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RBGO Gynecology and Obstetrics
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Editorial

347 Placenta Accreta Spectrum Disorders and Cesarean Scar Pregnancy Screening: Are we Asking the Right Questions?
Conrado Milani Coutinho, Laure Noel, Veronica Giorgione, Lígia Conceição Assef Marçal, Amar Bhide, and Basky Thilaganathan

Original Articles

Obstetrics

351 Comparison between Enzyme Immunoassays Performed on Samples of Dried Blood and Serum for Toxoplasmosis Prenatal Screening: Population-based Study
Bárbara Araújo Marques, Ericka Vianna Machado Carellos, Vânia Maria Novato Silva, Fernando Henrique Pereira, Maria Regina Lage Guerra, Jacqueline Araújo Domingos Iturra, José Nélio Januário, and Gláucia Manzan Queiroz de Andrade

Human Reproduction

357 Gynecological/Obstetric Background and Rheumatoid Arthritis: A Cross-sectional Study in Brazilian Patients
Anauá Fernanda dos Santos Cavalcante, Patrícia Martin, and Thelma Larocca Skare

Pediatric and Adolescent Gynecology/Obstetrics

362 Barriers to Puberty Talk between Mothers and Daughters: A Qualitative Study
Firoozeh Mirzaee, Malihe Pouredalati, Atefeh Ahmadi, and Masumeh Ghazaznfarpour

Basic and Translational Science /Mastology

368 Immunological Characteristics between $\alpha\beta$ T_{DC} and $\gamma\delta$ T_{DC} Cells in the Spleen of Breast Cancer-Induced Mice
Polyana Barbosa Silva, Márcia Antoniazi Michelin, Millena Prata Jammal, and Eddie Fernando Cândido Murta

Covid-19

374 Surgical Site Infection after Cesarean Delivery in Times of COVID-19
Vicente Sperb Antonello, Jessica Dallé, Ivan Carlos Ferreira Antonello, Daniela Benzano, and Mauro Cunha Ramos

377 Placental Sampling for Understanding Viral Infections—A Simplified Protocol for the COVID-19 Pandemic
Guilherme de Moraes Nobrega, José Paulo Siqueira Guida, Rodolfo Rosa Japecanga, Arthur Antolini-Tavares, Indira Mysorekar, and Maria Laura Costa

Review Articles

384 Clinical Features and Maternal-fetal Results of Pregnant Women in COVID-19 Times
Ana Paula Nogueira Godoi, Gilcelia Correia Santos Bernardes, Leilismara Sousa Nogueira, Patrícia Nessralla Alpoim, and Melina de Barros Pinheiro

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395 The Female Athlete Triad/Relative Energy Deficiency in Sports (RED-S)
Alexandra Ruivo Coelho, Gonçalo Cardoso, Marta Espanhol Brito, Inês Neves Gomes, and Maria João Cascais

403 Interventions among Pregnant Women in the Field of Music Therapy: A Systematic Review
Bruna Mayumi Omori Shimada, Magda da Silva Oliveira Menezes dos Santos, Mayara Alvares Cabral, Vanessa Oliveira Silva, and Gislaine Cristina Vagetti

Case Report

414 Lipschütz Ulcer: An Unusual Diagnosis that Should Not be Neglected
Daniela Alexandra Gonçalves Pereira, Eliana Patrícia Pereira Teixeira, Ana Cláudia Martins Lopes, Ricardo José Pina Sarmento, and Ana Paula Calado Lopes

Febrasgo Statement

417 Management of hypoactive sexual desire disorder in women in the gynecological setting
Lucia Alves da Silva Lara, Sandra Cristina Poerner Scalco, Andréa Cronemberger Rufino, Stany Rodrigues Campos de Paula, Eduardo Siqueira Fernandes, Joice Martins de Lima Pereira, Siglia Sousa de França, Sheila Reis, Suzane Beirão de Almeida, Fabiene Bernardes Castro Vale, Théo Lerner, Yara Maia Villar de Carvalho, Carmita Helena Najjar Abdo, and Flávia Fairbanks Lima de Oliveira

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Editorial

Placenta Accreta Spectrum Disorders and Cesarean Scar Pregnancy Screening: Are we Asking the Right Questions?

Conrado Milani Coutinho[1] Laure Noel[2] Veronica Giorgione[3] Lígia Conceição Assef Marçal[1] Amar Bhide[2] Basky Thilaganathan[2,3]

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According to the World Health Organization, approximately 295,000 women died in 2017 during the antenatal and postpartum period. The vast majority (94%) of these cases occurred in low- and middle-income countries, with an estimate of 810 daily deaths from preventable causes.[1] Obstetric hemorrhage is the leading cause of maternal mortality worldwide and, among its key etiologies, placenta accreta spectrum (PAS) disorders have been increasing in prevalence concurrently with the global rise in the proportion of Cesarean deliveries and rates have currently being reported between 0.01% to 1.1% of pregnancies.[2–5] Accurate screening and diagnosis of PAS is of utmost importance for timely antenatal referral to tertiary hospitals and management by specialized multidisciplinary teams, which has been associated with a reduction in its associated morbimortality.[6] Although ultrasound diagnosis of PAS can be reliably done in centers with expertise, with an accuracy of approximately 90%,[7,8] in non-specialized facilities this rate falls to 50%, mainly due to insufficient clinical suspicion and/or knowledge of risk factors.[9,10] Therefore, effective and systematic screening and diagnostic protocols for PAS should be implemented in all maternal-fetal health care services in order to prevent adverse outcomes related to undiagnosed PAS disorders. The purpose of this article is to highlight the importance of basic questions that should be incorporated by all sonographers while performing routine obstetrical ultrasound to improve the detection of PAS.

What are the Relevant Risk Factors for Pas Screening?

Numeroushistorical risk factors have been associated with the occurrence of PAS, including maternal obesity, advanced maternal age and parity, previous uterine surgery (including illegal terminations of pregnancy), and use of assisted reproductive technologies.[11] However, there is no doubt that the concomitance of the only risk factor related to the ongoing pregnancy–a low-lying placenta–with a previous Cesarean birth are the main risk factors for PAS, occurring concurrently in more than 90% of confirmed cases.[11–13] The reasons for that are not difficult to understand. Although preliminary studies suggested that PAS resulted from an excessive trophoblastic invasion and/or substandard decidual function,[14,15] the hypothesis of placental implantation on or into an iatrogenically defective decidua is currently gaining acceptance,[16–18] making the case for a common pathophysiological pathway between development of an uterine niche, Cesarean scar pregnancy (CSP) and PAS. Furthermore, recent epidemiological studies have challenged the previous association of the number of previous Cesarean sections and the risk for PAS, confirming that there is a plateau of risk for PAS after the second Cesarean birth.[19,20] This can be explained by the higher position of a uterine niche after previous elective Cesarean section compared to emergency Cesarean birth resulting in a three-fold increased risk of developing PAS in future pregnancies with placenta previa.[19,21] Therefore, as most risk factors for PAS seem to be proxy markers for the two previously cited and in order to improve the identification of PAS cases in the antenatal period, we would like to emphasize the importance of asking two simple questions while performing every obstetrical ultrasound: "is the placenta low-lying?" and "did the patient have a previous Cesarean section?".

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Is First Trimester Ultrasound Screening for Paspossible?

If the answer is yes to the latter two questions, then it is indeed possible that the woman may have a PAS. As obstetrical ultrasound between 11 and 13 gestational weeks is conventionally performed for pregnancy dating, identification of multiple pregnancies, diagnosis of abnormalities and screening for trisomies and preeclampsia, this would be the perfect timing to firstly assess the risk for PAS disorders. Several ultrasound markers have been proposed, such as low implantation of gestational sac on or into a previous Cesarean scar, reduced myometrial thickness, placental lacunae, enhancedmyometrial vascularity and abnormal uterus-bladder interface, many of them in common with the diagnostic features of a CSP (►**Figure 1**). A 2018 systematic review and meta-analysis[22] concluded that at least one PAS sign can be identified during the first trimester in 91.4% of confirmed cases and that a low anterior implantation of the gestational sac or the placenta close to or within a previous Cesarean scar is the most commonly observed sign (82.4% of cases), with a sensitivity of 44.4% (95% CI, 21.5-69.2%) and a specificity of 93.4% (95% CI, 90.5%-95.7). In 2019, a prospective screening study[23] assessed the performance of a two-stage PAS screening strategy in 22,604 pregnancies. Patients were first evaluated between 11-13 weeks and those presenting low-lying placenta and a history of uterine surgery were referred to a specialized clinic at 12-16 weeks. For the 6% (1298 cases) of pregnant women with at least one marker and considered to be at high-risk, the diagnosis of PAS was suspected in 14 cases and confirmed in 13. There were no cases of PAS in the low-risk patients. Performance of screening was not assessed due to the low number of PAS cases. These findings support the relevance of being aware of the position of the gestational sac/placenta in the first trimester scan in patients with a history of Cesarean sections, especially for the high-positioned scars secondary to elective procedures. On the one hand, the first trimester diagnosis of a CSP/PAS is desirable and should be pursued, mainly for being a condition associated with increased maternal morbimortality with a need for referral to specialized multidisciplinary centers for appropriate counselling and management.[24] On the other, this first trimester screening strategy would label 6% of women as being at high-risk for PAS, resulting in additional expenditure, use of human and logistical resources, and the negative psychological burden on the family – with less than 1 in 100 of these 'high-risk' women actually having a PAS. Additionally, although termination of pregnancy is usually discussed with these families, the natural history of CSP is not yet fully understood. Recent studies tried to discriminate the outcomes of CSP based on ultrasound signs. Among them, placental implantation "in the niche" instead of "on the scar",[25] residual myometrial thickness below 2 mm,[25] and identification of the pregnancy in the "high-risk-for-PAS triangle",[26] would be predictive of worse surgical outcomes and more advanced third-trimester sonographic staging of PAS (►**Figure 1A**). However, the rarity of this condition precludes the assessment of strong associations with outcome from the previous studies. Therefore, it is imperative to establish a collaborative approach to gather global experience among specialists conducting CSP cases. With this purpose in mind, we encourage clinician to upload CSP cases onto the international CSP Registry (https://csp-registry.com) (►**Figure 1B**).

Contingent Second and Third Trimester Screening for Pas

The rationale for a mid-trimester screening for PAS is to take advantage of the conventional 18-23 weeks anatomical ultrasound evaluation and the already implemented screening for placenta previa in non-specialized facilities. With the two proposed questions in mind, upon identification of a low-lying placenta (first question) on routine mid-trimester scan, all sonographers should enquire the patient about a previous Cesarean section (second question). The order of these questions is extremely important for the feasibility of the screening program, as the proportion of patients with

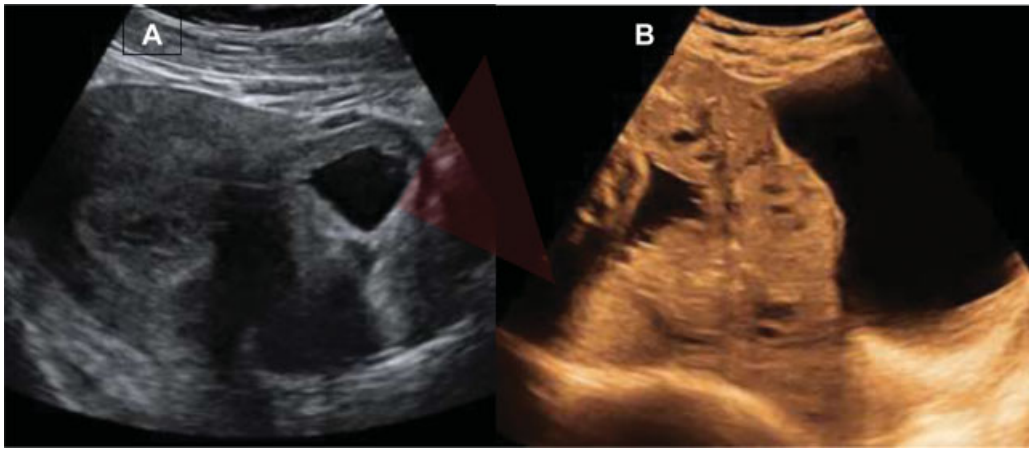

Fig. 1. A. Sagittal first trimester transabdominal ultrasound image of a Cesarean scar pregnancy highlighting the "high-risk-for-placenta accreta spectrum triangle" (implantation on the lower anterior quarter of the uterus, and into the Cesarean scar niche); B. Sagittal third trimester transabdominal ultrasound image of a placenta accreta spectrum disorder on a placenta previa completely covering the cervical internal os (arrow).

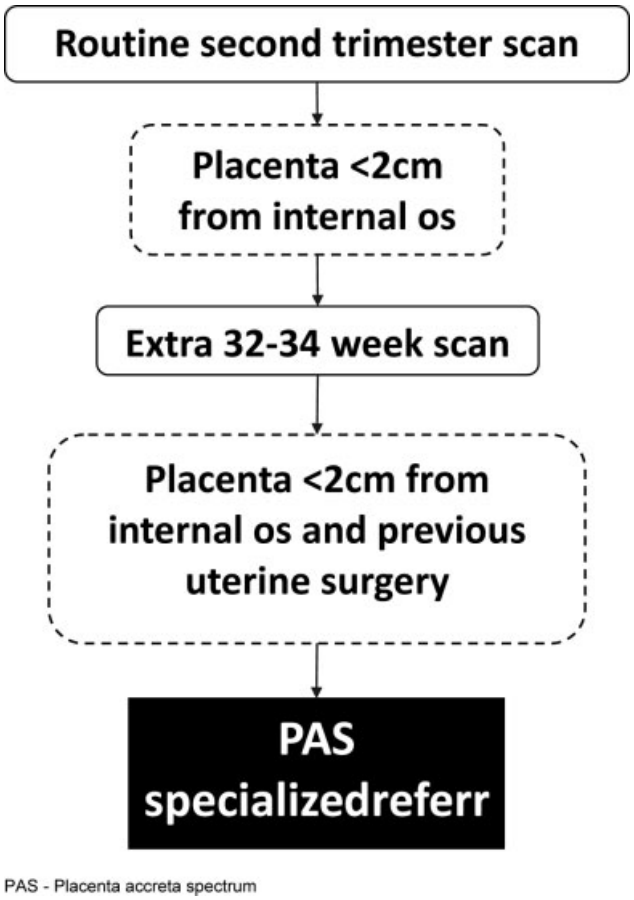

Fig. 2. Flowchart illustrating the screening pathway for PAS starting from the mid-trimester and highlighting the importance of the implementation of two simple questions on routine scanning: (1) "is the placenta low-lying?" and (2) "did the patient have a previous cesarean section?"

previous uterine surgery is incomparably higher than those with persistent low-lying placenta in the third trimester. This strategy has been explored by a retrospective study encompassing 57,179 women scanned between 18-23 gestational weeks.[27] For the 7.8% of patients with a low-lying placenta, a 32 week scan was arranged to assess placental position. Only 220 (0.4%) had a diagnosis of persistent placenta previa. 75 (0.1%) of them had a previous uterine surgery and were therefore referred for assessment by the PAS diagnostic service. In total, 21 out of 22 PAS cases were correctly identified by this screening program, with a sensitivity of 95.45% (95% CI, 77.16-99.88%) and a specificity of 100% (95% CI, 99.07-100%) (►**Figure 2**). PAS was confirmed based on clinical and histopathological criteria, as recommended by the International Federation of Gynecology and Obstetrics (FIGO).[28] From a public health perspective, this contingent PAS screening strategy is feasible in lower-resource medical settings with basic obstetric ultrasound facilities, not requiring additional visits beyond those that are routinely indicated. Furthermore, comparing to the first trimester screening, only 0.1% of patients would need to be referred to a specialized PAS diagnostic service (with one in three having a confirmed PAS), as opposed to a 6% figure between 11-13 weekswith less than one in 100 subsequently diagnosed with PAS. The success of such a screening strategy relies on an established regional referral service, with access to fetal medicine specialists properly trained to diagnose PAS disorders and dedicated, highly specialized multidisciplinary team at tertiary level hospital, where safe delivery can be arranged.[29]

Two simple questions asked by the sonographer at every obstetric ultrasound examination have the potential to alter the course of pregnancies at risk for PAS: (1) "is the placenta low-lying?" and (2) "did the patient have a previous cesarean section?". Suspicionfor CSP during the first trimester scan should trigger referral to specialized centers and careful counselling taking into consideration the lack of data regarding the natural history of CSP. Contingent screening for PAS in women with persistent placenta previa in the third trimester and a history of previous Cesarean section is feasible, effective and does not put additional burden on the public health system. In parallel with the establishment of specialist referral centers, the implementation of these simple questions and screening strategy have the potential to improve antenatal PAS detection rates and decrease maternal morbidity and mortality secondary to undiagnosed PAS.

Conflicts to Interest

The authors have no conflict of interests to declare.

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Comparison between Enzyme Immunoassays Performed on Samples of Dried Blood and Serum for Toxoplasmosis Prenatal Screening: Population-based Study

Comparação entre ensaios imunoenzimáticos realizados em amostras de sangue seco e soro para triagem pré-natal da toxoplasmose: Estudo populacional

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Abstract

Objective Most prenatal screening programs for toxoplasmosis use immunoassays in serum samples of pregnant women. Few studies assess the accuracy of screening tests in dried blood spots, which are of easy collection, storage, and transportation. The goals of the present study are to determine the performance and evaluate the agreement between an immunoassay of dried blood spots and a reference test in the serum of pregnant women from a population-based prenatal screening program for toxoplasmosis in Brazil.

Methods A cross-sectional study was performed to compare the immunoassays Imunoscreen Toxoplasmose IgM and Imunoscreen Toxoplasmose IgG (Mbiolog Diagnósticos, Ltda., Contagem, Minas Gerais, Brazil)in dried blood spots with the enzyme-linked fluorescent assay (ELFA, BioMérieux S.A., Lyon, France) reference standard in the serum of pregnant women from Minas Gerais Congenital Toxoplasmosis Control Program.

Results The dried blood spot test was able to discriminate positive and negative results of pregnant women when compared with the reference test, with an accuracy of 98.2% for immunoglobulin G (IgG), and of 95.8% for immunoglobulin M (IgM).

Conclusion Dried blood samples are easy to collect, store, and transport, and they have a good performance, making this a promising method for prenatal toxoplasmosis screening programs in countries with continental dimensions, limited resources, and a high prevalence of toxoplasmosis, as is the case of Brazil.

Keywords
- prenatal care
- prenatal diagnosis
- dried blood spot testing
- toxoplasmosis
- congenital toxoplasmosis

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Resumo

Objetivo A maioria dos programas de triagem pré-natal para toxoplasmose utiliza imunoensaios em amostras de soro de gestantes. Poucos estudos avaliam a acurácia dos testes de triagem em amostras de sangue seco, que são de fácil coleta, armazenamento e transporte. Este estudo teve como objetivo determinar o desempenho e avaliar a concordância entre um imunoensaio em sangue seco e um teste de referência em soro de gestantes de um programa de rastreamento pré-natal de base populacional para toxoplasmose no Brasil.

Métodos Realizou-se um estudo transversal para comparar os imunoensaios Imunoscreen Toxoplasmose IgM e Imunoscreen Toxoplasmose IgG (Mbiolog Diagnósticos, Ltda., Contagem, Minas Gerais, Brazil) em sangue seco com o padrão de referência ensaio fluorescente ligado a enzimas (*enzyme-linked fluorescent assay*, ELFA, BioMérieux S.A., Lion, França) no soro de gestantes do Programa de Controle de Toxoplasmose Congênita de Minas Gerais.

Resultados O exame em sangue seco foi capaz de discriminar os resultados positivos e negativos das gestantes quando comparado ao teste de referência, com acurácia de 98,2% para imunoglobulina G (IgG), e de 95,8% para imunoglobulina M (IgM).

Conclusão O sangue seco apresenta bom desempenho e é uma amostra de fácil coleta, armazenamento e transporte, o que o torna um método promissor para programas de triagem pré-natal de toxoplasmose em países com dimensões continentais, recursos limitados, e alta prevalência de toxoplasmose, como é o caso do Brasil.

Palavras-chave

- cuidado pré-natal
- diagnóstico pré-natal
- teste em amostras de sangue seco
- toxoplasmose
- toxoplasmose congênita

Introduction

Surveillance of acute toxoplasmosis infections in pregnant women is performed through periodic serological tests obtained from venipuncture. Dried blood spot testing has been successfully used in neonatal screening programs, and may be a promising alternative for the prenatal diagnosis of toxoplasmosis. Collection of dried blood spots is performed through capillary puncture, and the samples are stored in filter paper cards, which are stable and can be transported at low cost, enabling the performance of tests in patients from distant and economically-disadvantaged regions.

In Brazil, studies[1–3] from prenatal screening programs for toxoplasmosis based on dried blood spot testing reported a low prevalence of positive immunoglobulin M (IgM) samples, of 0.4% and 0.7%, leading to questions about the accuracy of this test. A systematic review[4] assessed the serological methods used in prenatal screening programs for toxoplasmosis worldwide and did not find publications with dried blood samples.

The present study was conducted in Minas Gerais (MG), the second most populous state in Brazil, located in the Southeastern region of the country. The Minas Gerais Congenital Toxoplasmosis Control Program (MG-CTCP) was implemented by the state's Department of Health, in partnership with Núcleo de Ações e Pesquisa em Apoio Diagnóstico (NUPAD, in Portuguese) of the School of Medicine at Universidade Federal de Minas Gerais (UFMG) in February 2013. The MG-CTCP included prenatal screening for toxoplasmosis of all pregnant women from 853 municipalities in the state by using dried blood on filter paper, which made universal screening possible in MG. There were $\sim$ 270,000 pregnant women in MG in 2014. More than 95% of them received prenatal care, and 74% attended 7 or more visits. Approximately 70% of pregnant women in the state are users of the Brazilian Unified Health System (Sistema Único de Saúde, SUS, in Portuguese), and NUPAD tested around 60% of them in 2014.

The primary objective of the present cross-sectional study was to compare the performance of a dried blood test used in the MG-CTCP with that of a commercial serological test considered a reference for the diagnosis of infection. The secondary objective was to determine the prevalence of toxoplasmosis among the pregnant women in the MG-CTCP.

Methods

A cross-sectional study was performed with pregnant women in the MG-CTCP who were cared for in one of the 853 municipalities in the state of MG. The sample size was calculated as 1,000 pregnant women to find a minimum number of 5 acute infections among them, as it is estimated that 40% of pregnant women in Brazil are susceptible to toxoplasmosis, and a cumulative incidence of seroconversion in pregnancy from 4.8 to 5.7 per 1000 is described.[5,6] Additionally, we planned to include 40 pregnant women with positive IgM results via filter paper, detected by the MG-CTCP in the same period, which is enough to reduce the low-frequency bias expected in the 2×2 tables. The Basic

Health Units (BHUs) included were randomly selected in each of the 13 macroregions of MG, considering the proportion of pregnant women with an acute infection profile (positive IgM and immunoglobulin G [IgG] anti-*Toxoplasma gondii*) among all pregnant women in the MG-CTCP in the year before the study, and the average amount of screenings performed per month and per BHU in the same period, according to the NUPAD database. A macroregion with a higher prevalence of probable acute infections had a larger number of patients screened than a region with a lower prevalence. Likewise, a BHU that performed a higher number of screening tests in the base period had a higher probability of being drawn within each macroregion. This method is called sampling with probabilities proportional to size. By rounding off issues in the monthly average number of examinations conducted by the BHUs, the total amount of samples was calculated as 1,038.

