manuscript can be obtained.

15. Author ORCID numbers. After authors' names their initials should be listed and then the 16-digit ORCID numbers.

16. Author contributions. To qualify for authorship, one must made substantial intellectual contributions to the study on which the article is based (see Policy Statements - Authorship at www.wame.com). The author should participate at least in one of these three categories:

a. research question, conception and design, data acquisition and analysis,

b. statistical analysis, interpretation of data, provision of funding, technical or material support, overall supervision of the project.

c. drafting or critical revision of the manuscript.

In some research projects experts (such as biostatisticians or epidemiologists) may participate and although they may not be equally familiar with all aspects of the work (for example, some clinical variables or laboratory measurements), they may be qualified as the authors. A statement acknowledging contribution to the manuscript should be signed by all the authors. It will be published in the section "Author contributions." The corresponding author is responsible for the integrity of the work as a whole. It is dishonest to omit mentioning the investigator who had important engagement with some aspects of the work.

A pre-defined list of possible author contributions is given below. For each author one or more of the following 14 potential functions should be selected:

- Conceptualisation
- Methodology
- Software
- Validation
- Formal analysis
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- Resources
- Data curation
- Writing - original draft
- Writing - review and editing
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After each of the applicable function, a colon (:) should be written, followed by the initials of the authors pertaining to this particular contribution (eg, Conceptualisation: AB, CD, Supervision: AB, XY, VW, etc).

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c. The name of the corresponding author and a statement that they empower him/her to sign all the requested statements and to communicate with the Editorial Office on their behalf.

This statement should be signed by all the authors.

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a. A statement that the submitted text is the result of the original work of the authors.

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e. A brief description of the study, with accent on its novelty and why the authors believe that it should be published in *Scripta Medica*.

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19. Submission of manuscripts. Manuscripts

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The title should identify the main topic or the message of the paper. The standard title of a research paper is a phrase (rarely a sentence) that identifies the topic of the paper; it should be concise and precise, informative and descriptive.
The title of a descriptive paper should include the necessary description, function, purpose, animal species or population. When a method is described, the title should indicate whether it is new or improved.

Abstract and key words. Structured abstract should be included in papers reporting original research. Abstracts are limited to 300 words in four labelled paragraphs: Background/Aim, Methods, Results and Conclusion. The abstract should state concisely the objectives of the study, methods used, results obtained and adequately answer the research question. The abstract should provide pertinent information when read alone. Abstracts should not contain any citing of references. Below the abstract, authors should provide 3-5 key words, according to the terms from the Medical Subject Headings – MeSH (www.nlm.nih.gov/mesh).

Introduction. Generally, this section provides the motivation for the paper (ie, what is missing or unknown in the research literature at this time), an overview of the scientific theory or conceptual models on which the research was based and the purpose of the study and why it is important. Cite only relevant references.

Methods. This section accurately describes the procedures used to carry out the study; it should be complete enough to permit others to replicate the study. Describe the methodological design, subjects, data sources, data collection methods and any statistical and analytical procedures. These five parts may not be needed in all papers. Short papers may include these details in different paragraphs, but titled subsections may be used in longer papers. The Methods section should describe how the research was structured, how subjects or groups of subjects (defined by sex, age and other characteristics) and how the subjects were chosen and assigned to

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these groups. Identify all drugs and chemicals by generic names, exact drug dosages and routes of administration. Variability should be expressed in terms of means and standard deviations (SD). A p-value can be used to disprove the null hypothesis, but the authors should also give an estimate of the power of the study and state the exact tests used for statistical analysis.

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Discussion. Discussion should be always written as a separate chapter and not together with the Results. Briefly state the principal finding that relates to the purpose or research question posed in the Introduction and follow the interpretation of the results obtained. Compare your findings with work reported previously by others. Discuss the implications of your findings and their limitations with respect to the methods used.

Conclusion. The conclusion should be brief and clear, answering the aim of the research and should not consist of repetition of the main results. Avoid unqualified statements and conclusions not completely supported by your data.

References. The reference list is the responsibility of the authors. List all the papers or other sources cited in describing previous or related research. Cite references in the text sequentially in the Vancouver numbering style, as superscripted number after any punctuation mark. For example: …as reported by Vulić and colleagues.[12] When two references are cited, they should be separated by comma, with a space. Three or more consecutive references are given as a range (eg, …as was published earlier.[12-14]). References in tables and figures should be in numerical order according to where the item is cited in the text. For citations according to the Vancouver style, see *Uniform Requirements for Manuscripts Submitted to Biomedical Journals*; this source gives the rules and formats established by the International Committee of Medical Journal Editors (www.icmje.org). The standardised abbreviations of the titles of scientific journals cited should be used. If there are six authors or fewer, list all six by last name, space, initials, comma. If there are seven or more, list the first three in the same way, followed by et al. For a book, list the editors and the publisher, the city of publication, and year of publication. For a chapter or section of a book, give the authors and title of the section, and the page numbers. For online material, please cite the URL and the date you accessed the website. Online journal articles can be cited using the doi number. Do not quote references within the Abstract and Conclusion section. All titles of cited manuscripts should be in English (the name of the original language should appear in brackets). Every effort should be done to add the doi number after the reference; if not available, PMID number should be listed. See examples below that conform to the Uniform Requirements for Manuscripts Submitted to Biomedical Journals:

- Miller AL, Evanson NK, Taylor JM. Use of donepezil for neurocognitive recovery after brain injury in adult and pediatric populations: a scoping review. Neural Regen Res. 2024 Aug 1;19(8):1686-95. doi: 10.4103/1673-5374.389628.
- International Committee of Medical Journal Editors (ICMJE). International Committee of Medical Journal Editors (ICMJE). Uniform Requirements for Manuscripts Submitted to

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Biomedical Journals: writing and editing for biomedical publication. Haematologica. 2004 Mar;89(3):264. PMID: 15020262.