Pregnant women who refused to participate in the study, as weell as those who presented unsuitable samples, were excluded. Pregnant women were included consecutively at the time of the first blood collection. Capillary and venous blood samples were collected simultaneously or with a difference of up to 24 hours, and were respectively transported on Whatman (Merck KGaA, Darmstadt, Germany and/or its affiliates. Sigma-Aldrich, Inc.) 903 filter paper at room temperature, and in styrofoam boxes with ice to the NUPAD reference laboratory. Free and informed consent was obtained for all pregnant women who agreed to participate in the study.

The present study was blinded regarding the professionals in charge of conducting the serological tests, but not regarding the main researchers, who needed to adopt clinical procedures of interest to the patient.

Immunoassays were used to determine anti-*T. gondii* specific IgM and IgG antibodies: the Imunoscreen (Mbiolog Diagnósticos, Ltda., Contagem, Minas Gerais, Brazil) in dried blood samples in the NUPAD laboratory, and the enzyme-linked fluorescent assay (ELFA, BioMérieux S.A., Lyon, France) in serum, using the automated VIDAS system in two laboratories outsourced by NUPAD (A and B). The IgG avidity test was performed using the serum of all pregnant women with reagent IgG results: chemiluminescence and ELFA by laboratories A and B respectively.

The Statistical Package for the Social Sciences (SPSS, SPSS, Inc., Chicago, IL, US) software, version 18, was used to set up a database and perform the statistical analyses. The performance of the Imunoscreen test in filter paper was evaluated in comparison with the reference standard (ELFA), and its sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated. For each of these parameters, additional analyses were performed, excluding the indeterminate results, or considering them as positive or negative. The receiver operating characteristic (ROC) curve was used to describe the performance of dried blood tests to classify pregnant women as infected or not by *T. gondii*.

The Chi-squared test for adherence was used to evaluate whether the distribution of samples received from each macroregion matched the distribution of the expected samples. The prevalence of toxoplasmosis was determined by the proportion of pregnant women with positive IgG anti-*T. gondii* results compared with the total number of pregnant women screened.

The Kappa coefficient was used to evaluate the agreement between the results of the tests in filter paper and serum samples. Since the Kappa depends on the agreement that goes beyond the coincidence of random evaluations, it is possible to find low values of this measurement due to the low prevalence of the event. It is usually associated with low levels of reproducibility, not due to substantial errors related to the test.[7] Therefore, the prevalence was calculated using adjusted Kappa for IgM.

The research project was approved by the Ethics in Research Committee of UFMG (CEP-257.199, 26/04/2013) and received funding from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazilian National Council for Scientific and Technological Development) (Case 456491/ 2014–7). Activity planning was supported by the institutions involved.

Results

From July 1, 2014, to December 31, 2014, we received 750 samples of pregnant women participating in the MG-CTCP. Of these, 40 were excluded because they were inappropriate; therefore, 710 pregnant women remained in the study. The Chi-squared test for adherence showed that the distribution of the 710 included samples was statistically similar to the distribution of the expected 1,038 samples concerning their origin by macroregion ($p = 0.126$). The prevalence of toxoplasmosis among the 710 pregnant women studied was of 45.2%. None had positive IgM test results. In total, 33 pregnant women with positive IgM samples in filter paper, detected in the MG-CTCP in the same screening period, were included in the study, totaling 743 pregnant women. The age of the sample ranged from 12 to 47 years (median: 24 years). The first examination was performed at a median gestational age of 10 weeks, and the median interval in days between the collection of dried blood and serum samples was zero. Considering the ELFA method as a reference test, the relative sensitivity, specificity, PPV, and NPV of the Imunoscreen test for the detection of IgM and IgG antibodies in dried blood were calculated (►**Table 1**).

No significant difference in IgM sensitivity of the filter paper test was observed when indeterminate results were excluded (58.3%; 95% confidence interval [95%CI]: 42.2–72.9%) or included among the positive results (58.1%; 95% CI: 43.3–71.6%) ($p = 1.000$) (►**Table 2**). However, a significant reduction in IgG sensitivity of the Imunoscreen test was observed when indeterminate results were included among the negative results (56.8%; 95%CI: 40.9–71.3%; $p < 0.001$).

The Kappa coefficient of agreement in serum and dried blood was calculated for IgM, IgG, and IgM when adjusted for prevalence (►**Table 2**). When the indeterminate results were included as positive, a good agreement was found between the tests for IgM, and a very good agreement for IgG. When prevalence-adjusted Kappa for IgM was used, a very good

Table 1 Distribution of IgM and IgG results in dried blood and serum samples in 743 pregnant women participating in the comparison between the Imunoscreen test (dried blood) and the ELFA (serum) reference test

Tests			Result by the ELFA[B] method in serum			
		Results	Positive	Indeterminate	Negative	Total
Result by the Imunoscreen[A] method in filter paper	IgM	Positive	21	2	10**	33
		Indeterminate	1	1	1	3
		Negative	15*	3	689	707
		Total	37	6	700	743
	IgG	Positive	321	0	9##	330
		Indeterminate	49	0	2	51
		Negative	12#	1	346	359
		Total	382	1	357	740

Abbreviations: ELFA, enzyme-linked fluorescent assay; IgG, immuniglobulin G; IgM, immunoglobulin M.
Notes:
*False negative IgM.
**False positive IgM.
#False negative IgG.
##False positive IgG.
[A]Imunoscreen test. According to the manufacturer, the test presents 100% of sensitivity and 98.1% of specificity for IgM, and 100% and 99.1% respectively for IgG.
[B]Enzime-linked fluorescent assay (ELFA). According to the manufacturer, the test presents 81.8% to 90.9% of sensitivity for IgM, and 93.8% to 98.4% for IgG, and a specificity of 100.0% for IgM and of $>$ 99.0% for IgG. The IgM results are interpreted as negative if lower than 0.55, indeterminate if between 0.55 and 0.64, and positive if $\geq$0.65. For IgG, the result is negative if lower than 4.0, intermediate if between 4.0 and 7.9, and positive if $\geq$ 8.0.

Table 2 Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and Kappa concordance coefficient for the Imunoscreen test (dried blood) compared with the ELFA (serum) reference test, considering indeterminate results in the sample analyzed to be positive

Imunoscreen test (dried blood) compared with the ELFA (serum)	IgM	IgG
Sensitivity – % (95%CI)	58.1 (43.3–71.6)	96.6 (94.3–98.0)
Specificity – % (95%CI)	98.4 (97.2–99.1)	96.9 (94.6–98.3)
PPV – % (95%CI)	69.4 (54.4–84.5)	97.1 (95.4–98.8)
NPV – % (95%CI)	97.5 (96.3–98.6)	96.4 (94.4–98.3)
Kappa coefficient of agreement (95%CI)	0.61 (0.55–0.68)	0.93 (0.92–0.95)
Kappa adjusted for IgM prevalence (95%CI)	0.92 (0.91–0.94)	–

Abbreviations: 95%CI, 95% confidence interval; ELFA, enzyme-linked fluorescent assay; IgG, immunoglobulin G; IgM, immunoglobulin M.

agreement between the tests was found, regardless of the inclusion of indeterminate results.

There were 15 false-negatives for IgM in filter paper. In each case, dried blood and serum samples were collected on the same day or up to 24 hours after. In 10 out of 13 cases with information on gestational age, the sample was collected in the first trimester of gestation. All samples tested positive for IgG, except for a dried blood sample with indeterminate IgG. There was low avidity of IgG only in 1 case. In addition, 14 samples presented serum IgM values extremely close to the cut-off of the test. Toxoplasma infection was excluded in all the children of these mothers after the follow-up.

There were 10 false-positive results for IgM and 9 false-positives for IgG. There were also 12 false-negative results for IgG in filter paper, all of which had low values of IgG in serum, high IgG avidity, and negative IgM. Serology was repeated during the routine prenatal follow-up for all of these 12 subjects.

The ROC curves showed that the Imunoscreen test could discriminate positive and negative pregnant women when compared with the reference test, with the area under the curve equal to 98.2% for IgG and to 95.8% for IgM.

The IgM screening was performed as part of the MG-CTCP in newborns of susceptible mothers or of mothers with suspected acute infection during pregnancy. The results of neonatal screening were available for 316 of the 419 newborns of 412 pregnant women with positive IgM in pregnancy; all of them were negative.

Discussion

This population-based study compared serologies performed on two types of blood samples (dried blood on filter paper and serum) in a large cohort of pregnant women participating in a longitudinal toxoplasmosis screening program.

The prevalence of toxoplasmosis found, 45.2%, was compatible with the overall results of the MG-CTCP, but lower than previous findings in the city of Belo Horizonte and in Brazil.[8,9]

None of the 710 pregnant women initially included in the study had positive IgM results. In Brazil, some studies report a rate of up to 8% of acute infection in pregnancy, but the IgM antibody is generally observed in ~ 0.5% to 2.0%.[10,11] Boa-Sorte et al.[12] used the Imunoscreen test and reported 1.88% (95%CI: 0.6–2.71) of positive IgM results among pregnant women, which suggests that the sample of the present study may have been insufficient to identify acute cases. As the parameters of sensitivity, specificity, and ROC curve are independent of the prevalence of the studied event, it was possible to add IgM reagent samples to the total of samples originally calculated and to carry out an analysis of IgM results.[13]

The test under study was compared with a reference test which, although presents high sensitivity and specificity, may not represent all of the true positive and negative results of the individuals. The unavailability of a "gold standard" test for the detection of IgM antibodies to toxoplasma makes it difficult to evaluate new tests, and may perpetuate inherent errors in the reference test. Several investigators have already shown that the accuracy of the tests differ markedly, depending on the use of selected or routine sera.[14] Therefore, positive IgM test results should be confirmed by additional tests (such as the IgM immunosorbent agglutination assay [ISAGA] and differential agglutination, for example) in laboratories experienced in the diagnosis of toxoplasmosis, or by demonstration of a significant increase in antibody titers in serial serum samples with intervals of at least 3 weeks that run parallel to prevent misinterpretation.[6,15]

There were 12 false-negative IgG results in filter paper. When analyzed, the serum results were compatible with those of long-term acquired infection, that is, low IgG, non-reactive IgM, and high avidity of IgG. As these pregnant women were considered susceptible according to the filter paper results, the serology was repeated during the routine prenatal follow-up, which allowed for diagnostic opportunities. In total, 9 cases of false-positive IgG results in filter paper were observed, but a serological follow-up was not performed, since those pregnant women were considered infected before pregnancy. Although they represented only 1.2% of the total cases, this occurrence should be minimized as much as possible.

A total of 10 pregnant women with false-positive IgM results in filter paper had mandatory confirmation of serology in the serum samples, and the results could be correctly reclassified.

When the indeterminate results were included as positive (►**Table 2**), a good agreement was found between the Imunoscreen and the reference tests for IgM, and a very good agreement for IgG, a plausible option at the MG-CTCP workflow, which repeats all the exams with indeterminate results. When the prevalence-adjusted Kappa for IgM was used, as this antibody has low prevalence (1% to 2%) in the population, as observed in the present study, a very good agreement between the tests was found, regardless of the inclusion of indeterminate results.[15] However, some authors argue that the value of Kappa obtained with adjustment according to prevalence may not reflect reality, and may suggest that corrections must be made regarding sample size.[16] On the other hand, lower Kappa values for IgG and lower IgG sensitivity in filter paper were observed when indeterminate results were included among the negatives, with significant difference. Likewise, most of the indeterminate IgG antibodies detected corresponded to positive values in low titers, from past infections, or even from early infections in a few cases. This finding suggests the need for adequacy in the cut-off initially used; however, this did not represent a problem for the MG-CTCP, since indeterminate results were always confirmed with a new serum sample.

Conclusion

Considering the high prevalence of toxoplasmosis in Brazil, the risk of acute infection in pregnant women, and the severity of congenital toxoplasmosis, screening for infection enables adequate prevention and may be cost-effective. The good performance of dried blood samples makes this a promising method for countries with continental dimensions, limited resources, and high prevalence of toxoplasmosis, such as Brazil.

Contributors

All of the authors contributed to the project and data interpretation, the writing of the article, the critical review of the intellectual content, and approved the final version to be published.

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Conflict of Interests

The authors have no conflict of interests to declare.

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Gynecological/Obstetric Background and Rheumatoid Arthritis: A Cross-sectional Study in Brazilian Patients

Antecedentes ginecológicos/obstétricos e artrite reumatoide: Um estudo transversal em pacientes brasileiros

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Abstract

Objective To study a sample of rheumatoid arthritis (RA) patients for their gynecological/obstetric history and compare them to controls to determine their influences on number of pregnancies, menarche, menopause and reproductive years following RA onset.

Methods This is a cross-sectional study of 122 RA patients and 126 controls. Patients and controls were questioned about age of menarche, age of menopause, number of pregnancies and abortions. Reproductive years were calculated as the difference between age at menopause and age at menarche. For comparison, we used the Mann-Whitney, unpaired *t*, chi-squared, and Spearman tests. The adopted significance was 5%.

Results In the RA patients with disease beginning in the postmenopausal years, the period of reproductive years (age at menopause – age of menarche) showed a positive correlation with age at disease onset (rho = 0.46; 95% confidence interval [CI] = 0.20–0.55 with $p = 0.0008$). The number of pregnancies was higher in patients with postmenopausal disease onset when compared with those with premenopausal disease onset (median of 3 with interquartile range [IQR] = 2–4 versus median of 2 with IQR = 1–3; $p = 0.009$), and RA patients had more pregnancies than controls ($p = 0.0002$).

Conclusion The present study shows that, in our population, the duration of reproductive years and the number of pregnancies are linked to the onset of RA.

Keywords
- rheumatoid arthritis
- pregnancies
- menarche
- menopause
- postmenopause

Resumo

Objetivo Estudar uma amostra de pacientes com artrite reumatoide (AR), com investigação da história ginecológica e obstétrica, comparando-a com controles, visando conhecer suas influências no número de gestações, menarcas, menopausa e anos reprodutivos no início da AR.

Métodos Trata-se de um estudo transversal de 122 pacientes com AR e 126 controles. Pacientes e controles foram questionados sobre idade da menarca, idade da menopausa, número de gestações e abortos. Os anos reprodutivos foram calculados com a

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diferença entre a idade da menopausa e a idade da menarca. Para comparação, foram utilizados Mann Whitney, Teste t não pareados, Teste qui-quadrado e teste de Spearman. A significância adotada foi de 5%.

Resultados Nas pacientes com AR e início da doença na pós-menopausa, o período de anos reprodutivos (idade da menopausa - idade da menarca) apresentou correlação positiva com a idade de início da doença (rho = 0,46; intervalo de confiança de 95% [IC95%] = 0,20–0,55 com $p = 0,0008$). O número de gestações foi maior nas pacientes com início da doença no período pós-menopausa quando comparadas às pacientes em pré-menopausa (mediana de 3 com intervalo interquartil [IIQ] = 2–4 versus mediana de 2 com IIQ = 1–3; $p = 0,009$). Nas pacientes com AR, foi observado um maior número de gestações do que no grupo controle ($p = 0,0002$).

Conclusão O presente estudo mostra que, em nossa população, a diminuição dos anos reprodutivos e o alto número de gestações estão relacionados ao surgimento da AR.

Palavras-chave
- artrite reumatoide
- gestação
- menarca
- menopausa
- pós-menopausa

Introduction

Rheumatoid arthritis (RA) is the most common connective tissue disease, with a prevalence of 1% in the general population.[1] Similar to other connective tissue diseases, RA has a female predominance; the ratio ranges from 4 women to 1 man, when the disease begins in the reproductive years, to 2 to 1, when it initiates after 60 years.[2,3] Hormonal influences have been considered to play a role in this female preponderance as estrogens are considered to be agents with proinflammatory activity and capable of activating B cells.[2,4]

Rheumatoid arthritis and female reproduction have been linked in the literature for decades, and the decrease in its symptoms during pregnancy is well recognized, with exacerbation of the disease in the postpartum period.[5] The gynecological and obstetrical history have been studied in this context; however, contradictory results have been obtained. According to an epidemiological investigation in a Swedish cohort, having more than one pregnancy amplified the risk of anti-citrullinated-protein antibody (ACPA)-negative RA in females of reproductive age.[6] Nevertheless, Guthrie et al.[7] studied 310 RA patients and 1,418 controls and found that parous women were around 40% less likely to receive the RA diagnosis.[6,7] Another study, by Peschken et al.,[8] also showed that greater parity reduced the chances of being diagnosed with RA. In addition, a relationship between higher number of pregnancies and delayed onset of RA has been found.[7,9]

Age at menopause has also been considered a risk factor in cases of postmenopausal RA, with data suggesting that early menopause increases the risk of this disease.[10,11] A Norwegian study evaluating 156 women with RA found reduced parity compared with the control group, suggesting decreased fertility in RA patients.[12]

Rheumatoid arthritis is a disease with genetical and environmental factors that may combine with hormonal factors to influence the risk of developing it.[2,13] Therefore, conclusions on the features that can influence the onset of RA, such as gynecological and obstetrical history, may vary depending on the studied geographical region. Herein, we studied a sample of patients with RA, regarding their gynecological/obstetric history, and compared them to controls in order to determine the influence of the number of pregnancies, menarche, menopause, and reproductive years in the onset of RA, specifically in our region (south of Brazil).

Methods

This is a cross-sectional study approved by the local Committee of Ethics in Research under protocol number 31727220.3.0000.0103 from the Mackenzie Presbyterian Institute; written consent was obtained from all patients. It studied 122 RA patients and 126 controls. To be included, RA patients had to fulfill at least 6 points in the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria for RA.[1,14] Patients with disease onset prior to 16 years of age (juvenile form) or having any other associated inflammatory disease were excluded. This is a sample that encompasses all female patients with RA diagnosis from a single rheumatology unit from a university hospital that has a specialized rheumatoid arthritis outpatient clinic and that visited for regular consultations during the period of June to July 2020.

Patients and controls were questioned about the age of menarche, age of menopause, number of pregnancies, and number of abortions. Pregnancies and abortions were classified as appearing prior to or after RA onset. Reproductive years were calculated as the difference between age at menopause and age at menarche. The charts of RA patients were reviewed for clinical data (age of disease onset, and presence of RA clinical criteria) and serological data (rheumatoid factor). The control females were hospital employees and their relatives.

The results were analyzed with the help of the software Graph Pad Prism version 6.01 (Graph Pad Software, San Diego, CA, USA). To analyze the data distribution, the Shapiro-Wilk's test was used. The central tendency of parametric data was expressed in mean ± standard deviation (SD) and of non-parametric data as median and interquartile range (IQR). For comparison of numerical data (age, age of menarche, menopause, number of pregnancies, and number of abortions), we

used the Mann-Whitney or unpaired *t*-tests. To compare nominal/categorical data (number of children/female and number of individuals at menopause), we used the chi-squared test. The correlation of the number of reproductive years with age at disease onset in RA patients with postmenopausal onset was done with the Spearman test. The adopted significance was 5%.

Results

The RA Studied Sample

The group of 122 RA patients had 290 children (2.3/patient). The description of the RA studied sample is on ►**Table 1**.

In this sample, only 12 children from 12 mothers were born after the RA diagnosis had been made. No differences were found in the age of menarche, menopause, number of children and of abortions when RA patients with positive rheumatoid factor (RF) were compared with those with negative RF (►**Table 2**).

►**Table 3** shows the comparison of patients with pre and postmenopausal disease onset.

In the subset of patients with RA that had postmenopausal disease onset , the period of reproductive years (age at menopause – age of menarche) showed a positive correlation with age at disease onset (rho = 0.46; 95% confidence interval [CI] = 0.20–0.55 with $p = 0.0008$).

Table 1 Description of the studied sample of rheumatoid arthritis

Variables	
Mean age (years) ± SD	57.2 ± 10.2
Median disease duration (years) (IQR)	9.5(6.0–17.0)
Mean age at diagnosis (years) ± SD	45.2 ± 12.0
RA beginning in post-menopause period (n)	49/121 (40.4%)
Median age at first son (years) (IQR)	22 (20–25)
Median pregnancies (n) (IQR)	3.0 (2.0–4.0)
One child	23/122 (18.8%)
Two children	32/122 (26.2%)
Three children	32/122 (26.2%)
Four children or more	35/122 (28.6%)
Median abortions (n) (IQR)	0 (0–0)
Positive rheumatoid factor (n)	78/122 (63.9%)

Abbreviations: IQR, interquartile range; n, number; SD, standard deviation.

Table 2 Comparison of gynecological/obstetric background in rheumatoid arthritis patients according to the presence of rheumatoid factor

Variables	With positive RF N = 78	With negative RF N = 44	*p*-value
Median age at first pregnancy (IQR)	23.0 (19.0–26.0)	22.0 (20.0–25.0)	0.50
Disease onset prior to menopause	44/78 (56.4%)	28/44 (63.3%)	0.43
Median pregnancies (n) (IQR)	2.5 (2.0–4.0)	3.0 (2.0–3.7)	0.25
Median number of abortions	0 (0–0) 0–2	0 (0–0.75)	0.79

Abbreviations: IQR, interquartile range; n, number; RF, rheumatoid factor.

Table 3 Comparison of rheumatoid arthritis patients with disease onset prior and postmenopause

Variables	Fertile women N = 72	Women at menopause N = 50	*p*-value
Age at disease onset (n)	40 (31–45.7)	56 (50–60)	< 0.0001
Median age of menarche (years) (IQR)	13 (12–14)	13 (12–15)	0.57
Median pregnancies (n) (IQR)	2 (1 -3)	3 (2–4)	0.009
Number of abortions (n) IQR	0 (0–1)	0 (0–0)	0.96
Rheumatoid factor	44/72 (61.1%)	34/50 (68%)	0.43

Abbreviations: IQR, interquartile range; n, number.

Comparison of Obstetrical and Gynecological History in RA and Controls

The comparison of these two samples is on ►**Table 4**.

When the age of menopause of controls was compared with the age of menopause in RA patients with

Table 4 Comparison of gynecological/obstetric history of rheumatoid arthritis patients with controls

Variables	RA patients N = 122	Controls N = 126	*p*-value
Median age (years) (IQR)	57.0 (51.0–64.2)	55.0 (50.0–60.5)	0.10
Postmenopausal females (n)	78/122 (63.9%)	81/126 (64.2%)	0.95
Median menarche age (years)	13.0 (12.0–14.7)	12.5 (12.0–14.0)	0.02
Median age at menopause (years (IQR)	48.0 (45.0–51.0)	50.0 (47.0–51.5)	0.02
Median pregnancies (n) (IQR)	2.0 (2.0–3.0)	2.0 (1.0–2.0)	0.0002
Pregnancies number/female			< 0.0001
Nulliparas (n)	0	12/125 (9.6%)	
One child (n)	26/122 (21.3%)	28/125 (22.4%)	
Two children (n)	40/122 (32.7%)	55/125 (44.0%)	
Three children (n)	30/122 (24.5%)	19/125 (15.2%)	
Four children or more (n)	26/122 (21.3%)	10/125 (8.0%)	
Median abortions (n) (IQR)	0 (0–0)	0 (0–1)	0.07

Abbreviations: IQR, interquartile range; n, number.

postmenopausal disease onset, we found that in controls the median age of menopause was 50 years (IQR = 47.0–51.5), while it was 46.0 (IQR = 42.7–50.0) in RA patients, with $p = 0.003$.

Discussion

Our results have shown that RA patients had earlier menopause and later menarche than controls, suggesting that reproductive years in RA females are diminished. They also show that, if the number of reproductive years increases, the disease onset is delayed. These results point to a protective role of the length of reproductive years in the occurrence of this disease. Finding an early age for menopause in RA is consistent with previous results. A study with the Nurses' Health Study (NHS) cohort showed that early menopause increased the risk of RA (HR = 2.1).[11] Another study found that menopause onset at an age earlier than 40 years old more than doubled the risk of RA (odds ratio [OR] = 2.5).[15]

Although estrogen is broadly considered to have a proinflammatory activity, its action is much more complex; this hormone may have diverse effects on the immune system. according to its concentration, on tissue receptor expression, and even on the female's reproductive stage.[2] Estrogens at periovular to pregnancy serum levels are capable of increasing B-cell responses, driving antibody secretion in healthy and autoimmune situations.[16] At similar levels, it stimulates the secretion of IL (interleukin) -4 and IL-10 and inhibits tumor necrosis factor (TNF) production, downregulating T cell-dependent immunity.[16] The secretion of IL-1B (a proinflammatory cytokine) by monocytes and macrophages is increased at periovulatory to early pregnancy levels; however, it is inhibited at late pregnancy levels.[16]

Binding to the different receptors may also modulate the influence of estrogen on the immune system. Synovial cells of RA joints have both estrogen receptors, ER-α and ER-β, with higher density of ER-β, which is usually upregulated under hypoxic and inflammatory circumstances such as arthritis.[17] Studies in animal models have shown that the use of selective ER-β estrogens may have a repressive effect in the transcription of proinflammatory genes[16]; this has led to the attempt to use them in the treatment of RA, unfortunately with negative results.[18]

The number of pregnancies has been considered protective for RA by some authors but not by others.[7,8,10] In the present work, we found that RA patients have significantly more children than controls. In addition, the number of pregnancies was higher in patients with postmenopausal diagnosis. Altogether, these data suggest that the number of pregnancies is linked positively to this disease appearance. Nevertheless, it is important to remember that the premenopausal women are still fertile, and the number of pregnancies may increase, blurring this difference. Another point to pay attention to and that may have caused possible interpretation bias within this data are that we did not collect information on breast feeding. Although controversial, some authors found that long duration of breast feeding was associated with increased risk of developing RA.[17,19,20] Prolactin is considered both a hormone and a cytokine; it has an immune stimulatory effect, inhibiting the negative selection of autoreactive B lymphocytes and promoting autoimmunity.[21]

The present work has several limitations: its cross-sectional design is one of them; another one is not having data on breast feeding. Also, it would have been interesting to analyze the influence of the gynecological/obstetric background according to the anti-citric citrullinated peptide (CCP) positivity. However, it does highlight the importance of menarche and menopause ages in the risk of developing RA, showing that more studies in this area could bring important information for the understanding and treatment of this disease.

Conclusion

In conclusion, the present study shows that, in our population, the decrease in reproductive years and the high number of pregnancies are linked to the onset of RA.

Collaborations

All of the authors contributed with the project and data interpretation, the writing of the article, the critical review of the intellectual content, and with the final approval of the version to be published.

Conflict of Interests

The authors have no conflict of interests to declare.

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Barriers to Puberty Talk between Mothers and Daughters: A Qualitative Study

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Abstract

Objective The aim of the present study is to explain the barriers to puberty talk between mothers and daughters.

Methods In the present study, the conventional content analysis method was used. The present study was conducted from September 2018 to August 2019 in Iran. The study population consisted of mothers and adolescent girls. The data was collected using purposeful sampling method. The sample consisted of 4 mothers and 6 girls that were interviewed using semistructured interviews. Data collection continued until data saturation was achieved. Data analysis was conducted as described by Graneheim et al. using NVivo 11 software.

Results In the present study, after exploring the views of the participants about barriers to puberty talk between mothers and daughters, one dominant theme emerged. Puberty talk is seen as an "inappropriate talk with a girl." There were several subthemes, including "lack of mother's awareness regarding the school role, the busy schedule of the mother, and the adoption of alternatives to mother's talk with girls".

Conclusions Different sociocultural factors affect puberty talk between mothers and adolescent girls. It is important that mothers and policy makers take these barriers into account.

Keywords
- content analysis
- puberty
- girls
- mothers
- qualitative study

Introduction

The World Health Organization (WHO) defines adolescence as the period between childhood and adulthood in the age range between 10 and 19 years old. It marks the start of reproductive age. Adolescence is a period of life characterized with typical health, progressive, and authorized requests. Various dimensions of this period include biological processes, such as physical growth and weight gain, changes in body structure, and the development of the reproductive and sexual system. These typical descriptions are affected by socioemotional processes in different societies worldwide.[1–3]

Psychological problems, infectious diseases, unsuccessful marriages, premature and risky pregnancies, injuries and deaths of mothers and children, as well as lasting physical and psychological problems can have a huge bearing in this period.[4] The fundamental health concerns caused by rapid changes during puberty are the source of the curiosity of adolescents. These concerns are related to physical, psychological, and mental changes, which highlight the psychosocial feature of sexual maturity.[5]

Since adolescence is a time of vulnerability, obtaining health-related information from different sources is crucial to healthy choices made by adolescents.[6] There are three main

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sources of puberty information, including "mass media and their message about puberty," "menstruation and related facts," and "female-centered education through school and extracurricular programs, relevant books, and capacity building in the society by educating teachers and parents".[7]

The interaction of parents (as proximal social determinants of health) and adolescents, and the subjective norms/values of the parents and their role in the growth characteristics of adolescents can affect either positively or negatively their general health and welfare.[8,9] A study by Rembeck et al.[10] of the experiences of two girls, during adolescence exhibited that forging an active and close relationship with one's mother is essential in relieving stress and obstacles caused by the physical and sensitive changes in participants, which underscores the role of mothers as the first and most reliable source of support.

The results of a study performed in the USA showed that only 2% of teenagers gained their information from health staff.[11] Thus, available resources, convenience, sources of reference, educational meetings, and types of services can affect health practice and, therefore, improve or discourage health behaviors.[12] In girls going through menarche, mothers were the major source of information. Other sources, such as relatives (including an older sister) and teachers were scantly mentioned, and there were some regional disparities. In 41 studies, mothers were introduced as the source of information, with half of the girls reporting their mothers as the main source.[13] The Indonesia Demographic and Health Survey in 2012 reported that a quarter of the adolescent girls did not talk about menstruation with anyone before menarche, and that 17% of them were not aware that menstruation was a physical sign of puberty.[13]

The study by Olfati et al.[14] in Iran showed that the knowledge the girls regarding puberty and their health attitudes and behaviors are significantly lower than expected due to the lack of proper information offered by parents to girls. The results of a study on Egyptian teenage girls exhibited that their level of knowledge, beliefs, and health presentation about puberty and menstruation was low.[15] Also, another study in Baghdad found that teenage girls were not sufficiently aware of puberty and menstruation, and this restrained their health practice due to their beliefs.[15]

The points discussed above suggest that puberty talk between mothers and daughters is very important, but that this communication is affected by a variety of cultural, social, and economic factors, among others. However, some of these factors are still unknown. Despite multiple quantitative research and other studies conducted in Iran and in other countries,[16–18] some facets remain relatively unknown. In other words, people in different societies face various challenges. There are few resources available about barriers to puberty talk between mothers and daughters. Therefore, the present study was conducted to contribute to the literature in this field.

Methods

The present study was conducted using qualitative and descriptive interviews with adolescent girls and mothers to explore their perspective on the meaning of puberty. This design offers a deeper insight into this issue, which is obscure and relatively unknown.

Setting and Participants

The sample consisted of six girls and four mothers. They were recruited through verbal invitation, with a letter that explained the goals of the research. They were also assured of the confidentiality and anonymity of data reporting and management. The participants could withdraw at any moment during the process of data collection. Prior to the study, an informed consent form was obtained from the participants. The inclusion criteria were: speaking Farsi, experience of puberty, and having a daughter who has experienced puberty. Reluctance to continue the interview was excluded from the present study. A total of 6 women and 11 girls were invited to participate in the study; 2 women refused to participate in the survey due to time restrictions, and 1 girl withdrew from the study due to discomfort with an interview.

Data Collection

The interviews were conducted by an M.P, who is a midwife and expert in the subject. She collected data using face-to-face interviews with the participants. The present study was conducted from November 2018 to July 2019. For this purpose, a semistructured questionnaire was designed. The interviews with the girls began with an open-ended question about how the girls experienced puberty. Some of interview questions included: "Would you like to explain puberty to me?" or "Which factors do affect puberty talk between your mother/ girl?" There were some probe questions used during interviews, such as: "Can you elaborate more on this subject?" or "Can you give me an example for this issue?" All participants were interviewed at school, in a private room. Individual interviews lasted between 30 and 45 minutes. All interviews were voice-recorded using an MP3 recorder (model ICD-PX470, Sony electronic inc. Made in China), and then were transcribed verbatim. The interviews were recorded in Persian language and then translated into English. The participants were selected from among diverse individuals. Data collection was sustained until saturation was achieved. that is, after 8 interviews. The last two interviews did not add any extra information.

Data Analysis

Data collection and analysis were conducted simultaneously. The conventional content analysis method was used for data analysis, as described by Graneheim et al.[19] The interviews were reread and relistened to reinforce deeper engagement with the data. Codes were involved to small parts of the transcripts using NVivo 11. Emerging themes were identified and checked to determine their relevance regarding the data. Initial themes were refined and divided into subthemes by reviewing the data to establish clear patterns. Quotes that explained the themes were assigned a code. A total of 1,235 codes was achieved after merging 3 subthemes, and 1 theme was obtained.

Rigor and Reflectivity

In the present study, we used several methods to ensure methodological rigor. All researchers were conversant with research methods and expert in the field of midwifery and puberty-related matters. Field notes were obtained from each interview (data triangulation). Throughout the study, we reflected on the analytic process (the investigator's triangulation). We also held meetings with the research team to discuss scientific and organizational aspects of the study (peer debriefing). The writing of the present article was guided by the combined criteria for reporting qualitative research (COREQ).

Results

The study participants comprised 10 mothers and girls (4 mothers with adolescent girls at the age of puberty and 6 girls). The mothers were in the age range between 39 and 51 years old, and the girls were between 13 and 15 years old. The demographic characteristics of the participants are presented in ►**Table 1**. Quotes are shown in italics. The statements belonging to mothers and girls are marked with M and G, respectively. In exploring the views of the participants about the barriers to puberty talk between mothers and girls, one dominant theme emerged: "Inappropriate relationship with girls."

Other subthemes included "lack of mother's awareness regarding the role of schools, busy schedule of mothers, and replacement of communication with mother."

Table 1 Characteristics of the Participants*

Characteristics	Value
Mothers	
Employment status	
Employed	2(50)
Housewife	2(50)
Race	
Iranian	4(100)
Number of children	
>2	2(50)
≥ 2	2(50)
Income status	
$>$ \$45	1(25)
\$45–100	2(50)
$\geq$ \$100	1(25)
Civil status	
Married	4(100)
Level of education	
Diploma	1(25)
B.A degrees	2(50)
M.A degrees	1(25)
Experience of raising an older daughter	
Yes	2(50)
No	2(50)
Girls	
puberty experiences, y	
>3	2(33.33)
≥ 3	4(66.67)
Nationality	
Iranian	6(100)

*Values are expressed as N (%).

Inappropriate Relationship with the Girl

In the present study, mothers and girls admitted that one of the main barriers to the puberty talk between mothers and daughters was an inappropriate relationship. This dominant theme comprised three subthemes, which are described below (**Chart 1**).

Lack of Mother's Awareness Regarding the Role of the School

Lack of mother's awareness regarding the role of the school was one of the main barriers to appropriate puberty talk between mothers and daughters. This category covered two issues: "lack of mother's awareness regarding the role of the school's health counselors and delegation of maternal duties to the school's health educators."

In the view of mothers, schools provide complete information about puberty to their daughters, and they do not need to discuss these issues with their daughters. In other words, they delegated their maternal job to the school. This overreliance on the school undermined the interaction of mothers and adolescent daughters.

Chart 1 "Inappropriate relationship with girl" as a dominant theme in barriers to puberty talk between mothers and daughters

Theme	Subtheme	Category
Inappropriate relationship with girls	Mother's unawareness of the role of the school	Mother's lack of awareness regarding the role of school health educators.
		Delegating maternal duties to the school's health educators.
	Busy schedule of the mother	Employed mothers
		Mother's responsibility to take care of other children
	Using alternatives to mother's talk with girls	Communication with peers
		Communication with an older sister
		Communication on the Internet
		Generational gap and disparity of views

The statement by one of the mothers (M3, 41 years old, employed) lends credit to this point: "I think the school offers girls all the necessary information about puberty and, hence, I do not need to talk to my daughter about it."

Mothers with a Busy Schedule

According to the participants, one barrier to this mother-daughter communication was the busy schedule of the mothers. "Employed mother and the responsibility of taking care of other children" fall into this category. In the case of employed mothers, they are too busy to talk about puberty with their daughters due to their occupational concerns. When there are many children and mothers must take care of other children, they also do not have enough time to talk with their daughters about puberty.

For example, one of the girls (G2, 14 years old, 4 years of puberty experience) asserted, "…my mother does not have time to talk about this sort of things to me. She is employed and, when coming back from work, she is too tired to talk." On the other hand, a mother (M2, 49 years old, housewife) stated: "I have two other children to look after, and this leaves me no time to talk about puberty with my daughter."

Using Alternatives to Mother's Talk

"Generation gap in terms of views, talking with peers or an older sister, and online communications" are other categories of this subtheme.

According to the participants, the generation gap between mothers and daughters is the cause of divergent beliefs between the two generations. As a result, they prefer to discuss these puberty issues with their peers as a way of substituting the relationship with their mothers.

As stated by one of the girls (G4, 15 years old, 6 years of puberty experience), "I can easily talk about puberty with my peers because we understand each other."

Intellectual differences between generations present another barrier to proper puberty talk between mothers and girls.

However, there are disparity in values, beliefs, and lifestyles due to the development of the society.

This divergent lifestyle is viewed as a barrier to puberty talk between mothers and daughters.

This point is raised by one of the girls (G6, 15 years old, 5 years of puberty experience): "Due to the intellectual and generational difference between my mother and I, we have trouble understanding each other, since we have different values and attitudes."

Mothers who had older girls suggested that their adolescent girls preferred to talk with their older sisters because the older sister could resonate with their experience more effectively. Girls feel less ashamed and embarrassed to talk with their sister rather than with their mother.

This point was also underscored by another girl (G1, 13 years old, 2 years of puberty experience): "I have an older sister and I ask her most of my puberty questions, because I'm ashamed of talking to my mother about these things."

Girls' access to the cyberspace is another barrier to effective talk between mothers and their daughters. The girls can easily obtain necessary information about puberty from the Internet.

In this regard, one of mothers (M1, 51 years old, housewife) mentioned: "Today, children have access to the Internet, and my daughter gets all the information she needs from the Internet, so she talks less about these issues with me."

Discussion

All family members are involved in teaching puberty subjects to adolescents, but the role of the mother is more significant than that of others, as adolescent girls often learn healthy behaviors from their mothers.[20–22] Some studies have also reported that parents are the key sources of information about reproductive health for their children. According to the study by Nwagwu in Nigeria, the major sources of information about reproductive health of adolescent girls are parents (56.1%), friends (53.18%), books (45.56%), teachers (44.15%), the Internet (45.19%), and health centers (54.14%).[23]

Many studies in Iran and in other parts of the world have shown that peers and the media are the primary sources of information about the reproductive health of adolescent,[24,25] which is at odds with evidence from India, Nagpur, Tigray, and Nigeria, where mothers are the major source of information.[26–28]

In our study, communication through cyberspace and peers were major sources of information, which is consistent with the aforementioned studies. We found that these forms of communication substituted puberty talk between mothers and daughters. Kumar et al.[29] demonstrated that mothers and sisters are the main source of information about puberty for 75 and 8.64% of girls, respectively. The findings of our study revealed that girls preferred talking to their older sister, which is aligned with the aforementioned results.

Mothers can answer many of the questions of the girls regarding reproductive subjects, which can improve the quality of the mother-daughter relationship and mitigate the load of physical, psychological, and social problems and unhealthy behaviors associated with adolescence.[30] It seems that mothers are the best reference to provide basic information about reproductive health to their daughters, but the sense of embarrassment, insufficient knowledge, superstitions, and misunderstandings of mothers about reproductive health can deter them from imparting their knowledge to the adolescents.[31] Mothers need to be sufficiently aware of the physical and emotional changes of their teenagers.[32]

In the study by Shahhosseini et al.,[33] healthcare providers highlighted the role of mothers as the most reliable source of information for adolescents. From the perspective of mothers, describing menstruation to adolescent girls is their duty, but they pointed to the difficulty of explaining menstruation to the girls.[34] Contrary to these findings, our results suggested that the lack of awareness by part of the mothers regarding the role of school health educators and delegating maternal duties to school health educators deter them from getting involved in in puberty education of their daughters. In line with our findings, another study has shown that students are not happy about receiving sexual education from their families.[24]

In the study by Kamalikhah et al.,[24] parents were weighed barrier to discussions about sexual reproductive health. Talking about sexual health issues with adolescents was associated with a sense of embarrassment in some mothers.[35] As a result, the relationship between parents and adolescents should be fostered through cooperative training programs. Some studies have stressed the effects of formal education on health issues of female students.[35]

An inappropriate mother-teenager relationship can hamper a teenage girl's access to the most vital sources of information, paving the way for flawed sources of information, which can provoke a plethora of health problems.

Our results suggested that employed mothers usually do not have enough time to talk with girls about puberty, since they spend most of their time at work. Also, mothers who take care of other children at home lack time to talk about puberty with their daughters.

Unlike the results of our study, the results of a study in Nigeria revealed a significant statistical association between the employment of mothers and the practice of good menstrual hygiene.[36]

The findings of our study have shown that the generation gap between mothers and daughters is also a barrier to proper communication between mothers and girls. This intellectual distance may lead girls to seek guidance from friends or sources other than their mothers regarding puberty issues.

Therefore, it is necessary that health professionals adopt an approach that stresses training mothers and informing girls about puberty. These training programs for mothers should address how to forge a close mother-daughter relationship and how to remove barriers to the discussion of puberty or of the health consequences that originate from the lack of knowledge.

Conclusion

The present study was conducted in Iran, so the findings might have limited generalizability. However, this is an essential characteristic of all qualitative studies. However, the results of the present study could be generalized to societies with the same cultural backgrounds. Another limitation of the present study is the lack of interviews with counseling teachers who advise girls in schools. It is suggested that they be interviewed in future studies.

Collaborations

All authors contributed with the project and data interpretation, the writing of the article, the critical review of the intellectual content, and with the final approval of the version to be published.

Ethical Consideration

The present study was approved by ethics committee of the Kerman University of Medical Sciences (number 1395.837). At the outset of all interviews, the interviewer stressed that Participants could leave the study at any time. All interview data was anonymous and securely stored in the university data system. Only researchers with a password had access to the data.

Conflict of Interests

The authors have no conflict of interests to declare.

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Immunological Characteristics between αβ T_{DC} and γδ T_{DC} Cells in the Spleen of Breast Cancer-Induced Mice

Características imunológicas entre células T_{DC} αβ e T_{DC} γδ no baço de camundongos com câncer de mama induzido

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Abstract

Objective To evaluate the antitumoral role of γδ T_{DC} cells and αβ T_{DC} cells in an experimental model of breast cancer.

Methods Thirty female Balb/c mice were divided into 2 groups: control group ($n = 15$) and induced-4T1 group ($n = 15$), in which the mice received 2×10^5 4T1 mammary tumor cell line. Following the 28-day experimental period, immune cells were collected from the spleen and analyzed by flow cytometry for comparison of αβ T_{DC} ($TCR\alpha\beta^+$ $CD11c^+MHCII^+$) and γδ T_{DC} ($TCR\gamma\delta^+CD11c^+MHCII^+$) cells regarding surface markers ($CD4^+$ and $C8^+$) and cytokines (IFN-γ, TNF-α, IL-12 and IL-17).

Results A total of 26.53% of γδ T_{DC}- control group ($p < 0.0001$) - the proportion of αβ T_{DC} was lower in splenic cells than γδ T_{DC}; however, these 2 cell types were reduced in tumor conditions ($p < 0.0001$), and the proportion of IFN-γ, TNF-α, IL-12 and IL-17 cytokines produced by γδ T_{DC} was higher than those produced by αβ T_{DC}, but it decreased under conditions of tumor-related immune system response ($p < 0.0001$).

Conclusion Healthy mice engrafted with malignant cells 4T1 breast tumor presented T_{DC} with γδ TCR repertoire. These cells express cytotoxic molecules of lymphocytes T, producing anti-tumor proinflammatory cytokines.

Keywords
- immunology
- breast neoplasms
- immunotherapy
- T-lymphocytes
- receptors

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Resumo

Objetivo Esclarecer o possível papel antitumoral das células T_{DC} γδ e T_{DC} αβ em um modelo experimental de câncer de mama.

Métodos Trinta baços de camundongos Balb/c analisados por citometria de fluxo, separados entre grupo controle ($n = 15$) e o grupo tumoral induzido por 4T1 ($n = 15$).

Resultados Presença de 26,53% de T_{DC} γδ nos camundongos do grupo controle ($p < 0,0001$), proporção de T_{DC} αβ menor em células esplênicas do que T_{DC} γδ; no entanto, estes dois tipos de células são reduzidos em condições tumorais ($p < 0,0001$), e a proporção de citocinas IFN-γ, TNF-α, IL-12 e IL-17 produzidas pelas célula T_{DC} γδ foi maior do que as produzidas pelas células T_{DC} αβ, mas foram diminuídas sob condições de resposta ao sistema imunológico relacionada ao tumor ($p < 0,0001$).