- Hull J, Forton J, Thompson A. Paediatric respiratory medicine. Oxford: Oxford University Press; 2015.
- Bydder S. Liver metastases. In: Lutz S, Chow E, Hoskin P, editors. Radiation oncology in palliative cancer care. Chichester (UK): John Wiley & Sons, Ltd.; 2013. p. 283-298.
- Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic program- ming. In: Foster JA, Lutton E, Miller J, Ryan C, Tet- tamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. p. 182-91.
- Polgreen PM, Diekema DJ, Vandeberg J, Wiblin RT, Chen YY, David S, et al. Risk factors for groin wound infection after femoral artery catheterization: a case-control study. Infect Control Hosp Epidemiol [Internet]. 2006 Jan [cited 5 Jan 2007];27(1):34-7. Available from: http://www.journals.uchicago.edu/ ICHE/ journal/ issues/v27n1/2004069/2004069. web.pdf.

Review article

Review articles are written by individuals who have studied a particular subject or area extensively and who are considered experts. Narrative reviews and Systematic reviews should be identified as such in their titles. For these reviews, the word count should not exceed 2.500 words, excluding references and abstract. The manuscript may have up to 4 tables or figures and as many as 50 references. Any deviation from these limitations may be approved at the discretion of the Editor-in-Chief.

Current topic

Current topics are actually mini-reviews. They also review a certain topic, but less extensively and by quoting a considerably smaller number of original publications.

Special article

Special articles of 1500 words or less may be devoted to any medical problem, historic perspective, education, demography or contemporary issues. Up to 15 references may be cited and the piece may contain 2 tables or figures. An unstructured abstract in English (150 words or less) should accompany a specific article. Financial disclosure should be presented.

History of medicine

History of medicine articles deal with some historic aspects of medical research and practice. They should be considered as a type of a special article.

Case report

Case reports are most likely to be published if they describe any of the following: an unreported drug side effects (adverse or beneficial), drug interactions; a new, unexpected, or unusual manifestation of a disease; previously unsuspected causal association between two diseases; presentations, diagnosis and/or management of new and emerging diseases; an unexpected association between diseases or symptoms; an unexpected event in the course of observing or treating a patient, findings that shed new light on the possible pathogenesis of a disease or an adverse effect; a previously unknown disease. *Scripta Medica* does not publish instructive case reports, that is, presentations that make important teaching point of what is already well known but often forgotten.

Case reports (no longer than 750 words) should include the following: title, introduction, case history (including up to three figures) and discussion, references (up to six) and an unstructured abstract in English. The abstract may be a single paragraph containing no more than 100 words and followed by key words. Title should facilitate retrieval with electronic searching. Case presentation should include the history, examination and investigations adequately, description of treatments, all available therapeutic options that have been considered and outcomes related to treatments. Discussion includes the following: statement an unusual diagnosis, prognosis, therapy; report of a literature review of other similar cases; explain rationale for reporting the case; what is unusual about the case; could things be done differently in a similar case. There should also be a short conclusion.

Case reports may have as many as five authors. A very short case, about a particular disease can be submitted as a Letter to the Editor. Consent for publication must be obtained from the patients involved; if this is not possible, permission from a close relative or a legal guardian must be obtained before submission. Only non-identifiable data should be included, thus protecting the identity of the patient.

In a cover letter authors should indicate how the case report contributes to the medical literature. Submissions that do not include this information

will be returned to authors prior to peer review. For all case reports, informed written consent is required; the cover letter should state that consent was obtained. Authorship statement and financial disclosure should be presented.

Images in clinical medicine

The editors will consider original, clear and interesting images that depict new or "classic" clinical pictures submitted along with a descriptive paragraph of up to 200 words. The report may include two authors and three references. The authors must obtain a signed, informed consent from the patient or from a close relative or a legal guardian. The cover letter from the corresponding author should state that written consent was obtained.

Letter to the editor

If the letter refers to a recent journal article, it should not exceed 250 words, excluding references. All letters should be brief and to the point with no more than five reference citations. Figures or tables are not permitted in this format. Financial disclosure should be presented.

Editorial

Editorials are solicited by the editor to provide perspective on articles published in the journal and/or to express the general policies or opinions of the Editorial Board. Editor-in-Chief may invite a respectable scholar to write an editorial on a certain topic.

Submission of papers

Manuscripts, tables and figures should be submitted via the official journal website http://scriptamedica.com/submit-a-manuscript/, whenever it is possible, **all in one file. To assist the reviewing process, besides this full-text file, additional files should be uploaded, too:**

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(2) Cover letter, signed by the corresponding author (see above for details)

(3) Title page, containing the manuscript title, full names and surnames (in this order!) and affiliations of the authors and the following seven statements/assertions:

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- Acknowledgement
- Funding
- Conflict of interest
- Data access
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