Conclusão Camundongos saudáveis induzidos ao tumor de mama 4T1 apresentaram T_{DC} com repertório TCR γδ. Estas células expressam moléculas citotóxicas de linfócitos T, produzindo citocinas proinflamatórias anti-tumor.

Palavras-chave
- imunologia
- neoplasias mamárias
- imunoterapia
- linfócitos T
- receptores

Introduction

A new type of immune cell has been described, and these new cells have characteristics of innate and acquired immunity. T_{DC} cells were identified in mice and in humans as cells that express T cell receptors (TCRαβ) specific of T lymphocytes, and simultaneously express the CD11c markers and major histocompatibility complex class II (MHCII or HLA [Human Leukocyte Antigen] in humans), found in innate cells, mainly in dendritic cells. These molecules in the same cell confer unique characteristics and properties; they carry out functions of dendritic cells (DCs) that do not need to be activated by antigen-presenting cells (APCs). When stimulated by specific receptors, such as the family of Toll-like receptors, they can produce interleukin-2 (IL-12) cytokine, as well as process and present antigens.[1]

T lymphocytes respond in a specific manner to pathogens and cancer cells by the recognition of specific antigens due to TCR (T-cell receptor) in their membrane, similar to the role of the immunoglobulins in B cells. The TCR consists of 2 polypeptide chains; ∼ between 90 and 99% of all T cells have the αβ TCR, but a minority has γδ chains.[2,3] Both cells originate from common thymic precursors, but the biological roles and molecular understanding of these two subsets differ substantially. The T lymphocytes that express αβ TCR depend on the presentation of antigens in a defined HLA molecule to be activated, and usually are tolerant to self-peptides. On the other hand, the γδ T lymphocytes do not rely on the recognition of classic HLA molecules, and the identification of tumor antigen is made by ubiquitous changes observed across many individuals, which allows these cells to not undergo the rejection process, and, consequently, they can be transferred more easily between individuals. Unlike αβ T cells that have their biological role well-characterized in cancer immune surveillance, the protective role of γδ cells during tumor development has only been increasingly reported over the past two decades.

The presence of tumor-infiltrating γδ T lymphocytes has been associated with good prognosis in patients with melanoma[4] and gastric cancer,[5] and high levels of these types of circulating lymphocytes have been associated with reduced cancer risk, increased 5-year-disease-free and increased survival after bone marrow transplant for acute leukemia.[6]

The antitumoral ability of γδ T lymphocytes is associated with their synthesis of interferon γ (IFN-γ), and of tumor necrosis factor-α (TNF-α), as well as their cytotoxic potential. Other studies have also reported the role of interleukin-17 (IL-17) produced by γδ T cells, mainly when they act together with immunogenic cell death-inducing chemotherapeutic drugs.[7]

To clarify whether T_{DC} cells could also have the γδ chains and the possible antitumor role of this new cell population, we investigated the T_{DC} population comparing both αβ TCR and γδ TCR T_{DC}, as well as their cytokines in an experimental model of mice engrafted with malignant cells.

Methods

Animals

Thirty 8-week-old female Balb/c mice, kept in the sectoral vivarium of the Oncology Research Institute (IPON, in the Portuguese acronym) of the Universidade Federal do Triângulo Mineiro (UFTM, in the Portuguese acronym), were used. During the 28-day experimental period, the animals were divided into a control group (healthy mice) and a tumor group (4T1 breast tumor cell-engrafted mice). Each group consisted of 15 animals, was housed in plastic cages under a 12-hour light/dark cycle at 21 ± 3°C, with food and water available ad libitum. After the experimental period, the animals were euthanized by overdosing with 50 mg/kg of ketamine and 15 mg/kg of xylazine, and their spleens were removed for analysis. The present study was approved by the Ethics Committee on Animal Use of the UFTM, under number 379/2016 - CEUA/UFTM.

Tumor

The animals were selected at random, and the tumor-induced group was engrafted with 4T1 inoculated with 2×10^5 cells in

the last pair of breasts, on the left mammary gland. Tumor cells of the strain mentioned above are cells isolated from the Balb/c mice spontaneous tumor, with high proliferative, invasive, and tumorigenic power. The cells were maintained in culture in Roswell Park Memorial Institute (RPMI) medium and incubated at 37°C and 5% CO^2 (Water Jacket Incubator 3110, Thermo Fisher Scientific, Marietta, OH, USA). After the culture period, the cells were washed with 0.9% saline solution and centrifuged at 290xg at 4°C for 10 minutes and then inoculated in the group of tumor-induced by 4T1 mice.

Characterization of Immune Cells by Flow Cytometry

The spleens of the control group and of the tumor group were disclosed, filtered, and washed with saline solution, and after counting in a Neubauer chamber, 1×10^6 cells were placed in tubes suitable for the flow cytometry technique. The cells were then labeled with extracellular anti- γδ TCR- antibodies (T lymphocyte receptor), anti-CD11c (adhesion molecules), anti-IA (antigen-presenting molecule), anti-CD4 (helper T lymphocytes), and anti-CD8 (cytotoxic T lymphocytes) – all antibodies acquired from BD Biosciences. After the 30-minute incubation, the cells were washed and prepared to receive the intracellular antibody labels for anti-IFN-γ, anti-TNF-α, IL-12, and IL-17 proinflammatory cytokines. To block nonspecific binding, the antimouse IgG2b Immunoglobulin G2b) - (mouse) Rabbit Monoclonal Antibody (IgG2b), anti-rat IgG2a Immunoglobulin G2a - (mouse) Rabbit Monoclonal Antibody (IgG2a), and antirat IgG2b isotypes were used. The cells were read on the BD FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) cytometer, and the data analyzed using Flowing software.

The gating strategy used was the delimitation by size and granularity (FSCxSSC) of the spleen cells of the control and tumor groups. Subsequently, the double-positive labeling of CD11c and IA (MHCII [Major Histocompatibility Complex - class II]) was limited and, thus, the γδ TCR labeling traced the γδ T_{DC} cells. Within this population of γδ T_{DC}, we analyzed the phenotypic and cytokine markers of interest.

Statistical Analysis

Statistical analyzes and graphs were prepared using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). The Kolmogorov-Smirnov tests were used to verify the normality of the variables. Non-normal samples were analyzed by the Mann-Whitney test, both for comparison between the control group and the tumor-induced group from both profiles and for the comparison of αβ T_{DC} and γδ T_{DC} cell expression. The data obtained were represented with their corresponding median, minimum and maximum values. The difference found between the groups was considered statistically significant when $p < 0.05$.

Results

The flow cytometry profile shows the comparison of αβ T_{DC} ($TCR\alpha\beta^+CD11c^+$ $MHCII^+$) and γδ T_{DC} ($TCR\gamma\delta^+CD11c^+$ $MHCII^+$) cell infiltrates in the spleen of healthy mice engrafted by breast cancer 4T1 cells (►**Figs. 1 a, b, c** and **d**). When analyzing the frequency of the γδ T_{DC} cell profile (►**Fig. 1a**), a significant decrease was found in the tumor group, with a median of 18.11 (17.21–19.01) compared with the control group (26.53; 23.62–29.99) ($p < 0.0001$). The frequencies of both αβ T_{DC} and γδ T_{DC} cells were compared, and a significance was found in the tumor-induced group of both cell profiles, that is, there was a higher amount of αβ T_{DC} cells (47.74; 22.97–57.36) than of γδ T_{DC} cells (18.11; 17.21–19.01) in the spleen of the 4T1 tumor-induced mice group ($p < 0.0001$).

The mean fluorescence of auxiliary T lymphocyte (CD4) and cytotoxic T lymphocyte (CD8) markers present in the αβ T_{DC} and γδ T_{DC} cells of both groups (►**Fig. 1b**) was analyzed, and it was observed that the CD8 αβ T_{DC} cells showed a decrease in the tumor group (764.7; 485.8–1467) in comparison with the control group (1,650; 1,292–3,418) ($p = 0.0012$). Regarding CD4 γδ T_{DC} cells, a significant decrease was found in the tumor group (1,873; 1,421–2,325) compared with the control group (2,350; 2,140–2,561) ($p = 0.0009$), as well as for CD8 γδ T_{DC} cells, of which there was significant decrease in the 4T1 tumor group (329.0; 292.3–692.4) compared with the control group (1,630; 1,370–1,889) ($p < 0.0001$). When comparing both cell profiles, a decrease of CD8 γδ T_{DC} cells (329.0; 292.3–692.4) was found in comparison with CD8 αβ T_{DC} cells (764.7; 485.8–1467) ($p < 0001$) in the tumor group.

The mean fluorescence intensity of the cytokines produced by αβ T_{DC} and γδ T_{DC} cells were analyzed (►**Fig. 1c**). It was noticed that, for IFN-γ αβ T_{DC} cells, there was a decrease in the tumor group (1,554; 705.3–1,885) compared with the control group (4,720; 4,488–6,120) ($p < 0.0001$), as well as a TNF-α αβ T_{DC} decrease in the tumor group (1,655; 403.8–2,673) compared with the control group (2,877; 2,716–4,658) ($p < 0.0001$). Regarding IL-12 αβ T_{DC} cells, a decrease was observed in the tumor group (1,841; 360.7–2,728) compared with the control group (3,686; 2,028–5,163) ($p = 0.0002$), as well as for L-17 αβ T_{DC} cells, which also decreased in the tumor group (578.5; 326.3–873.8) compared with the control group (4,666; 1,117–6,436) ($p < 0.0001$).

In relation to the cytokines produced by γδ T_{DC} cells, a decrease in IFN-γ γδ T_{DC} cells was found in the tumor group (2,349; 1,261–3,429) compared with the control group (5,972; 5,649–6,297) ($p < 0.0001$). Regarding IL-17 γδ T_{DC} cells, a decrease in the tumor group (468.3; 307.1–692.2) was observed when compared with the control group (2,026; 1,563–2,489) ($p < 0.0001$).

Finally, the profiles of αβ T_{DC} and γδ T_{DC} cells were compared, and an increase in the IFN-γ γδ T_{DC} cells of the control group (5,972; 5,649–6,297) was found, when compared with IFN-γ αβ T_{DC} cells (4,720; 4,488–6,120) ($p = 0.0005$). An increase was also found in the TNF-α γδ T_{DC} cells of the control group (5,972; 5,649–6,297) compared with TNF-α αβ T_{DC} cells (2,877; 2,716–4,658) ($p < 0.0001$), as well as an increase in TNF-α γδ T_{DC} cells in the tumor group (2,349; 1,261–3,429) in relation to TNF-α αβ T_{DC} cells (1,655; 403.8–2,673) ($p = 0.0157$). There was a decrease in IL-12 γδ T_{DC} cells in the control group (1,526; 1,290–1,793) compared with IL-12 γδ T_{DC} cells (3,686; 2,028–5,163) ($p < 0.0001$). When comparing IL-17 γδ T_{DC} cells, a decrease was found in

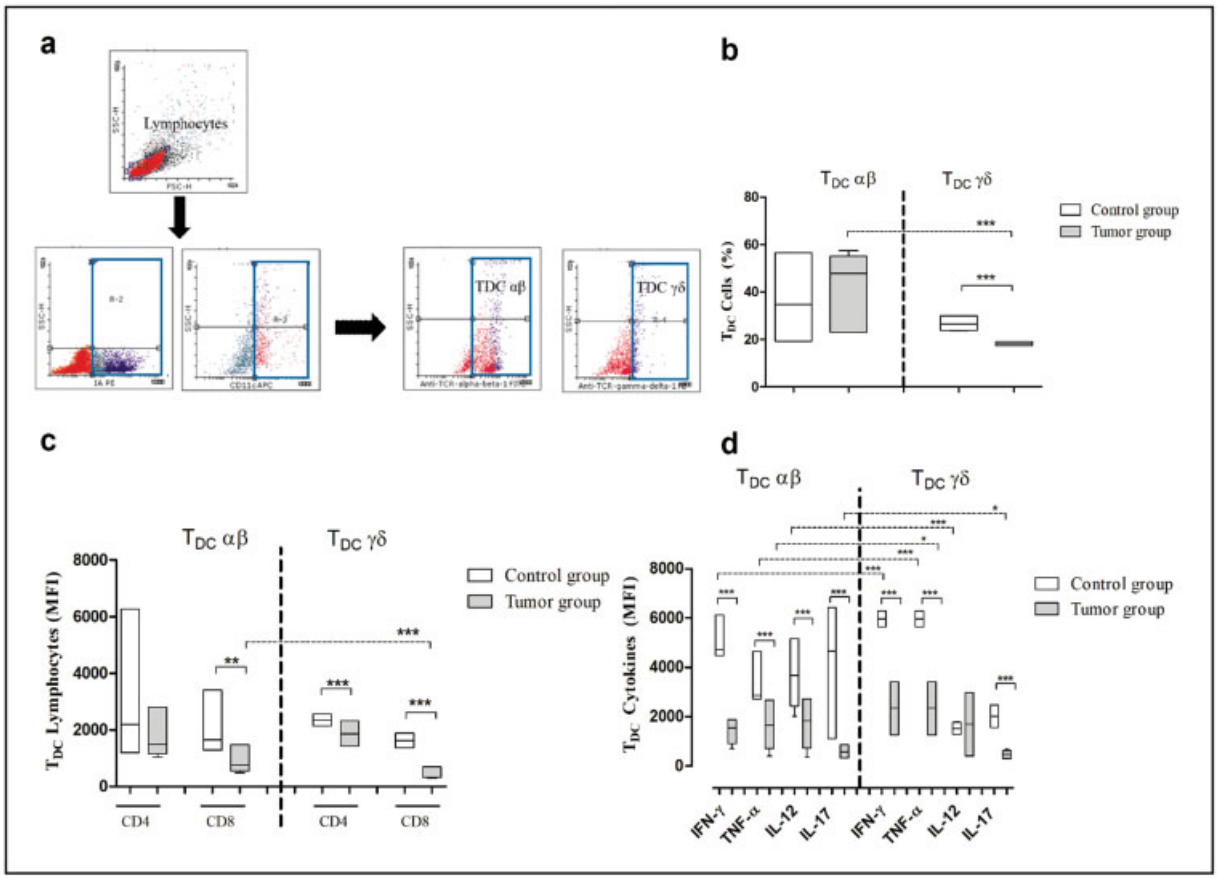

Fig. 1 Comparison of immunological characteristics between αβ T_{DC} and γδ T_{DC} cells in the control and in the tumor group. (a) Representative graph of flow cytometric analysis to identify frequency of αβ T_{DC} and γδ T_{DC} cells in the spleen of the control and of the tumor group. (b) Frequency analysis of αβ T_{DC} and γδ T_{DC} cells in the spleen of the control and of the tumor group. (c) Mean fluorescence intensity of αβ T_{DC} $CD4^+$/γδ T_{DC} $CD8^+$ and γδ T_{DC} $CD4^+$/γδ T_{DC} $CD8^+$ cells in the spleen of the control mice and of the tumor group. (d) Mean fluorescence intensity of αβ T_{DC} IFN-γ, αβ T_{DC} TNF-α, αβ T_{DC} IL-12, and αβ T_{DC} IL-17 and γδ T_{DC} IFN-γ, γδ T_{DC} TNF-α, γδ T_{DC} IL-12 and γδ T_{DC} IL-17 cells in the spleen of the control and of the tumor group. Representative graphs of two independent experiments, $n = 15$ each (median with range). The results were analyzed by the Mann-Whitney test to compare the mean fluorescence intensity of subtypes αβ T_{DC} and γδ T_{DC} cells (statistical differences represented by the dashed line). Differences were considered statistically significant at $p < 0.05$ (5%). $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$.

the tumor group (468.3; 307.1–692.2) in relation to IL-17 αβ T_{DC} cells (578.5; 326.3–873.8) ($p = 0.0157$).

Discussion

Kuka et al[1] described T_{DC} cells ($TCR\alpha\beta^+CD11c^+MHCII^+$) as a cell subtype with properties common to polyclonal T αβ cells and dendritic cells. These rare cells have a morphological similarity to dendritic cells that express intermediate levels of CD11c and present Major Histocompatibility Complex (MHC) class II antigenic molecules. Besides, these cells are also characterized by the expression of costimulatory molecules (CD80, CD86) and lymphocyte surface markers (CD3, CD4, and TCR α/β).[1]

The frequency of αβ T_{DC} cells described by Kuka et al[1] is of ~ 0.04% in the spleen of healthy mice. In our study, it was identified an average of 34.64% in healthy mice and of 47.74% in the group of 4T1 breast tumor cell-engrafted mice. Kuka et al[1] identified and characterized T_{DC} cells by flow cytometry by analyzing the total cells. Our study delimits an area (gate) referring to lymphocytes, size and granulation of this cell type. T_{DC} cells have similar morphology and size to T lymphocytes.[1]

The presence of the cell profile for γδ T_{DC} ($TCR\gamma\delta^+CD11c^+MHCII^+$) was verified in the same conditions, and the presence of a percentage of 26.53% of γδ T_{DC} in the control group and of 18.11% in mice with breast cancer ($p < 0.0001$) was found.

The present study reports that between 1 and 4% of all T cells present in the thymus, in secondary lymphoid organs, and in the lungs of adult mice are γδ T lymphocytes. In mucous membranes, such as the intestinal membrane, there are 25 to 40% of this cell type, where the most significant amount is concentrated,[8] in addition to presenting subtypes as well as phenotypic and functional dieting properties.[9]

In our studies, the effect of a systemic immune response under the influence of tumor cells, which decreased both αβ T_{DC} and γδ T_{DC} cells, was observed. However, when comparing these two cell profiles – αβ T_{DC} and γδ T_{DC} – there was a higher amount of αβ T_{DC} in the 4T1 tumor-induced tumor group than γδ T_{DC} ($p < 0.0001$).

The αβ T cell repertoire is higher in T lymphocytes and, most of the time, they have protective antitumor activity, mainly related to human melanoma tumors.[10]

A study with human blood samples from 38 patients diagnosed with breast cancer compared with healthy controls showed that the proportion of γδ T cells in the circulating blood of healthy controls is 1.6 times greater than in breast cancer patients.[11] These data corroborate with our study, since γδ T_{DC} cells are present in more significant quantities in the control group ($p < 0.0001$).

Concerning γδ T cells, in an antitumor immune response, pioneering studies on the immunoprotective role of these cells in mice were performed in murine models with skin cancer, which were chemically induced by carcinogens or by subcutaneous transfer of melanoma tumor lineage. From these studies, relevant roles of γδ T in antitumor immunity have been described, with mechanisms mediated by the NKG2D C-type lectin-like receptor expressed on NK (NKG2D) receptor by dendritic epidermal T cells (DETCs) Vγ5 + residing in tissues.[12,13]

Studies comparing tumor progression in mice with deficient γδ T cells (due to genetic inactivation of the γδ TCR receptor) versus mice with sufficient γδ T cells (wild) have firmly established the protective role of γδ T cells[10] because it was found that γδ T cells prevented the progression of chemically induced papilloma to cutaneous squamous cell carcinomas. In contrast, αβ cells seemed to favor tumor progression[14]; the same happened with spontaneous B cell lymphomas,[15] prostate cancer[16] and in the transplantable model of melanoma B16-F0.[17] Besides, some studies show, in the context of infections by cytomegalovirus and malaria, that γδ T cells can be activated later, in the form of direct cytotoxicity, by the action of granzyme B and through stimulating effects such as the secretion of cytokines IFN-γ and TNF-α, or by the direct presentation of antigen.[18]

Most γδ T cells, unlike αβ T lymphocytes, do not exhibit CD4 or CD8 coreceptors, so antigen recognition is not restricted to antigen-presenting molecules.[8] Thus, the expression of αβ T_{DC} and γδ T_{DC} cells related to helper T lymphocyte (TCD4) and cytotoxic (TCD8) markers was compared, revealing that the proportion of CD8 γδ T_{DC} is less expressed in splenic cells than CD8 αβ T_{DC}, but these two cell types are decreased in tumor conditions ($p < 0.0001$).

A recently conducted study comparing subsets of γδ T lymphocytes in 40 patients with Chron disease demonstrated a significant decrease in this cell population, concluding that this condition can affect the immune responses against this disease.[19] We believe that, like cancer, the suppressive conditions provided by them can have the same result with αβ and γδ $T_{DC,}$ leading to a deficiency of this mechanism.

The central cytokines produced by γδ T_{DC} presented a higher proportion of this cell type than those produced by αβ T_{DC}. That is, there is a higher production of the IFN-γ, TNF-α, IL-12 cytokines in the control group, and a lower proportion of IL-17 γδ T_{DC} in the group of mice with breast cancer. However, this condition decreases when there is a systemic immune response related to tumors ($p < 0.0001$).

It is inferred that γδ T_{DC} cells are similar to the mechanisms exerted by γδ T cells. Studies show that this cell type is an important precursor source of IFN-γ and TNF-α, which inhibits tumor growth and angiogenesis. Also, the study performed with the combination of concanavalin A (ConA) and interleukin-2 (IL-2) demonstrated the potential for polarization and plasticity of γδ T_{DC} cells, which induced the intense proliferation of these cells and the consequent production of interleukin-12 (IL-12) and interleukin-2 (IL- 18).[20]

In inflammatory conditions, a situation observed in some cancers and infections, they favor the polarization of γδ T cells toward an IL-17 producing phenotype.[21] A recent study of transcriptome sequencing in ∼ 18,000 tumor masses in humans revealed that, among tumor-infiltrating leukocytes, γδ T cells were strongly associated with a good prognosis.[22] In our study, it was observed that γδ T_{DC} in a systemic immune response is suppressed regarding αβ T_{DC} cells in tumor conditions ($p = 0.0157$).

A specific type of γδ T cells (γδ $TCD27^{+}$) from mice secrete the IFN-γ cytokine, responsible for inhibiting tumor angiogenesis and improving the expression of MHC class I by tumor cells, thus promoting efficiency in the responses of CD8+ T cells.[23] In our studies, it was observed that γδ T_{DC} cells expressed IFN-γ in more significant quantities in healthy mice ($p < 0.0001$). In the study with a model of adoptive transfer of γδ T cells in mice against melanoma B16-F0, it was observed that a specific subtype of γδ T $Vγ4^{+}$ (but not $Vγ^{+}$ T cells) had the protective function dependent on its high eomesodermin expression and IFN-γ production.[24]

Even though IFN-γ is the main cytokine produced by mouse γδ T cells, IL-17 is involved in the protective responses of γδ T cells in some cancer models.[25] Interleukin-17-producing γδ T cells cooperated in mediating bladder cancer regression.[26] In another study, IL-17-producing γδ T cells are associated with chemotherapeutic agents (such as doxorubicin) in various models of epithelial tumor transplantation and demonstrated a better antitumor response.[27] In our study, it was identified that IL-17-producing γδ T_{DC} is less frequent in the breast cancer-induced group compared with IL17-producing T_{DC} αβ ($p < 0.0001$). Thus, it can be inferred that, in a systemic antitumor response, these cells may be suppressed by tumor escape mechanisms to antitumor immune responses.

Therefore, according to the data found in the present study, we can conclude that γδ T_{DC} has immunological characteristics shared with conventional effector αβ T_{DC} cells. The healthy mice engrafted with 4T1 breast tumor presented T_{DC} with γδ TCR repertoire. These cells express T helper and cytotoxic T lymphocyte molecules, producing antitumor proinflammatory cytokines, suggesting that γδ T_{DC} could have an antitumor role, and even be used in the future in antitumor immunotherapy. However, new studies investigating its function in other tumor types are necessary.

Contributors
All authors were involved in the design and interpretation of the analyses, contributed with the writing of the manuscript, read, and approved the final manuscript.

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Conflict to Interests

The authors have no conflict of interests to declare.

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Surgical Site Infection after Cesarean Delivery in Times of COVID-19

Infecção de sítio cirúrgico após cesariana em tempos de COVID-19

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Abstract

Objective To analyze effects of the COVID-19 pandemic on the consumption of personal protective equipment and products (PPEP), as well as the frequency of surgical site infection (SSI) among non-COVID-19 patients submitted to cesarean sections.

Methods A retrospective study was conducted in a maternity unity of a public teaching hospital which was not part of the reference service for COVID-19 treatment. It compared PPEP consumption and the occurrence of SSI after cesarean sections in monthly periods before and after the occurrence of the first case of COVID-19 in Porto Alegre, state of Rio Grande do Sul, Brazil. Personal protective equipment and products consumption was measured as units of masks, gloves, gowns, and caps, and use of alcohol-based products or soap for hand sanitation as ml/patient/day. The SSI index was calculated as the proportion of cases of SSI over the number of cesarean sections performed monthly during the study period.

Results There was an increase in all measured items of PPEP, with consumption of disposable masks with a median of 1,450 units in the pre-COVID period, and of 2550 in the post-COVID period (a 75.9% increase). A decrease of 49% in SSI was detected, with a median of 1.74 in the pre-COVID period and of 0.89 in the post-COVID period.

Conclusion The increase in consumption of PPEP could be a result of safer practices adopted by healthcare workers with the advent of COVID-19, of which the following reduction in the occurrence of SSI could be a direct consequence. Despite the severity of the crisis, one could state that extreme situations can lead to valuable reflections and opportunities for improvement.

Keywords

- surgical wound infection
- cesarean section
- personal protective equipment
- coronavirus

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Resumo

Objetivo Analisar os efeitos da pandemia de COVID-19 sobre o consumo de equipamentos e produtos de proteção individual (EPPI), assim como a frequência de infecção de sítio cirúrgico (ISC) em pacientes não infectadas por COVID-19 submetidas a cesarianas.

Métodos Foi realizado um estudo retrospectivo em uma maternidade de um hospital público de ensino que não fazia parte do serviço de referência para o tratamento do COVID-19. Foram comparados o consumo de EPPI e a ocorrência de ISC após cesárea nos períodos mensais antes e após a ocorrência do primeiro caso de COVID-19 em Porto Alegre, RS, Brasil. O consumo de EPPI foi medido em unidades de máscaras, luvas, aventais e gorros, e o uso de produtos à base de álcool ou de sabonete para higienização das mãos em ml/paciente/dia. O índice SSI foi calculado como a proporção de casos de ISC sobre o número de cesarianas realizadas mensalmente durante o período do estudo.

Resultados Houve aumento em todos os itens medidos do EPPI, com o consumo de máscaras descartáveis apresentando uma mediana de 1.450 no período pré-COVID e de 2550 no período pós-COVID (aumento de 75,9%). Detectou-se também diminuição de ISC, com medianas de 1,74 no período pré-COVID e de 0,89 no período pós-COVID, com redução de 49% no valor da mediana.

Conclusão O aumento do consumo de EPPI pode ser resultado de práticas mais seguras adotadas pelos profissionais de saúde com o advento do COVID-19, do qual a redução na ocorrência de ISC pode ser uma consequência direta. Apesar da gravidade da crise, pode-se afirmar que situações extremas podem gerar reflexões valiosas e oportunidades de melhorias.

Palavras-chave

- infecção da ferida cirúrgica
- cesariana
- equipamento de proteção pessoal
- coronavírus

Introduction

The pandemic of COVID-19 reached 30,675,675 cases and 954,417 deaths worldwide as of the 21st of September 2020. Brazil was hit later but, as of the same date, 4,495,183 cases and 135,793 deaths were reported, with an underreporting of unknown magnitude.[1] The World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) issued guidelines on personal protective equipment and products (PPEP) for healthcare workers during the care of suspected or confirmed COVID-19 infection.[2,3] Since the spectrum of the disease ranges from absence of symptoms to the demand of critical care, universal protection for healthcare workers in all settings became a standard of care.[4–6] Due to the high infectivity and potential morbidity and mortality associated with COVID-19, increasing adherence of healthcare workers to safety recommendations was foreseeable.[5,6] The present study aims at analyzing the possible effects of the COVID-19 pandemic on the consumption of PPEP, as well as the frequency of surgical site infection (SSI) among non-COVID-19 patients submitted to cesarean sections.

Methods

A retrospective study was conducted in a public maternal hospital that was not part of the reference services for COVID-19 treatment, comparing PPEP consumption and the occurrence of SSI after cesarean sections in periods determined as pre-COVID and post-COVID, based on the occurrence of the first documented case of COVID-19 in our city. The PPEP consumption was measured as units of masks, gloves, gowns, and caps, and use of alcohol-based products and soap for hand sanitation as ml/patient/day. The SSI index was calculated as the proportion of cases of SSI over the number of cesarean sections performed monthly during the study periods. The SSI was based on the criteria established by the CDC, including cases of superficial incisional SSI, deep incisional SSI, and organ/space infections.[7] Data from April 2019 to February 2020 (pre-COVID) and from March to July 2020 (post-COVID) were compared. All cases of cesarean sections performed in both periods were included, except for five patients, in whom COVID-19 was suspected. Variables were described as median, minimum and maximum. Percentages were accompanied by confidence intervals (CIs). The analysis was performed with IBM SPSS Statistics for Windows, Version 20 (IBM Corp., Armonk, NY, USA). The present study was approved by the Hospital Ethical Review Board (registered under the number 50047715.9.0000.5530).

Results

There was an increase in the consumption of all measured PPEP items (from 22.2% in the median value of consumption of surgical caps to 75.9% in the median of disposable masks), when comparing the pre-COVID to the Pos-COVID period (►**Table 1**). A decrease in the SSI was detected, with a

Table 1 Consumption of personal protective equipment and products and surgical site infection after cesarean section index in the pre-COVID-19 and post-COVID-19 periods by healthcare workers

Factor	Pre-COVID*	Post-COVID**	% variation***
Disposable masks (units/month)	1,450 (900–1,700)	2,550 (2,300–4,500)	75.9%
Disposable Gloves (units/month)	15,000 (13,000–17,700)	19,300 (17,000–22,000)	28.7%
Gowns (units/month)	140 (40–1,000)	200 (160–300)	42.9%
Caps (units/month)	1,800 (1,300–2,200)	2,200 (2,000–2,500)	22.2%
Alcohol-based products for hand hygiene (ml/pct-day/month)	22.90 (5.56–61.18)	33.96 (15.92–60.25)	48.3%
Soap for hand hygiene (ml/pct-day/month)	21.96 (6.11–42.25)	27.67 (17.66–30.65)	26.0%
Postcesarean SSI ((n of SSI/total of procedures)* 100)	1.74 (0–4.90)	0.89 (0.00–1.55)	48.9%
Cesarean	102 (92–126)	127 (105–150)	24.5%

Abbreviation: SSI, surgical site infection.
*Pre-Covid-19: April 2019 to February 2020.
**Post-Covid-19: and from March to July 2020. There was a significant increase in the absolute number of cesarean sections during this period because two maternity unitis in Porto Alegre were closed and obsteric cases were redirected to our hospital.
***Date presented as median (minimum-maximum); %variation = ((final-initial)/initial)*100.

reduction of 49% in the median of postcesarean SSI. The proportion of cesarean sections had no significant variation between both periods Pre-COVID and Post-COVID in the study.

Discussion

Being a retrospective study, a definitive cause-effect relationship cannot be established; nonetheless, the conspicuous association among the studied variables allows the inference that the decrease in consumption of PPEP was probably a result of the adoption of safer practices by healthcare workers after the advent of COVID-19, and that the reduction in the occurrence of SSI was probably its resulting consequence. Such a sizable reduction leads us to believe that the former adherence of healthcare workers was less than ideal, despite consistent education efforts. We believe that this reduction may resemble the one obtained by the introduction of hand washing proposed by Semmelweiss in the XIX century.[8]

Conclusion

Our data suggests that the advent of the COVID-19 epidemic had a positive impact on the adherence to safety measures and infection control among patients submitted to cesarean section. Despite the severity of the crisis, one could state that extreme situations can lead to valuable reflections and opportunities for improvement. A careful trend follow-up, after the eventual mitigation of the COVID-19, will be most needed.

Conflict of Interests

The authors have no conflict of interests to declare.

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Placental Sampling for Understanding Viral Infections — A Simplified Protocol for the COVID-19 Pandemic

Coleta placentária para entender infecções virais – Um protocolo simplificado para a pandemia de COVID-19

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Abstract

Objective The coronavirus disease 2019 (COVID-19) is a pandemic viral disease, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The impact of the disease among the obstetric population remains unclear, and the study of the placenta can provide valuable information. Adequate sampling of the placental tissue can help characterize the pathways of viral infections.

Methods A protocol of placental sampling is proposed, aiming at guaranteeing representativity of the placenta and describing the adequate conservation of samples and their integrity for future analysis. The protocol is presented in its complete and simplified versions, allowing its implementation in different complexity settings.

Results Sampling with the minimum possible interval from childbirth is the key for adequate sampling and storage. This protocol has already been implemented during the Zika virus outbreak.

Conclusion A protocol for adequate sampling and storage of placental tissue is fundamental for adequate evaluation of viral infections on the placenta. During the COVID-19 pandemic, implementation of this protocol may help to elucidate critical aspects of the SARS-CoV-2 infection.

Keywords
- COVID-19
- placenta
- pregnancy
- systematic sampling
- viral infections

Resumo

Objetivo A doença do novo coronavírus (COVID-19) é uma doença viral pandêmica causada pelo coronavírus da síndrome respiratória aguda 2 (SARS-CoV-2). O impacto da doença entre a população obstétrica ainda é incerto, e o estudo da placenta pode fornecer informações valiosas. Assim, a coleta adequada do tecido placentário pode ajudar a caracterizar algumas propriedades das infecções virais.

Métodos Um protocolo de coleta placentária é proposto, objetivando a garantia de representatividade da placenta, descrevendo a maneira de conservação adequada das amostras, e visando garantir sua integridade para análises futuras. O protocolo é

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apresentado em suas versões completa e simplificada, permitindo sua implementação em diferentes configurações de infraestrutura.

Resultados A amostragem com o intervalo mínimo possível do parto é essencial para coleta e armazenamento adequados. Esse protocolo já foi implementado durante a epidemia de vírus Zika.

Conclusão Um protocolo para coleta e armazenamento adequados de tecido placentário é fundamental para a avaliação adequada de infecções virais na placenta. Durante a pandemia de COVID-19, a implementação deste protocolo pode ajudar a elucidar aspectos críticos da infecção por SARS-CoV-2.

Palavras-chave
- COVID-19
- placenta
- gestação
- coleta sistemática
- infecções virais

Introduction

Coronavirus disease 2019 (COVID-19) is a severe and highly relevant viral disease in the global scenario. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (family *Coronaviridae*, genus *Betacoronavirus*), the etiological agent of the disease, causes asymptomatic or a mild respiratory infection in the majority of cases.[1–4] However, people with underlying risk factors, such as increased age, cardiovascular disease, and diabetes, present higher rates of clinical complications and severe acute respiratory syndrome (SARS).[4]

In viruses of the same genus, such as SARS-CoV (subgenus *Sarbecovirus*) and Middle East respiratory syndrome coronavirus (MERS-CoV) (subgenus *Merbecovirus*), as well as in other respiratory disease viruses, there is an increased risk of morbidity and mortality during pregnancy.[3,5–8] The impact of COVID-19 on the obstetric population and the gestational consequences of SARS-CoV-2 are a great concern for investigation.[9–11] Data from the United Kingdom show that most women admitted with SARS-CoV-2 infection during pregnancy were in the late second or third trimester, which replicates the pattern seen for other respiratory viruses, with women in later pregnancy being more severely affected, a third of whom had preexisting comorbidities.[12] In United States, reports show higher rates of hospitalization (31.5%), intensive care unit (ICU) admission (1.5%), and mechanical ventilation (0.5%) in pregnant women, when compared with nonpregnant women (5.8%, 0.9%, 0.3%, respectively).[13] Thus, recent data from Brazil have demonstrated an increased risk of severity among pregnant women, with high numbers of maternal death, and significant cases without adequate respiratory support and with no intensive care admissions.[14]

To understand the different facets of COVID-19 during pregnancy, the placenta can serve as a valuable source of information about maternal and fetal conditions. The placenta is a complex and unique interface between maternal and fetal vascular beds, mediating the exchange of nutrients and others residues, allowing the fetal uterine existence and maintaining a highly reliable homeostasis.[15,16] The broad spectrum of placental functions depends on its tissues and cellular stratification, which form a selective biological barrier, called the blood-placental barrier.[17] Those tissues may be affected by viral infections, such as parvovirus B19, rubella virus, cytomegalovirus, herpes simplex viruses, and Zika virus (ZIKV), and the consequences of the placental tissues' immune response and destruction during different periods of pregnancy can lead to severe consequences on gestational and neonatal outcomes.[18]

The current evidences about vertical transmission are uncertain and the preponderance of evidence so far does not indicate a significant role for vertical transmission.[5,10,19] However, to understand the impact of COVID-19 on maternal morbidity and mortality is crucial, and the evaluation of the placental tissue may provide data about pathways related to the viral infection within the placenta.[20] Recent placental histopathology results from SARS-CoV-2-positive women did not demonstrate a specific pathology or pathological pattern; however, nonspecific histomorphologic changes suggestive of maternal/fetal vascular malperfusion have been reported.[21] Viral particles in the organ has been detected, although aspects of the effects and pathways of infection by SARS-CoV-2 and how it occurs on placental tissues remain largely unknown up to this date.[21–24]

Our group has previously shown, during the ZIKV outbreak in Brazil, that the placenta is a possible site for viral persistence and that viral detection relies on adequate and appropriate sampling and storage.[25] Thus, here we detail sampling procedures and also propose a simple protocol that can be performed in the delivery room, to guarantee representative tissues of placenta, allowing further investigation consequences of viral infection in pregnancy, including SARS-CoV-2 infection.

Methods

Placental Sampling Protocol

The placental sampling protocol aims to represent the various tissues that constitute the placenta, and also the umbilical cord, at the time of childbirth. The sampling includes 4 regions of the placenta: the basal plate, the chorionic villus, the chorionic plate, and the amniotic membrane (►**Fig. 1**). To preserve the best sampling quality, collection should be performed in the shortest possible interval from childbirth. Due to different conditions for sampling in different facilities, two versions of the protocol are proposed, the complete (►**Fig. 1A**) and the simplified (►**Fig. 1B**). All procedures must be performed following the local biosafety rules. The current manuscript is a protocol description. Each study that implements it must necessarily undergo appropriate ethical

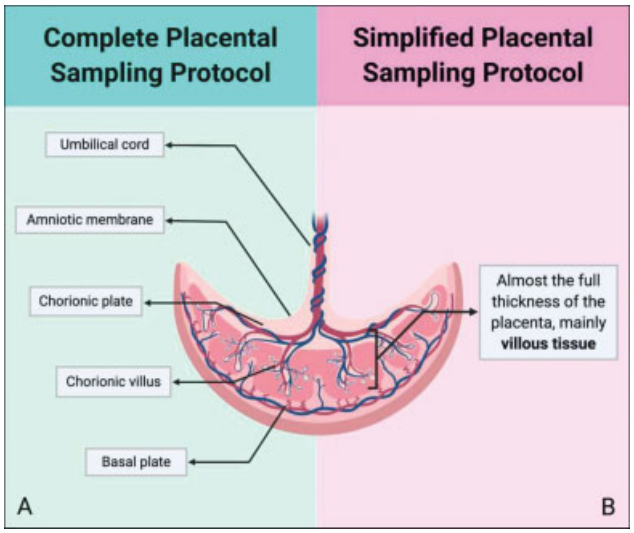

Fig. 1 Cross section of the placenta showing its components in different versions of the protocol—complete (A) and simplified (B) placental protocol version.

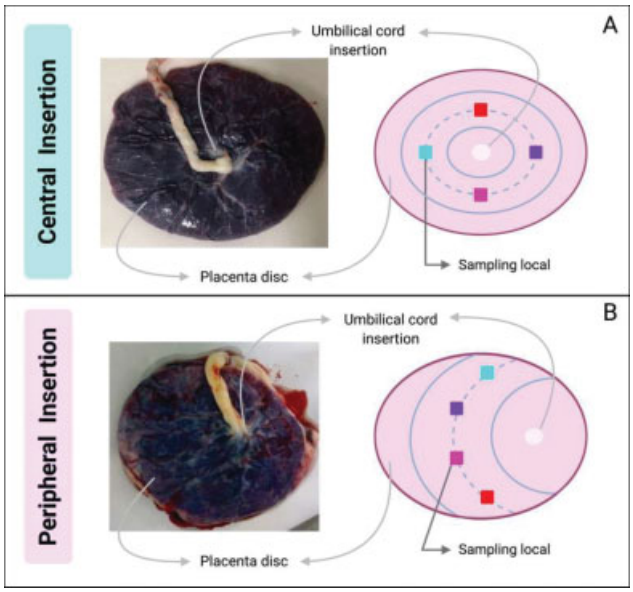

Fig. 2 Placental sampling based on umbilical cord insertion site — central (A) and peripheral (B) insertion.

approval. The protocol was approved by the ethics committee of the coordinating center (#4.047.168) and of each participating center, with implementation in 5 obstetric reference centers of the *Brazilian Network of COVID-19 during Pregnancy* (REBRACO, in the Portuguese acronym) up to now.[26] The latest World Health Organization (WHO) recommendations for sampling COVID-19 patients include a biosafety level 2 (BSL2) facility with all adequate caution.[27] Specific procedures with high viral load, like viral isolation, must be conducted in biosafety level 3 (BSL3) facilities.[28]

Complete Placental Sampling

After childbirth, the placenta should be immediately prepared for sampling or preserved in a cool refrigerator (4°C) in a sterile container for a maximum of 2 hours after childbirth until sampling is possible. The processing of the placenta must be done in an adequate sterile hood; the materials and equipment necessary for adequate placental sampling are described in Supplementary Data S1 (online only). Placental samples will be stored in cryotubes and histology cassettes. All storage materials must be properly identified before the procedure, with patient identification and corresponding placental region.

The first step is to have the placenta washed, with sterile saline or sterile phosphate buffer saline, inside a tray, and any solid residues or visible blood clots must be removed. After cleaning, the placenta is placed on a surface with sterile absorbent paper with the basal plate facing up. The choice of the sampling locals to ensure representativeness is based on the insertion of the umbilical cord (►**Fig. 2**). In placentas with umbilical cord centrally inserted, three imaginary concentric circles (one coincident with the placental disc borders, one marginal to the umbilical cord, and a third placed between those two previously described) should be projected, and the sampling places are positioned in the intermediate circle. Four points are chosen in the intermediate circle for sampling, equidistant from each other (►**Fig. 2A**). In placentas with peripheral cord insertion, three concentric semi-circles starting from the cord insertion site should be considered, and sampling will be performed in the intermediate circle (►**Fig. 2B**). The areas where the sampling take place must not contain macroscopic anomalies, such as areas of detachment or extensive calcification.

After the sampling areas are defined, tissues are sampled in the following order: basal plate, chorionic villus, amniotic membrane, chorionic plate, and umbilical cord (►**Fig. 1A**). The basal plate corresponds to the maternal face of the placenta; at the sampling places, an incision should be made with the scalpel 5 mm deep, seeking to avoid contamination with villi. Chorionic villus corresponds to the tissue underlying the basal plate; superficial tissue must be despised because it may contain traces of the basal plate. The amniotic membrane corresponds to the thin and transparent membrane that lines the chorionic plate; to acquire it, this layer must be detached from the chorionic plate. The chorionic plate corresponds to the fetal face of the placenta; it is necessary to dissect the amniotic membrane previously and collect the tissue below, ~ 2 mm thick, and visible calibrated blood vessels should be avoided. Finally, for the umbilical cord, samples are obtained sectioning it transversely, to obtain two samples.

Samples of $\sim 15 \times 15 \times 15$ mm (except for the amniotic membrane, where the sample is $\sim 10 \times 5 \times 2$ mm) are initially obtained, from each placental region, and, further, each of these pieces are divided in 3 equal parts (technical triplicate). Umbilical cord samples ~10 mm thick and divided in 3 equal parts are also obtained.

Each tissue sampling has two storage destinations: histology cassettes for formalin fixation and cryotubes for cryopreservation. One of three parts of each sample replica will be stored in a cassette, that will in the end contain four samples, one from each previously selected region — again focusing on representativeness (►**Fig. 3A**). Two of three parts of each replica will be placed in two cryotubes, each containing 4 samples, one from

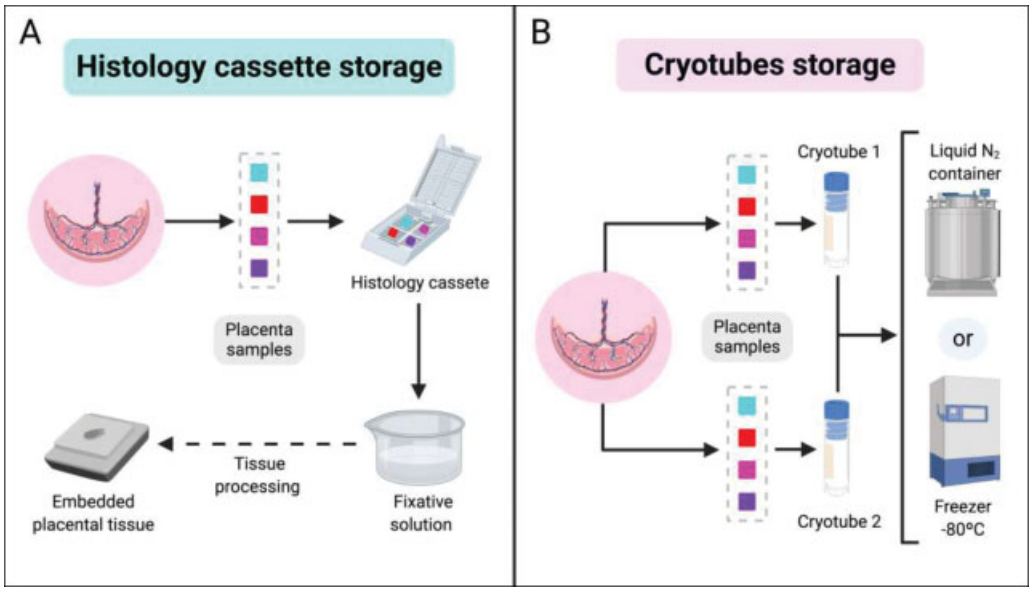

Fig. 3 Tissue sample storage process for each sampling site. (A). Histology cassettes storage. (B). Cryotube storage.

each previously selected local, guaranteeing the representativeness of the placenta (►**Fig. 3B**). The umbilical cord samples follow the same storage method: the histology cassette and each cryotube present two fragments of total section of the tissue.

Simplified Placental Sampling

After childbirth, the placenta can be immediately sampled in the operating room, by the responsible delivery team, after adequate training. This can mitigate any concerns regarding biosafety standards, materials usage, and adequate use of appropriate personal protective equipment (PPE) (Supplementary Data 1). All storage material must be properly identified with the patient coding, as previously proposed for the complete placental sampling.

The selection of sampling areas to ensure representativeness is the same as that applied for the complete placental sampling, and it is based on the insertion of the umbilical cord, defining four places (►**Fig. 2**). When obtaining such samples, there is no detail on the different regions, and the samples must contain almost the full thickness of the placenta, removing the more superficial maternal tissue and focusing on collecting the villous tissue in deeper regions (►**Fig. 1B**). Samples of $\sim 15 \times 15 \times 15$ mm are obtained from each local.

The samples collected are stored in cryotubes, each containing a sample from a previously selected area, and properly preserved after childbirth. To guarantee representativeness of the placenta, analysis with samples obtained with simplified placental sampling must use material from the four sampling areas for each assay (►**Fig. 4**). After sampling in the operating room, the placenta should be sent for routine pathological analysis.

Storage and Cautions

Placenta samples in histology cassettes must be placed in a fixative solution, such as 10% buffered formalin, and then be processed for embedding, which can be made in paraffin or other material (►**Fig. 3A**). Cryotubes containing samples must be preserved immediately at very low temperatures to maintain sample integrity. Cryotubes must be stored in a liquid nitrogen (N_2) container or directly in -80°C freezer. Low temperatures must be maintained in the final accommodation (►**Fig. 3B**). Biosafety guidelines must be followed during all manipulation of samples: sampling, freezing, storing, and processing.[27,28] All disposable materials used during the sampling process must be considered as infectious waste. Other materials must be cleaned and sterilized, preferably in an initial 10% sodium hypochlorite solution.[27]

Histopathological Analysis

The placenta should be further considered for histopathological examination. After the sampling protocol process is finished, the placenta should be placed in a container with an adequate volume of buffered formalin and sent for histopathological analysis. The sampling and analysis of normal and abnormal findings should follow the Amsterdam Placental Workshop Group Consensus Statements, to enable comparison and international standardization of report results.[29] A consistent understanding, with basic gross examination and histologic patterns of injury is important to maximize the diagnostic, prognostic, and therapeutic benefit of placental examination.[30]

Results and Discussion

Adequate placental sampling is key to the evaluation of different insults that may affect the placenta, the woman, and the fetus. We described a placental sampling protocol, which has already been implemented in our setting and allowed us to provide some evidence regarding ZIKV infection during pregnancy.[25] Conserving the integrity of placental samples enables future analysis, using molecular biology and biochemistry techniques.

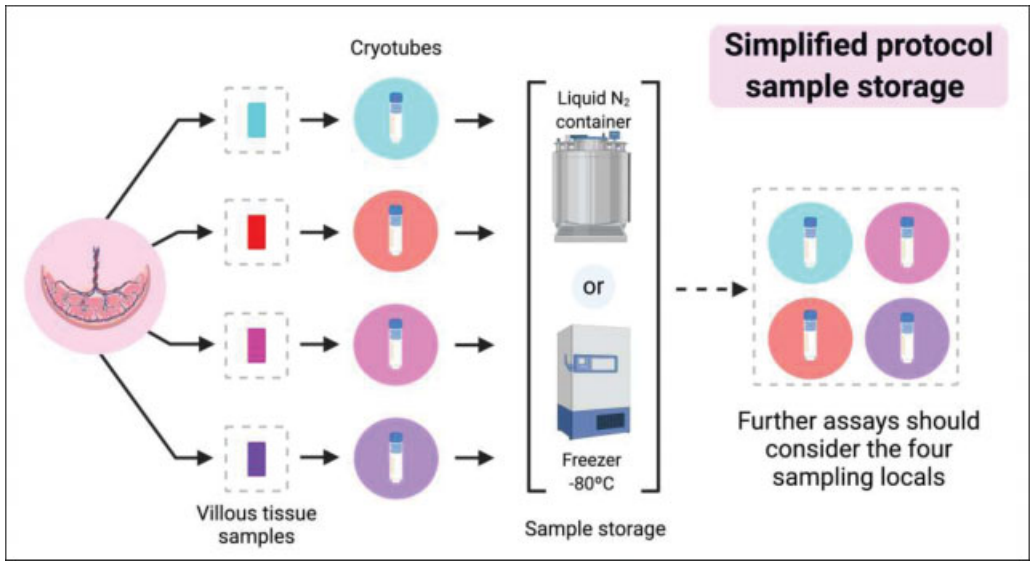

Fig. 4 Placenta sample storage process for simplified protocol version.

Immunohistochemistry, immunofluorescence, and a series of different stains, such as the commonly used hematoxylin and eosin stain, can be performed in the histological samples obtained from paraffin-embedded tissue cassettes. Using samples preserved in cryotubes, after specific treatments and extractions, experiments and assays based on proteins (such as Western-blot and proteomic analyzes) or nucleic acids (such as qPCR and next-generation sequencing), or even lipids and other biomolecules, can be implemented. We highlight that some samples are more appropriate than others for specific assays. As a relevant example, formalin-fixated and paraffin-embedded samples could lead to some methodological difficulties for the detection and testing of ribonucleic acid (RNA) viruses, while cryogenic stored samples are more adequate for such experiments.

In addition to maintaining the placental characteristics most similar to those at the moment of childbirth, the sampling is also representative of the placenta as an organ. The samples are collected from areas where the thickness of the placenta is regular, according to its distance from the umbilical cord's spot of insertion. By sampling in random equidistant regions, representativeness of the entire placenta is obtained, thus reducing the interference of specific site features (outliers) and bias in future analyses.

Due to the recommended sampling and subsequent adequate storage, the samples maintain the integrity of the biomolecules. In a previous study from our research group, in which the detailed placental protocol sampling was applied, it was possible to extract whole RNA molecules from samples preserved in -80°C freezers for periods of up to 2 years. This research made it possible to identify the ZIKV genome in placenta samples. This study suggested that, a simplified protocol, mainly with villous tissue samples, if respecting representativeness and adequate storage of the material, could be effective for viral detection.[25] However, inadequate placental sampling can be distracting and generate misleading results; for that reason, all studies involving placental samples should detail the procedure.[31]

The infection routes of SARS-CoV-2 regarding vertical transmission remain unclear, and there is limited information about COVID-19 during pregnancy and its consequences.[10,11,32–34] Some cellular components have been considered as putative binding receptors for viral entry, such as the membrane protein angiotensin-converting enzyme 2 (ACE2), which is widely expressed in the surface of trophoblasts and endothelial cells.[35–37] Recent studies suggest the ACE2 as part of the viral adsorption, and due to its expression in placental cells, it could possibly lead to a placental infection.[38,39] Early studies published had not reported detection of the SARS-CoV-2 genome by reverse transcription-polymerase chain reaction (RT-PCR) assays in placental samples; however, details regarding the methodological process (sampling method, processing time, sample storage) are not clear and, therefore, did not rule out the possibility of viral presence at the maternal-fetal interface, which has now been shown.[40,41] Recent results demonstrated the presence and infection of the virus in the placental tissue, mainly in the chorionic villi, an area emphasized in the current protocol.[22–24] A study involving 19 pregnant women infected with SARS-CoV-2 indicated the viral infection in villi syncytiotrophoblast and cytotrophoblasts by *in-situ hybridization* technique (nucleic-acid based technique), with a specific target for the SARS-CoV-2 RNA.[23]

As well as the ZIKV, SARS-CoV-2 contains a positive-sense single-stranded RNA genome.[1,3,42] Given the previous experience to detect the ZIKV genome in placentas sampled by this protocol,[25] investigation of the COVID-19 virus could benefit from this protocol. Therefore, the implementation of the simplified protocol focusing on the chorionic villi can enable a greater scope of biological material sampling in different reference centers, mainly in countries with a severe pandemic scenario in the obstetric population, such as Brazil.[14,26]

Conclusion

The placenta has a key role in the understanding of maternal-fetal complications. The implementation of the protocol in different settings would standardize placental sampling and storage, improving techniques description and results, and even providing the exchange of samples through different settings and global locations. The adequate storage of the samples would allow accurate and biologically relevant results in future studies to understand possible critical aspects of viral infections, such as pathogenesis, transmission routes, and functional changes related to infection by SARS-CoV-2 in the placenta.

Contributions

G. M. N. and M. L. C. structured the manuscript and did the majority of the writing, conception, and design. J. P. S. G., R. R. J., A. A. T. and I. U. M. were essential for developing the article and conducting a critical review of the intellectual content. All authors declare that they have seen and approved the final version of the manuscript.

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Conflict of Interests

The authors have no conflict of interests to declare.

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Clinical Features and Maternal-fetal Results of Pregnant Women in COVID-19 Times

Características clínicas e resultados materno-fetais de mulheres grávidas com COVID-19

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Abstract

Objective Coronavirus disease 2019 (COVID-19) is a disease caused by a newly discovered coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which usually leads to non-specific respiratory symptoms. Although pregnant women are considered at risk for respiratory infections by other viruses, such as SARS and Middle East respiratory syndrome (MERS), little is known about their vulnerability to SARS-CoV-2. Therefore, this study aims to identify and present the main studies on the topic, including the postpartum period.

Methods In this narrative review, articles were searched in various databases, organizations, and health entities using keywords compatible with medical subject headings (MeSH), such as: *COVID-19*, *pregnancy*, *vertical transmission*, *coronavirus 2019*, and *SARS-CoV-2*.

Results The review of the scientific literature on the subject revealed that pregnant women with COVID-19 did not present clinical manifestations significantly different from those of non-pregnant women; however, there are contraindicated therapies. Regarding fetuses, studies were identified that reported that infection by SARS-CoV-2 in pregnant women can cause fetal distress, breathing difficulties and premature birth, but there is no substantial evidence of vertical transmission.

Conclusion Due to the lack of adequate information and the limitations of the analyzed studies, it is necessary to provide detailed clinical data on pregnant women infected with SARS-CoV-2 and on the maternal-fetal repercussions caused by this infection. Thus, this review may contribute to expand the knowledge of professionals working in the area as well as to guide more advanced studies on the risk related to pregnant women and their newborns. Meanwhile, monitoring of confirmed or suspected pregnant women with COVID-19 is essential, including in the postpartum period.

Keywords
- SARS-CoV-2
- COVID-19
- pregnancy
- pregnancy complications
- high risk pregnancy

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Resumo

Objetivo A *Coronavirus disease 2019* (COVID-19) é uma doença causada por um coronavírus recém descoberto, o *severe acute respiratory syndrome coronavirus 2* (SARS-CoV-2), que geralmente leva a sintomas respiratórios não específicos. Embora mulheres grávidas sejam consideradas em risco de infecções respiratórias por outros vírus, como SARS e *Middle East respiratory syndrome* (MERS), pouco se sabe sobre sua vulnerabilidade ao SARS-CoV-2. Portanto, este estudo tem como objetivo identificar e apresentar os principais estudos sobre o tema incluindo o período pós-parto.

Métodos Nesta revisão narrativa, foram pesquisados artigos em diversas bases de dados, organizações e entidades de saúde, utilizando palavras-chave compatíveis com o MeSH, tais como: *COVID-19*, *gravidez*, *transmissão vertical*, *coronavírus 2019*, e *SARS-CoV-2*.

Resultados A revisão da literatura científica sobre o assunto revelou que as gestantes com COVID-19 não apresentaram manifestações clínicas significativamente diferentes das não gestantes, porém existem terapias contraindicadas. Em relação aos fetos, foram identificados estudos que relataram que a infecção por SARS-CoV-2 em mulheres grávidas pode causar sofrimento fetal, dificuldades respiratórias e parto prematuro, mas não há evidências substanciais de transmissão vertical.

Conclusão Devido à falta de informações adequadas e às limitações dos estudos analisados, é necessário fornecer dados clínicos detalhados sobre as gestantes infectadas pelo SARS-CoV-2 e sobre as repercussões materno-fetais causadas por esta infecção. Assim, esta revisão pode contribuir para ampliar o conhecimento dos profissionais que atuam na área, bem como para orientar estudos mais avançados sobre o risco relacionado à gestante e seu recém-nascido. Enquanto isso, o monitoramento de gestantes confirmadas ou suspeitas com COVID-19 é essencial, incluindo o pós-parto.

Palavras-chave

- SARS-CoV-2
- COVID-19
- gravidez
- complicações na gravidez
- gravidez de alto risco

Introduction

Coronaviruses (CoVs) are a large viral family, known since the mid-1960s, that cause respiratory infections in humans and animals. Some coronaviruses can cause severe respiratory syndromes, such as severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS). Severe acute respiratory syndrome is caused by the SARS-associated coronavirus (SARS-CoV), with the first reports being made in China in 2002. Middle East respiratory syndrome, in turn, is a respiratory disease caused by the MERS-CoV. It was identified in 2012, and, since 2016, it has been drastically reduced after public health efforts to prevent transmission of MERS-CoV.[1,2] Recently, a new coronavirus has been identified, SARS-CoV-2, and it is associated with the coronavirus disease 2019 (COVID-19).[1,3–5]

The most common symptoms at onset of COVID-19 illness are fever, cough, and fatigue, while other symptoms include sputum production, dyspnea, headache, hemoptysis, and diarrhea.[3,6] Some patients with COVID-19 have laboratory changes such as lymphopenia, thrombocytopenia, and elevation of C-reactive protein (CRP). D-dimer elevation can also be identified and serves as an indication of a worse prognosis of COVID-19, although this is already a parameter normally increased in pregnant women.[7,8] Changes in radiographs are common in symptomatic patients with saturation $< 95\%$, manifesting as pneumonia.[7]

Many patients can be asymptomatic, which facilitates virus spread.[7] According to the World Health Organization (WHO), on August 10, 2020, there were 19,718,030 people infected globally, with 728,013 confirmed deaths.[9] In Brazil, on the same date, the number of people infected was 3,035,422, with 101,049 deaths,[10] and among these, 199 were puerperal women.[11]

Severe acute respiratory syndrome coronavirus and MERS-CoV have caused adverse maternal-fetal outcomes, such as maternal death, intrauterine fetal growth restriction, spontaneous abortion, and premature birth. Thus, considering that these viruses are similar, as they belong to the same genus *Betacoronavirus*, one can admit an adverse potential in pregnant women infected with SARS-Cov-2;[3,4,12] however, due to its recent discovery, little is known about its relationship with pregnancy. Therefore, this review aimed to analyze reports related to SARS-CoV-2 infection in pregnancy and postpartum as well as its consequences in the maternal-fetal sphere in order to assist in the management of these patients.

Methods

The PubMed, Scopus, Embase, MedRxiv, Science Direct, and Web of Science databases were searched electronically, as well as the websites for national and international health organizations.

Only articles published in English and Portuguese were considered. As search strategy, combinations of words related to coronavirus were used, including *severe acute respiratory syndrome*, *SARS*, *vertical transmissio*n, *SARS-CoV-2*, *COVID-19*, and *pregnancy*, until July 29, 2020.

Results

Clinical, Laboratory, and Imaging Features in Pregnant Women with Suspected or Proven COVID-19

It is known that pregnant women have a higher risk of severe morbidity and mortality when affected by other respiratory infections, such as influenza and SARS-CoV. Therefore, they should be considered a population at risk for COVID-19.[13–15] Adverse maternal-fetal outcomes (*e. g.* premature birth) have been reported in the literature. However, this information is based on limited data and it is not clear that these results are related to maternal infection.[13]

The Brazilian Ministry of Health included high-risk pregnant women in the risk group for complications caused by SARS-CoV-2 infection. It also emphasizes that urgent measures for specific clinical management must be respected for this population, such as early medication and not delaying radiographic exams regardless of the gestational period.[15,16] In addition, the possibility of worsening the infection caused by SARS-CoV-2 in pregnant women cannot be ruled out.[17] The coronavirus clinical management protocol (COVID-19) in primary health care of the Brazilian Ministry of Health has also emphasized the relocation of health professionals who are pregnant, especially if their pregnancy is high-risk. Furthermore, this protocol also establishes that both pregnant and puerperal women should receive priority care.[15]

Therefore, pregnant women with SARS-CoV-2 infection, even with a mild course, should be monitored including bi-monthly fetal growth ultrasound monitoring and Doppler assessment, due to the potential risk of restricted fetal intrauterine growth.[18] Due to the delay in reverse transcript polymerase chain reaction (RT-PCR) tests, chest computed tomography (CT) in the third trimester may be an effective way to screen for COVID-19 pneumonia in pregnant women, particularly in areas with outbreaks in progress.[19]

In a study by Ellington et al.,[20] data were collected from 91,412 women diagnosed with COVID-19, aged 15 to 44 years, 8.98% of whom were pregnant. Symptoms were reported by 97.7% of pregnant women and 96.2% of non-pregnant women. However, the risk of hospitalization was 5.4 times higher for pregnant women, while the risk of admission to the intensive care unit (ICU) and mechanical ventilation was 1.5 and 1.7, respectively, compared to the group of non-pregnant women.[20] In addition to the common laboratory findings in people with COVID-19, all pregnant women with SARS-CoV-2 pneumonia also presented D-dimer levels above the normal range, even considering the normal elevation usually found in pregnancy. Two (29%) patients had different degrees of abnormal liver function, as well as an increase in alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST). Interleukin-6 was tested in four patients, all with levels above the normal range. Two patients had chronic diseases (polycystic ovaries and hypothyroidism) and three had co-infection (two due to H1N1 and one due to *Legionella pneumophila*). According to chest computed tomography (CT), 6 (86%) patients had bilateral pneumonia, and the rest (14%) had unilateral pneumonia. After the follow-up period, all patients were discharged from the hospital. Four neonates were released without testing for SARS-CoV-2, and there were no signs of fever or pathological jaundice after 28 days. Three neonates were under observation and were tested, the result was positive in 1 of them 36 hours after birth, even with negative viral tests of cord blood and placenta. The neonate with a positive test did not have a fever or cough, had mild signs of breathing difficulty and a chest X-ray revealed mild pneumonia. After 28 days of life, the baby had two negative results on the molecular test and was discharged.[8]

Wu et al.[21] evaluated 23 pregnant women with COVID-19, most of whom were asymptomatic ($n = 15$). Among the asymptomatic pregnant women, six were at risk of miscarriage or premature rupture of the membrane. When comparing the average hospital stay, asymptomatic patients had a shorter hospital stay (14 days) than symptomatic patients (25.5 days).[21]

Physiological gestational changes and pathological disorders, such as endocrine and/or vascular disorders, which occur during high-risk pregnancies, may influence the pathogenesis and/or clinical presentation of SARS-CoV-2 infection in pregnant women.[22] The human placenta expresses an excessive amount of the angiotensin-converting enzyme 2 (ECA2),[23] which is the SARS-CoV-2 cell receptor,[24] whose main function is to regulate blood pressure and fetal development.[23] Thus, a possible intrauterine infection by COVID-19 can alter the ACE2 expression and trigger hypertensive complications during pregnancy, such as preeclampsia.[22]

Hypertensive syndromes are the most frequent complications in pregnancy and are the leading cause of maternal death in Brazil, mainly in its severe forms, such as preeclampsia and hemolysis, elevated liver enzymes, low platelet count (HELLP) syndrome.[25] ►**Figure 1** shows the most frequent complications in pregnancy.

In a study by Mendoza et al.,[26] 42 pregnant women with gestational age greater than 20 weeks and diagnosed with COVID-19 were classified as severe and non-severe according to the type of pneumonia. Eight pregnant women developed severe pneumonia requiring admission to the intensive care unit (ICU), and 6 of these women had characteristics of preeclampsia. When analyzing the criteria for preeclampsia/HELLP syndrome, it was found that only one case had all the requirements (increased lactate dehydrogenase [LDH], placental subperfusion, and abnormal angiogenic state). Thus, the authors suggested that the other 5 cases of preeclampsia can be explained by complications related to COVID-19.[26]

Although there are few specific data on SARS-CoV-2, other analyzed viruses and respiratory viruses can bring serious conditions to pregnant women and should guide the care of pregnant women with COVID-19 until additional data becomes available. Given the above, the consensus among experts was that pregnant women should be isolated to avoid contamination.[1]

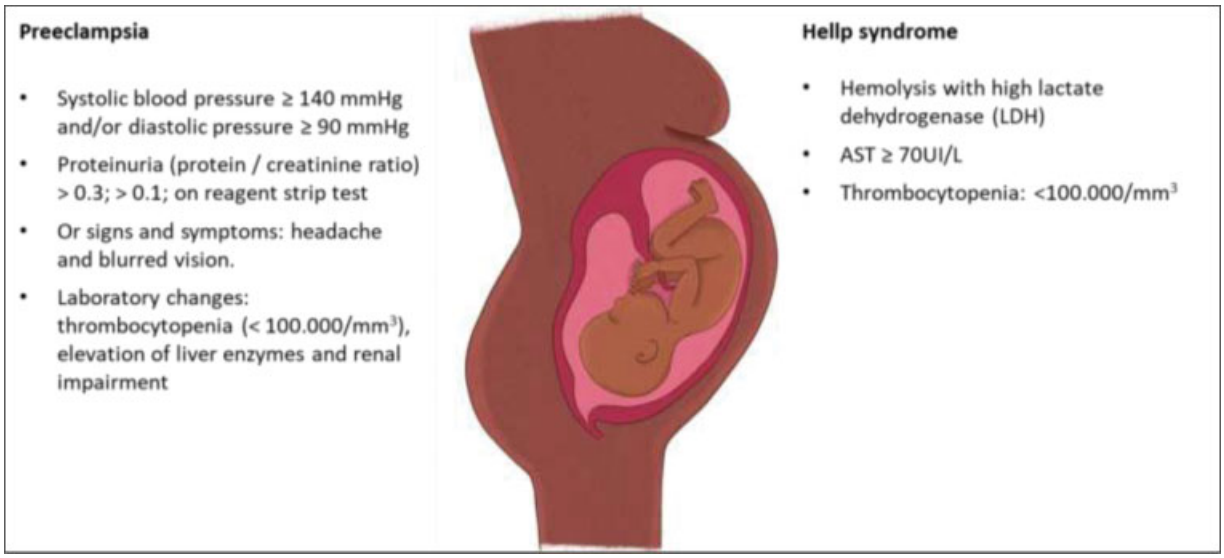

Fig. 1 Most frequent complications in pregnancy.

According to Zhang et al. (2020),[27] even the infection with mild symptoms of COVID-19 reduces lung function. Therefore, with early isolation and drug treatment, cases are less likely to progress to severe pneumonia. However, vigilance should be increased, and, if necessary, pregnancy must be interrupted as soon as possible to prevent the development of the disease to severe and critical stages. At the same time, multidisciplinary cooperation is essential to jointly guarantee the safety of the mother-child binomial.[27]

Even during the pandemic, research is conducted on the impacts of COVID-19 infection on the clinical presentation and perinatal and/or puerperal outcomes; however, the data are still limited and are not conclusive regarding the risk of developing severe forms of COVID-19 associated with pregnancy. However, due to the physiological changes of the gestational period, pregnant women can be seriously affected by some infections. Therefore, it is important to adopt precautionary measures against COVID-19 and systematic monitoring of pregnant women, even if this monitoring occurs in the non-face-to-face care. ►**Table 1** shows the findings of the main studies involving pregnant women with COVID-19 and their newborns.

Management of Pregnant Women with COVID-19

Prenatal and postpartum care cannot be postponed or canceled. Therefore, maternity services must be adapted quickly to provide safe care, minimizing the risk of spreading COVID-19. Unfortunately, health services will suffer from lack of professionals, as they also become ill and/or need to isolate themselves during this pandemic period.[44,45]

According to the Brazilian Ministry of Health (2020),[46] prenatal consultations should take place in a timely manner for pregnant women who do not have flu-like symptoms, paying attention to the prevention of agglomerations and the best hygiene practices. Pregnant women with flu-like symptoms, on the other hand, must have their elective procedures (consultations and routine exams) postponed for 14 days and, when necessary, be seen in an isolated place from other patients.[46] However, it is worth mentioning that depending on the region of the country, there may be specific guidelines. As an example, in Minas Gerais (Brazil), the State Department of Health stated in a technical note issued on April 1, 2020 that in an area with a high flow due to the COVID-19 pandemic, the flexibility of prenatal consultations at usual risk may occur, at clinical criteria. However, the minimum number of consultations and examinations recommended by the Ministry of Health of Brazil and the World Health Organization must be maintained.[47]

In a randomized, double-blind study conducted in the United States and Canada, the use of hydroxychloroquine as postexposure prophylaxis was evaluated. The participants were divided into two groups, 414 received hydroxychloroquine and 407 received placebo. All participants had home or occupational exposure to patients diagnosed with COVID-19. The results of this study demonstrated that the use of hydroxychloroquine as postexposure prophylaxis has no benefits.[48]

A retrospective cohort study was carried out with 1,438 patients admitted to 25 hospitals in New York to assess the association between hospital mortality caused by SARS-CoV-2 and the use of hydroxychloroquine or azithromycin. The authors concluded that there were no statistically significant differences in mortality between groups.[49]

It is very important to carry out an adequate clinical evaluation, establish criteria and prioritize the use of drugs indicated by the WHO (through the Solidarity study) and the Brazilian Ministry of Health, even if there is still no specific treatment for COVID-19. Other drugs should be used in very severe cases and in the absence of response to therapies.[50]

It is worth mentioning that, for some drugs, there are already more robust reports, but still without solid evidence of use in critically ill patients and not in mild cases. Therefore, caution, equilibrium, and common sense, combined with controlled scientific studies should be used to deal with this pandemic therapeutically. ►**Tables 2** and **3** shows the therapies under study against COVID-19 infection.

Table 1 Main results published on pregnant women with COVID-19 and their newborns

Number of pregnant patients	Delivery route	Maternal symptoms	Maternal/fetal complications	Study	Date of publication
09	Cesarean section	Seven patients had fever, four had cough, and two had malaise	One had flu, one had gestational hypertension, one had pre-eclampsia, two had fetal distress, and three had a ruptured membrane	(Chen et al., 2020)[28]	March 07, 2020
17	Cesarean section	Four had fever, four had cough, one had fatigue, two had chest pain, one had dyspnea, and one had diarrhea	Three underwent emergency cesarean section	(Chen et al., 2020)[29]	March 16, 2020
13	Ten cesarean sections, five of which were emergency. Three pregnant women were still pregnant at the end of the study	Ten had fever, three had dyspnea, and one was asymptomatic	Three had fetal distress, one had a ruptured membrane, and one was stillborn	(Liu et al., 2020)[30]	March 5, 2020
01	Spontaneous vaginal	Fever, cough, headache, and myalgia	Gestational hypertension and hypothyroidism.	(Zambrano et al., 2020)[31]	March 25, 2020
07	Seven cesarean sections, two of which were emergency due to preeclampsia	Six had fever, one had cough, one had shortness of breath, and one had diarrhea.	Two patients had hypertension, blurred vision, and preeclampsia.	(Yang et al., 2020)[32]	June 2020
01	Vaginal delivery	Fever and dry cough	Premature rupture of the membrane	(Xiong et al., 2020)[33]	April 10, 2020
01	Emergency cesarean section	Fever	Intermittent fever in the postoperative period.	(Wang et al., 2020)[34]	March 12, 2020
23	Eighteen cesarean sections, two vaginal deliveries and three patients voluntarily terminated the pregnancy in the first trimester	Four patients had fever, six had cough, one had nasal congestion, and 15 patients were asymptomatic	One had fetal intrauterine hypoxia, two had a ruptured membrane, four had gestational hypertension, and three had threat of miscarriage	(Wu et al., 2020)[21]	April 8, 2020
03	Vaginal deliveries	Fever, cough, and chest tightness	No complications	(Khan et al., 2020)[35]	March 19, 2020
04	Three cesarean sections and one vaginal delivery	Three had fever, two had cough, two had myalgia/fatigue, and two had headache	Two newborns had edema and rash	(Chen et al., 2020)[36]	March 16, 2020
07	Cesarean	Six patients had fever, one had cough, one had shortness of breath, and one had diarrhea	Three patients had uterine scars	(Yu et al., 2020)[8]	March 24, 2020
15	Ten cesarean sections, a vaginal delivery, and four patients were still pregnant at the end of the study	Thirteen had fever, nine dyspnea, three myalgia, one diarrhea, one cough and one fatigue	Mild clinical manifestations	(Liu et al., 2020)[37]	July 2020
01	Emergency cesarean section	Fever	No complications	(Wang et al., 2020)[38]	February 28, 2020
09	Seven cesarean sections and two vaginal deliveries	Eight patients had fever, four had cough, one had diarrhea, and one had sore throat	Five neonates were cured, four remained in the hospital until the end of the study, and one died	(Zhu et al., 2020)[39]	February 09, 2020
01	Vaginal delivery	Fever, chills, dry cough, and myalgia	No complications	(Iqbal et al., 2020)[40]	April 01, 2020
05	Three vaginal deliveries, one cesarean section due to gestational diabetes and one emergency cesarean section due to fetal tachycardia	All pregnant women had postpartum fever	Two patients developed gestational diabetes, and one developed preeclampsia	(Chen et al., 2020)[41]	March 28, 2020
01	Cesarean section	Fever, nasal congestion, and respiratory distress	No complications	(Dong et al., 2020)[42]	March 26, 2020
02	Cesarean section	Two patients had fever, two had nasal congestion, and one had chills. One had fever, nasal congestion, sore throat, and a rash	No complications	(Fan et al., 2020)[43]	March 17, 2020
Case: 16 COVID-19	Caesarean section	Fifteen pregnant women with COVID-19 had mild	One patient in the control group was in a more serious	(Zhang et al., 2020)[27]	March 25, 2020

Table 1 (*Continued*)

Number of pregnant patients	Delivery route	Maternal symptoms	Maternal/fetal complications	Study	Date of publication
pregnant women Control: 45 healthy pregnant women		pneumonia, and one of them had severe pneumonia	condition. No patient in the case group was in critical condition		
8.207	Not informed	1,799 patients had cough, 1,190 had fever, 1,323 had myalgia, 989 chills, 1,409 had headache, 497 had diarrhea, 682 had nausea or vomiting, 350 had abdominal pain, 326 had runny nose, and 587 had new loss of taste or smell	Risk of hospitalization 5.4 times higher than non-pregnant women. Risk of being admitted to the ICU and receiving mechanical ventilation was 1.5 and 1.7, respectively, compared to the group of non-pregnant women	(Ellington et al., 2020)[20]	June 26, 2020

Abbreviations: COVID-19, coronavirus disease 2019; ICU, intensive care unit.

Table 2 Drugs recommended by the World Health Organization, in the Solidarity study, for the treatment of COVID-19, on July 6, 2020

Drug	Use in pregnant women	References
Remdesivir	It was not possible to evaluate the effectiveness and/or safety of its use in pregnant women. The Food and Drug Administration (FDA) has authorized its compassionate use for the treatment of severe COVID-19 in children and pregnant women.	(McCoy et al., 2020)[51] (Lim et al., 2020)[52]
Lopinavir/ritonavir with interferon beta-1	It presented a good safety profile in its use in pregnant women. The results of this study with Interferon beta 1 suggested that there was no increased risk of miscarriage or congenital anomalies.	(Tookey et al., 2016)[53] (Hellwig et al., 2020)[54]

Table 3 Drugs recommended by Brazilian Ministry of Health for the treatment of COVID-19

Drug	Use in pregnant women	References
Chloroquine	Chloroquine can induce adverse ophthalmic and cardiac effects on the fetus. It is also genotoxic. Use should be carefully assessed.	(Lacroix et al., 2020)[55]
Hydroxychloroquine	In the treatment of autoimmune diseases, it is not associated with any increased risk of birth defects, spontaneous abortions, fetal death, or prematurity. Hydroxychloroquine can induce adverse ophthalmic and cardiac effects on the fetus. It is also genotoxic. Use should be carefully assessed.	(Lacroix et al., 2020; Sperber et al., 2009)[55,56]
Azithromycin	In most studies, there were no significant associations between the use of azithromycin in pregnant women and congenital malformations.	(Keskin-Arslan et al., 2020)[57]

Maternal-fetal Care

According to the United States Center for Disease Control and Prevention (CDC), health professionals should follow some recommendations when performing obstetric procedures in pregnant patients with confirmed or suspected COVID-19 diagnosis, from prehospitalization to discharge of the mother and baby.[51–60] It is worth mentioning that, in Brazil, the diagnosis in pregnant women must follow the same protocol for the general adult population and that attention should be paid to the signs and symptoms that demonstrate clinical severity.[46]

Prehospital care includes notification of the obstetrics unit for the proper delivery room preparation, for the correct use of personal protective equipment (PPE) by the health professionals involved and conduct in accordance with biosafety rules.[46]

During hospitalization, care must be taken to avoid new infections, and newborn isolation should be discussed with health professionals. If the mother expresses the desire to breastfeed, she should be instructed on the precautions to be followed, such as proper hygiene, use of the breast pump or use of a mask, if she chooses to breastfeed.[46] The benefits of

Table 4 Main studies assessing the possibility of vertical transmission of SARS-CoV-2

Number of pregnant women	Age of pregnant women	Pregnancy period	Premature birth	Average birth weight	1-minute Apgar score	5-minute Apgar score	Vertical transmission signals	Study	Date of publication
1	30 years	35 weeks	One newborn	Not informed	Not informed	Not informed	RT-PCR not detected	(Li et al., 2020)[68]	26 de junho de 2020
9	26–40 years	36–39 weeks + 4 days	4 newborns	Two newborns had low birth weight	8–9	9–10	Six newborns had RT-PCR undetected at birth. It was not possible to investigate the other newborns at the time of birth.	(Chen et al., 2020)[28]	March 07, 2020
17	28.7–29.5 years	Three pregnant women: < 37 weeks 14 pregnant women: ≥ 37 weeks	Three newborns	Not informed	7–9	9–10	RT-PCR not detected	(Chen et al., 2020)[29]	March 16, 2020
13	22–36 years	25–38 weeks	6 newborns	Not informed	Nine newborns had an Apgar score of 10	Not informed	RT-PCR not detected	(Liu et al., 2020)[30]	March 05, 2020
01	41 years	31 weeks	One newborn	1,500 g	Not informed	Not informed	RT-PCR not detected in nasopharyngeal sample	(Zambrano et al., 2020)[31]	March 25, 2020
07	Not informed	36–37 weeks	Four late preterm infants	2,096 g ± 660 g	8–9	9–10	RT-PCR not detected	(Yang et al., 2020)[32]	June 2020
01	25 years	38 weeks + 4 days	No	3,070 g	9	10	RT-PCR not detected in samples of amniotic fluid, smear of the newborn's throat and rectum.	(Xiong et al., 2020)[33]	April 10, 2020
01	34 years	40 weeks	No	3,205 g	8	9	RT-PCR detected from pharynx swab collected 36 hours after birth. Cord and placenta samples were negative.	(Wang et al., 2020)[34]	March 12, 2020
23	21–37 years	Twenty pregnant women: > 28 weeks 3 pregnant women: < 12 weeks.	Not informed	Not informed	Not informed	9–10	RT-PCR not detected in 04 newborns. The SARS-CoV-2 infection was ruled out in the others using diagnostic criteria.	(Wu et al., 2020)[21]	April 08, 2020
03	27–33 years	34 weeks + 6 days 39 weeks + 1 day 38 weeks + 2 days.	One newborn	3,373 g	8–9	9–10	RT-PCR not detected	(Khan et al., 2020)[35]	March 19, 2020
04	28–34 years	37 weeks + 2 days 39 weeks 37 weeks + 3 days 38 weeks + 4 days	None	3,400 g	7–8	8–9	RT-PCR not detected in three newborns. The other parents did not authorize.	(Chen et al., 2020)[36]	March 16, 2020
07	29–34 years	37–41 weeks	None	3,264 g	8–9	9–10	Four newborns have not been tested. Of the three who were tested, one tested positive 36 hours after birth	(Yu et al., 2020)[8]	March 24, 2020
15	23–40 years	12–38 weeks	None	Not informed	8	9	RT-PCR not detected	(Liu et al., 2020)[37]	July 2020
01	28 years	30 weeks	One newborn	1,830 g	9	10	RT-PCR not detected	(Wang et al., 2020)[38]	February 28, 2020
09		31–39 weeks		2,423 g	7–10	8–10			

Table 4 (*Continued*)

Number of pregnant women	Age of pregnant women	Pregnancy period	Premature birth	Average birth weight	1-minute Apgar score	5-minute Apgar score	Vertical transmission signals	Study	Date of publication
	average age 30 years		Six newborns				RT-PCR not detected	(Zhu et al., 2020)[39]	February 09, 2020
01	34 years	39 weeks	None	Not informed	8	9	RT-PCR not detected	(Iqbal et al., 2020)[40]	April 1, 2020
05	25 to years	38 to 41 weeks	None	3,691 g	10	10	RT-PCR not detected	(Chen et al., 2020)[41]	March 28, 2020
01	29 years	34 weeks + two days	Not informed	3,120 g	9	10	RT-PCR not detected, High IgM and IgG	(Dong et al., 2020)[42]	March 26, 2020
02	29 and 34 years	36 weeks + five days 37 weeks	Not informed	3,145 g	9	10	RT-PCR not detected	(Fan et al., 2020)[43]	March 17, 2020
06	Not informed	Not informed	Not informed	Not informed	8–9	9–10	RT-PCR not detected. In two newborns high levels of IgM and IgG. And in three newborns normal IgM and elevated IgG.	(Zeng et al., 2020)[69]	March 26, 2020
Case: 16 COVID-19 pregnant women Control: 45 healthy pregnant women	Case: 24–34 years Control: 24–40 years	Not informed	One newborn from the case group	Case: 2,300–3,750 g Control: 2,180–4,100g	Not informed	Not informed	RT-PCR not detected in 10 newborns.	(Zhang et al., 2020)[27]	March 25, 2020

Abbreviations: COVID-19, coronavirus disease 2019; IgG, immunoglobulin G; IgM, immunoglobulin M; RT-PCR, reverse transcription polymerase chain reaction; SARS-CoV2, severe acute respiratory syndrome coronavirus 2.

breastfeeding outweigh any potential risks of transmitting the virus through breast milk.[25,61]

The American College of Obstetricians and Gynecologists recommends that doctors should follow the CDC's Interim Clinical Guidelines for the management of patients with confirmed coronavirus disease (COVID-19).[13] Recommendations are that even if the ideal maternal-newborn care plan is to maintain this binomial, the temporary separation of the newborn from a mother with confirmed or suspected COVID-19 should be strongly considered to reduce the risk of transmission to the newborn.[62]

The WHO recommends that the mother and the newborn should stay together and practice skin-to-skin contact, including hygiene and respiratory care for the mother, especially immediately after birth and during breastfeeding establishment, if the mother or her babies are cases suspected or confirmed of COVID-19.[63]

Delivery routes should be individualized based on the obstetric indications and preferences of the pregnant woman. Cesarean section is ideally performed only when clinically justified. Decisions on the corticosteroids use for fetal pulmonary maturation, emergency childbirth, and termination of pregnancy are challenging conditions and must be based on many factors, such as gestational age, severity of maternal condition, and fetal viability and well-being, within a multiprofessional assessment.[64,65]

Vertical Transmission of SARS-Cov-2

A systematic review article[66] included 24 studies that analyzed the effects of COVID-19 on pregnant women and newborns. Regarding clinical symptoms, fever was the most common symptom, occurring in 62.9% of patients, coughing in 36.8%, and sore throat in 22.6%. All deliveries were carried out in a negative pressure room, and care was taken to avoid contamination of the 94 newborns, 31 of whom were premature. The average birth weight was 3,127.6g. Two neonates tested positive for COVID-19. Amniotic fluid, placental fluid, umbilical cord, and gastric juice tested negative. There were three fetal deaths, two due to multiple organ failure and disseminated intravascular coagulation, and the other death because the neonate was cyanotic. No case of severe neonatal asphyxia was observed.

The placenta and decidua are the main interfaces between the mother and the fetus during pregnancy.[67] And, as already reported, the human placenta expresses ACE2[23] and, therefore, may be fundamental for the vertical transmission of SARS-CoV-2.[22] However, the COVID-19 impact on the intrauterine environment is still unclear, as well as whether vertical transmission occurs during a maternal infection. The main studies that have assessed the possibility of vertical transmission are described in ►**Table 4**.

The data to assess the COVID-19 severity in pregnant women are scarce, since most studies had a limited number of participants. It is important to keep in mind that the ideal is to do everything possible to minimize the chance of these patients contracting disease, and, if they do, the measures recommended by the Brazilian Ministry of Health and WHO should be adopted immediately.

Concluding Remarks

As previously mentioned, studies evaluating the consequences of COVID-19 in pregnant women are scarce and have a limited number of participants, which often generate

inconclusive data. Clinical manifestations in pregnant women are similar to those of non-pregnant patients, and there is still no scientific evidence of vertical transmission of SARS-CoV-2. When confirming or suspecting COVID-19 infection in pregnant women, professional follow-up is essential, and all precautions should be taken to minimize the impacts of the disease. Based on the clinical consequences due to the occurrence of pneumonia of other etiologies during pregnancy, there is a theoretical risk of COVID-19 determining unfavorable fetal repercussions. It is necessary that data on pregnant women infected with SARS-CoV-2 as well as its maternal-fetal repercussions are carefully and thoroughly analyzed and made available during the pandemic. Therefore, more detailed studies and specially designed to assess the effects of COVID-19 on pregnant women and their newborns are mandatory to fill this gap that still exists.

Conflict of Interests
The authors have no conflict of interests to declare.

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The Female Athlete Triad/Relative Energy Deficiency in Sports (RED-S)

A tríade da atleta feminina/déficit energético relativo no esporte (RED-S)

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Abstract

In a healthy athlete, the caloric intake is sufficient for sports energy needs and body physiological functions, allowing a balance between energy availability, bone metabolism, and menstrual cycle. On the other hand, an imbalance caused by low energy availability due to a restrictive diet, eating disorders or long periods of energy expenditure leads to multisystemic deregulation favoring the essential functions of the body. This phenomenon, described as the female athlete triad, occurs in a considerable percentage of high-performance athletes, with harmful consequences for their future. The present review was carried out based on a critical analysis of the most recent publications available and aims to provide a global perception of the topic relative energy deficit in sport (RED-S). The objective is to promote the acquisition of more consolidated knowledge on an undervalued theme, enabling the acquisition of preventive strategies, early diagnosis and/or appropriate treatment.

Keywords
- female athlete
- low energy availability
- amenorrhea
- bone health
- menstrual dysfunction

Resumo

Em uma atleta saudável, o aporte calórico é suficiente para a necessidade energética esportiva e para as funções fisiológicas corporais, permitindo um equilíbrio entre disponibilidade energética (DE), metabolismo ósseo e função menstrual. Por outro lado, um desequilíbrio devido à baixa disponibilidade energética (BDE) por dieta restritiva, perturbações alimentares ou grandes períodos de gasto energético conduz a uma desregulação multissistêmica priorizando as funções essenciais do corpo. Este fenômeno, descrito inicialmente como tríade da mulher atleta e, atualmente, como défice energético relativo no esporte (RED-S, na sigla em inglês) tem como pilares a BDE, disfunção menstrual e alterações na densidade mineral óssea (DMO), estando presente em uma percentagem considerável de atletas de alta competição, com consequências nefastas para o seu futuro a curto, médio e longo prazo. A presente revisão foi realizada a partir da análise crítica das publicações mais recentes disponíveis e pretende proporcionar uma percepção global do tema RED-S. O objetivo é promover a aquisição de um conhecimento mais consolidado sobre uma temática subvalorizada, possibilitando a aquisição de estratégias preventivas, diagnóstico precoce e/ou tratamento adequado.

Palavras-chave
- atleta feminina
- baixa disponibilidade energética
- amenorreia
- saúde óssea
- disfunção menstrual

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Definition: Female Athlete Triad versus Relative Energy Deficiency in Sport

The female athlete triad, initially described in 1993 and conceptually defined in 1997 by the American College of Sports Medicine (ACSM), was based on the presence of eating disorders, amenorrhea and osteoporosis. In 2007, 3 new components were defined: low energy availability (LEA), menstrual dysfunction, and changes in bone mineral density.[1–3] Afterwards, it was concluded that the existence of all elements for its diagnosis was not essential, since there is a very high variety in incidence for each one and that it is dependent on the type of sport, which can lead to underdiagnoses. Therefore, since 2014, after meeting the International Olympic Committee (IOC), it was changed for relative energy deficiency in sport (RED-S,) meeting the need for a holistic approach.[4,5]

This new concept allows the identification of energy deficiency as a key to the disruption of several physiological functions of different areas, such as reproduction, bone, endocrine, metabolic, hematological, growth and development, physiological, cardiovascular, gastrointestinal, and immunological, with consequences for the performance and health of the athlete in general.[4,6]

Low Energy Availability

Low energy availability, due to food scarcity or excessive energy expenditure, causes physiological adaptations to ensure life maintenance.[7] Therefore, there are different mechanisms favoring essential processes[8] instead of secondary functions such as growth, development, and reproduction.[9] Energy availability (EA) is calculated by subtracting the energy consumed (kcal) from the energy ingested (kcal) and dividing this value by the free fat mass (kg).[10] It consists of a theoretical concept, difficult to use on a routine basis. However, its knowledge and interpretation are important for a better evaluation of the athletes. The ideal EA should support the basic functions that allow a healthy state and adequate performance,[4] which is believed to be > 45 kcal/kg of free fat mass/day.[7] Several authors have attempted to define the threshold beyond which LEA leads to metabolic changes. However, due to the high interpersonal variability, it is only possible to predict that < 30kcal/kg of free fat mass/day, there is a high probability of physiological adaptation favoring vital systems.[4,7]

Pathophysiology

The new LEA concept highlights the complexity of this theme, which involves several hormonal pathways. There has been extensive research in this area in an attempt to identify the trigger of pathophysiological changes. However, these appear to result from multiple changes with different influences on different organs and systems.

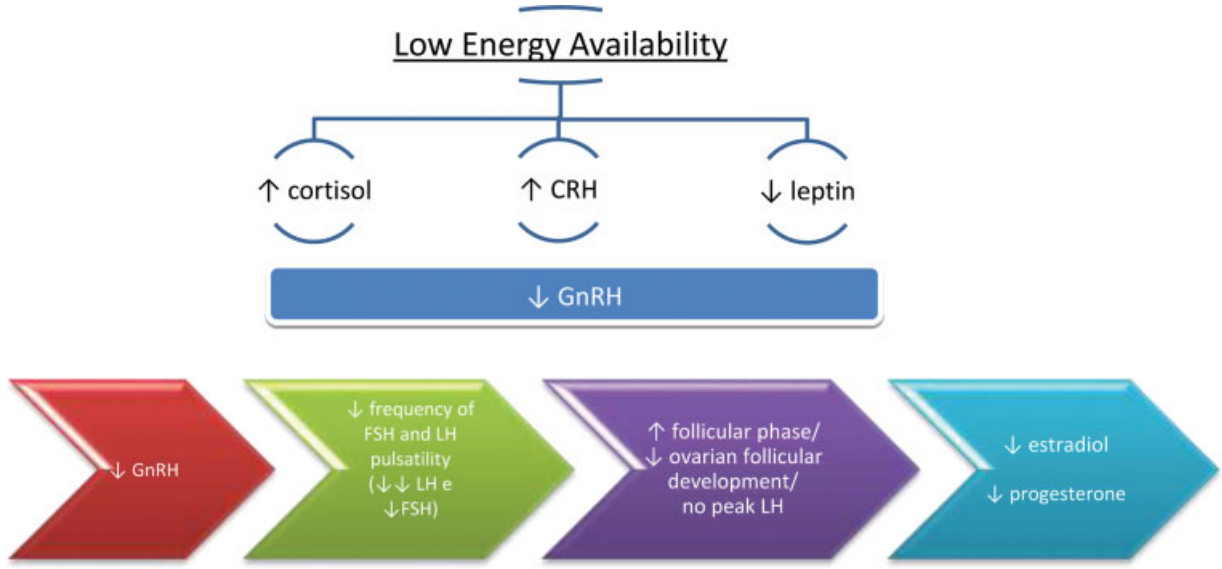
Adaptation to Energy Restriction

Low energy availability leads to a decrease in body fat mass with adaptation of normal adipose tissue activity and activation of different pathways after recognition as an internal stress state (namely activation of the hypothalamic-pituitary-adrenal [HPA] axis and the autonomic nervous system). These changes lead to neuroendocrine adaptations with energy redistribution in favor of vital systems preservation.[11,12] We can, therefore, identify:

- Decrease leptin[13,14]: anorexigenic hormone secreted by adipocytes and regulated by energy state. Negative impact on gonadotropin-releasing hormone (GnRH) secretion.[15]
- Increased ghrelin[13,16,17]: orexigenic hormone secreted by gastric oxyntic cells. Levels inversely related to fat mass. It has an effect on the hypothalamus and on the pituitary gland, negatively affecting the secretion of GnRH, of adrenocorticotrophic hormone (ACTH), of growth hormone (GH), of follicle stimulating hormone (FSH), and of luteinizing hormone (LH).
- Increased peptide YY: increased resistance to ghrelin. Associated with decreased release of GnRH and gonadotropins.[18]
- Decreased oxytocin[19]: apparently suppressive role in activity of the HPA axis and modifies the glucoregulatory response to caloric consumption.[20] It has antidepressant and anxiolytic effects.[21]
- Decreased insulin with increased sensitivity.[22] its decrease has an negative influence on GnRH signaling.
- Decreased insulin-like growth factor 1 (IGF-I): stimulates osteoblast function and bone formation. It mediates several actions by GH and may be responsible for increasing resistance to it.[23–27]
- Resistance to GH, despite its increase[23,24]: pituitary peptide, necessary for muscle and bone anabolism and metabolism of carbohydrates, proteins, and lipids.
- Thyroid function: adaptation due to decreased energy expenditure with a decrease in T3 and thyrotropin-releasing hormone (TRH). Thyroxine and thyroid-stimulating hormone (TSH) without changes/lower limit of normal.[8,28–31]
- Activation of HPA axis: An increase in basal cortisol leads to an increase in its nocturnal pulse amplitude, half-life, and area below the curve in amenorrhea athletes.[32,33] Increase in beta-hydroxybutyrate (ketone synthesized in the liver: carrier of energy to peripheral tissues, activity as an energetic metabolite, cellular signaling functions). Cellular function, regardless of sports practice.[6]

Hypothalamic Amenorrhea

As previously explained, exercise by its own has no suppressive effect on reproductive function. However, it can be the cause of menstrual disruptions by influencing energy availability. According to different studies, it is believed that functional hypothalamic amenorrhea occurs by the combination of different pathways in response to LEA, with a negative influence on GnRH: increased cortisol[34] and corticotropin-releasing hormone (CRH)[35] in response to stress and decreased leptin, with impact directly GnRH (►**Fig. 1**). Therefore, there is a decrease in the GnRH drive with



Figs. 1 and 2. Influence of low energy availability on the menstrual cycle.

reduction in frequency of FSH and LH pulsatility,[22,30,36] leading to changes in folliculogenesis and ovulatory function, resulting in lower estradiol and progesterone levels (►**Fig. 2**).[37]

It is important to emphasize that there is a wide spectrum of possible menstrual patterns, namely ovulatory eumenorrhea, subclinical menstrual dysfunctions, and amenorrhea.[34] A higher rate of amenorrhea occurs in sports whose lean phenotype is imposed (gymnastics, running, among others). The prevalence of hypothalamic amenorrhea can be as high as 69%, compared with 2 to 5% in the general population.[37–39]

Bone Metabolism

Bone development is negatively affected by LEA, with a decrease of different elements such as estrogens (inhibition of osteoclasts and growth of osteoblasts), IGF-1 (stimulation of osteoblastogenesis and promotion of bone formation), leptin (proliferation of osteoblasts) and T3 (proliferation of osteoblasts and promoting bone formation). Decrease in bone formation and bone turnover are the main consequences of changes in bone metabolism. This combination leads to loss of normal repair mechanisms for minor and major lesions, resulting in a higher risk of fracture.[40–43] In amenorrhea athletes, there is a decrease in bone mineral density (BMD), volumetric bone density, and strength associated with abnormal bone microarchitecture.[44–47] Even in weight-bearing exercises with theoretical benefit in BMD, changes are described mainly when associated with restrictive eating habits and low weight.[48,49]

Other Consequences

Cardiovascular: increase in total cholesterol, triglycerides, LDL and HDL.[50] Impairment of endothelial function and increased vascular resistance[51–53] associated with increased central fat.[54] Amenorrheic athletes can have lower heart rates and systolic blood pressure, due to disruptions of the normal renin-angiotensin-aldosterone response.[4] In more severe LEA states, severe bradycardia, hypotension, valve abnormalities, pericardial effusion, and arrhythmias can occur.[4]

Sports performance: impaired recovery with change in muscle mass and function[55]; interference in the glycogen reserve and protein synthesis.[56] The literature in this area is scarce, with only one study confirming a 10% decrease in the swimming speed of 400 m in athletes with amenorrhea versus eumenorrhea.[57]

Bone metabolism: Because it occurs mostly in adolescence, there is a proven risk of loss of bone mass with potential inability to reach the bone peak, which in 90% of individuals is reached by age of 18.[6]

Diagnosis

The diagnosis of RED-S does not imply the existence of concrete clinical or laboratory changes. It consists of an active search for athletes at risk due to insufficient energy availability, either due to low input or excessive expenditure.

The diagnosis requires a low threshold of suspicion and an approach based on a detailed medical history that should include questions about diet, dietary changes, weight fluctuations, exercise, training hours, sleep changes, stress, mood, cycle menstruation, fractures, and substance abuse. It is also important to address psychosocial issues such as the need for social approval, the claim to perfectionism, ambitions and expectations, which are more marked in athletes with amenorrhea. Family history of eating and reproductive disorders should also be explored.[34] In order to standardize screening and follow-up, the IOC created the RED-S clinical assessment tool (CAT), which should be part of the annual health assessment of the athlete and/or whenever there is evidence of eating disorder, menstrual dysfunction (secondary amenorrhea > 6 months or primary amenorrhea > 16

years), history of stress fracture, significant weight loss, change in height in relation to the target family height in adolescents, deficient performance, or evident mood change.[6,58]

Frequently, the first manifestation results in dysregulation of the menstrual cycle or in amenorrhea, and a functional etiology must be considered in the presence of oligomenorrhea and/or in the presence of amenorrhea for >3 months.[6,34]

Functional hypothalamic amenorrhea is characterized by the absence of menstrual cycles or by irregular cycles associated with estrogen deficiency due to insufficient stimulation or suppression of the hypothalamic-pituitary-ovary (HPO) axis in the absence of anatomical or organic pathology.[34]

As it is an exclusion diagnosis, it is crucial to consider the main causes of amenorrhea, such as drugs, intracranial prolactinoma/tumor, Kallmann Syndrome (anosmia and hyposmia), thyroid pathology, chronic pathology, and congenital pathology (i.e., imperforate hymen, Mullerian abnormalities/androgen insensitivity syndrome [AIS]).[59–63] To complement this study, a complete physical examination with search for excess androgens signs is essential, and the need for gynecological evaluation and imaging studies should be considered.[29,64–66]

In hypothalamic functional amenorrhea, bradycardia, orthostatic hypotension, BMI <18.5 kg / m2, parotid hypertrophy and/or signs of tissue hypoperfusion[65] are frequently accompanied by signs of hypoestrogenism, namely delayed puberty, breast atrophy, and vaginal atrophy.[6]

Complementary Diagnostic Tests

The initial study of amenorrhea includes the evaluation of hCG levels (to exclude pregnancy), FSH, prolactin and TSH. Some authors also recommend in the initial assessment the search of Free T4 (FT4), LH, estradiol and anti-Müllerian hormone (AMH).[34]

Additional studies depend on clinical suspicion. In chronic disease or eating disorder: blood count, liver and kidney function, electrolyte Panel, calcium, magnesium, phosphorus, glycemia, erythrocyte sedimentation rate, and C-reactive protein should be requested[34]; in the presence of signs of hyperandrogenism, total testosterone, DHEA-S, and 17OH-progesterone should be considered.[67,68]

Expected results in functional amenorrhea are decreased LH or low normal, normal FSH (usually higher than LH) and E2 <50pg/ml. The acute response after GnRH stimulation is preserved. Thyroid stimulating hormone, FT4, and testosterone are usually at the lower limit of normal.[30,31,34,69] Anti-Müllerian hormone does not change, and there seems to be no interference in the ovarian reserve.[70]

Bone mineral density assessment should be considered when menstrual dysfunction (6–12 months of amenorrhea/oligomenorrhea, primary amenorrhea), low BMI (<17.5 kg/m2) or significant weight loss (>5–10% of body mass within 1 month), in the presence of minor stress/post-trauma fracture, or in the event of an eating disorder. It is advisable to use the Z-Score scale in preference to T-Score, as the first is adapted to age and gender. Any location in the skeleton can be assessed; however, the spine is the most consensual in adolescents and young women with amenorrhea.[6,44,45,71–74]

The interpretation of densitometry in athletes must follow specific criteria, with BMD being considered lower than expected when Z-score$>$ -1; low BMD if Z-score between -1 and -1.9 with risk factors (nutritional deficiencies, hypoestrogenism or stress fractures), and osteoporosis when Z-score $<$ -2 with risk factors.[2,3,49]

Treatment

Due to the multifactorial etiology of female athlete triad - stress, weight loss, excessive exercise, and poor nutrition - a multidisciplinary team is essential for its approach.[2,3]

Nonpharmacological

Nonpharmacological treatment is always the first line of treatment, allowing resolution of most cases. As it is based on LEA, the aim is to restore normal balance with an individualized and dynamic nutritional, psychological, and sports plan that will allow the reestablishment of the hypothalamic-pituitary-ovary axis.[4] The increase of 5 to 10% of body weight or of 1 to 4 kg of weight with appropriate nutritional supply – 300 to 600 kcal caloric increase – distributed throughout the day and with protein and carbohydrate consumption preference.[6]

Vitamin Supplementation

Calcium and vitamin D have shown important benefits in decreasing the risk of stress fractures, as well as in their recovery, with supplementation recommended.[75,76] A daily dose of 1,300 mg of calcium (up to ,1500 mg/day) and of 800-1,000 IU of vitamin D (to achieve blood 25[OH]D concentration $>$ 75-100 nmol/L)[2,77,78] is recommended. The use of bisphosphonates should still be avoided, especially in young athlete women, considering its long half-life and its potential teratogenic effect in future pregnancies.

Pharmacological

Pharmacological treatment has a crucial role in selected cases. However, it should only be considered after failure in reestablishing menstruation after between 6 and 12 months of nonpharmacological therapy associated with a proven decrease in BMD. In presence of a young athlete with amenorrhea or oligomenorrhea, combined oral contraception is often used as an adjunct to normalize menstrual cycles. Despite the success in most cases, its use for this purpose is not recommended, as it may cover a possible physiological normalization and give false confidence to the athlete.[4] Corroborating the nonindication for its prescription, several studies report the lack of efficacy of oral estrogens in the recovery of BMD/bone protective effect.[79,80] This

is justified by their hepatic "first-pass effect," with potential suppression of liver production of IGF-1 impairing its bone trophic effect.[6,81] Currently, when it is necessary to initiate hormone replacement, the most accepted approach consists of transdermal estradiol therapy (E2) (which does not affect IGF-I secretion) associated with a cyclic oral progestative for a short period.[34] Nevertheless, it is always important to remind athletes that this has no contraceptive effect.[82] For contraceptive purposes, there is no contraindication for any method, although if there is a preference for combined contraceptive, we can offer the vaginal or transdermal route to avoid hepatic "first-pass effect". The only method that allows the perception of normal recovery are nonhormonal methods, such as nonhormonal intrauterine devices.

Investigational Therapy

Recombinant parathyroid hormone: can be weighted for short periods of time when BMD is very low or in cases of delayed fracture healing.[34] It is contraindicated in adolescents or young adults with open growth plates. It has shown improvement in BMD and faster recovery.[83,84]

Recombinant leptin: a promising therapy showing increased frequency and levels of LH pulse, improved follicular development, ovarian volume, E2 levels, increased T4, FT4, IGF-I, IGF-binding protein 3, bone alkaline phosphatase, and osteocalcin. However, decreased appetite and significant weight loss have been reported.[85] Similar results can be achieved with metreleptin regarding weight loss and fat mass.[86]

Recovery after Treatment

Nonpharmacological therapy can restore menstrual cycles to normal in months. However, some athletes may maintain folliculogenesis and altered follicular dynamics for years, with decreased gonadotropins and sex steroid hormones. In these cases, a luteal phase defect may occur with long menstrual periods associated with premenstrual spotting or short cycles due to decreased progesterone secretion.[87,88] Regarding bone metabolism, results can take several years to appear. Regardless of the positive correlation between increasing BMD and menstrual reestablishment, in many cases a full recovery is not achieved.[89]

Conclusion

Relative energy deficiency in sport consists of a low energy availability status mainly affecting young athletes, with potentially harmful and irreversible consequences on their health. Its prevalence is underestimated due to lack of and late diagnosis due to deficient knowledge of signs and symptoms. It is crucial and urgent to promote dissemination among different professionals, extending to athletes and their families, in order to increase alertness to this condition, allowing its prevention, early diagnosis, and adequate treatment.

Contributors
All authors were involved in the design and interpretation of the analyses, contributed to the writing of the manuscript, and read and approved the final manuscript.

Conflict to Interests
The authors have no conflict of interests to declare.

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Interventions among Pregnant Women in the Field of Music Therapy: A Systematic Review

Intervenções em gestantes na área da musicoterapia: Uma revisão sistemática

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Abstract

Objective To investigate in the literature the studies on the benefits of music therapy interventions among pregnant women in the prenatal, delivery and postpartum periods.

Data Sources The search for articles was carried out in the following electronic databases: VHL, LILACS, SciELO, Portal CAPES, PsycINFO, ERIC, PubMed/Medline, and journals specialized in this field: *Revista Brasileira de Musicoterapia* ("Brazilian Journal of Music Therapy") and *Voices*.

Study Selection Descriptors in Portuguese (*musicoterapia*, *gravidez*, *gestantes*, *revisão*), English (*music therapy*, *pregnancy*, *pregnant women*, *review*) and Spanish (*musicoterapia*, *embarazo*, *mujeres embarazadas*, *revisión)* were used. The search was delimited between January 2009 and June 2019. The process of selection and evaluation of the articles was performed through peer review.

Data Collection The following data were extracted: article title, year of publication, journal, author(s), database, country and date of collection, purpose of the study, sample size, type of care, intervention, instruments used, results, and conclusion. The data were organized in chronological order based on the year of publication of the study.

Summary of the Data In total, 146 articles were identified, and only 23 studies were included in this systematic review. The articles found indicate among their results relaxation, decreased levels of anxiety, psychosocial stress and depression, decreased pain, increase in the maternal bond, improvement in the quality of sleep, control of the fetal heart rate and maternal blood pressure, and decreased intake of drugs in the postoperative period.

Conclusion Music therapy during the prenatal, delivery and postpartum periods can provide benefits to pregnant women and newborns, thus justifying its importance in this field.

Keywords
- pregnancy
- music therapy
- music
- women's health

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Resumo

Objetivo Investigar na literatura os estudos sobre os benefícios das intervenções musicoterapêuticas em gestantes no pré-natal, parto e pós-parto.

Fontes dos dados A busca dos artigos foi realizada nas seguintes bases de dados eletrônicas: BVS, LILACS, SciELO, Portal CAPES, PsycINFO, ERIC, PubMed/Medline e revistas especializadas da área: *Revista Brasileira de Musicoterapia* e *Voices*.

Seleção dos estudos Utilizaram-se descritores em português (*musicoterapia*, *gravidez*, *gestantes*, *revisão*), em inglês (*music therapy*, *pregnancy*, *pregnant women*, *review*) e em espanhol (*musicoterapia*, *embarazo*, *mujeres embarazadas*, *revisión*). A busca foi delimitada de janeiro de 2009 até junho de 2019. Os processos de seleção e avaliação dos artigos foram realizados por revisão por pares.

Coleta de dados Os seguintes dados foram extraídos: título do artigo, ano da publicação, revista, autor(es), base de dados, país e data da coleta, objetivo do estudo, tamanho da amostra, tipo de atendimento, intervenção, instrumentos utilizados, resultados, e conclusão. Os dados foram organizados em ordem cronológica a partir do ano de publicação do estudo.

Síntese dos dados Foram identificados 146 artigos e incluídos apenas 23 estudos na revisão sistemática. Os artigos encontrados indicam em seus resultados relaxamento, diminuição dos níveis de ansiedade, de estresse psicossocial e de depressão, diminuição da dor, aumento do vínculo materno, melhora da qualidade do sono, controle da frequência cardíaca fetal e da pressão arterial materna, e diminuição da ingestão de fármacos no pós-operatório.

Conclusões A musicoterapia durante o pré-natal, parto e pós-parto pode trazer benefícios para a gestante e para o neonato, o que justifica sua importância nessa área.

Palavras-chave

- gravidez
- musicoterapia
- música
- saúde da mulher

Introduction

Pregnancy is a period characterized by physical, hormonal and emotional changes.[1] The birth marks a new phase in the life of the woman, the puerperium, which ends when the woman's body returns to the stage previous to pregnancy.[2]

Pregnant women are especially affected by stress during pregnancy, childbirth and the postpartum period. Several art forms have been studied in order to evaluate their relaxing potential and their effects on the physiology of individuals. Music has been a constant target of research regarding its effects on the most diverse groups of patients. The existing data point to its importance in improving the concentration, attention and physical endurance of the patients.[3] And it has been shown to be beneficial in the emotional, intellectual, psychological, physiological and social fields,[4] in addition to having specific beneficial effects regarding depression and normal postpartum pain, anxiety and greater satisfaction in the postpartum period.[5]

In pregnant women, these effects can be explained by a series of physiological mechanisms that are activated at the moment of listening to music, which remain activated for a prolonged period. As the main neurotransmitters related to music therapy, natural serotonin – which creates a state of relaxation – and acetylcholine have their potential boosted, with an effect of reducing the heart rate and blood pressure, and increasing blood flow to noble organs.[6] Listening to music also causes the glucocorticoids such as cortisol, which are strongly related to the state of stress, to have a reduced release, with a consequent benefit regarding fetal development, since they are able to cross the placental barrier and directly interfere in fetal physiology.[7]

A form of treatment that aims at the physical, mental and psychological integration of the patient, music therapy is also one of the methods used as a support in pregnancy. Some studies in the literature have shown that musical interventions have an insignificant effect on the reduction in stress during pregnancy[8] and in the decrease in pain during childbirth.[9] However, there a significant improvement in the levels of anxiety during pregnancy and labor has been observed.[8,9] A systematic review by Van Willenswaard et al.[8] points out that no study on music therapy was found during their detailed search, diverging from other systematic reviews that examined interventions made by a music therapist. In view of the divergent results in the literature, the importance of the present study is evident. Therefore, the aim of the current study was to investigate in the literature about the benefits of music therapy interventions among pregnant women in the prenatal, delivery and postpartum periods.

Methods

Type of study

The present is a systematic literature review performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement.[10] The

methodology of a systematic review has a high performance in identifying scientific evidence. According to the Oxford Center for Evidence-Based Medicine (OCEBM),[11] the typology of the systematic review is classified as level 1 out of 5 possible levels in the representation of evidence, as it makes it possible to establish a panorama on the studied topic. We used the The State of the Art through Systematic Review (StArt) software, developed by the Software Engineering Research Laboratory (Laboratório de Pesquisa em Engenharia de Software, LAPES, in Portuguese) of Universidade

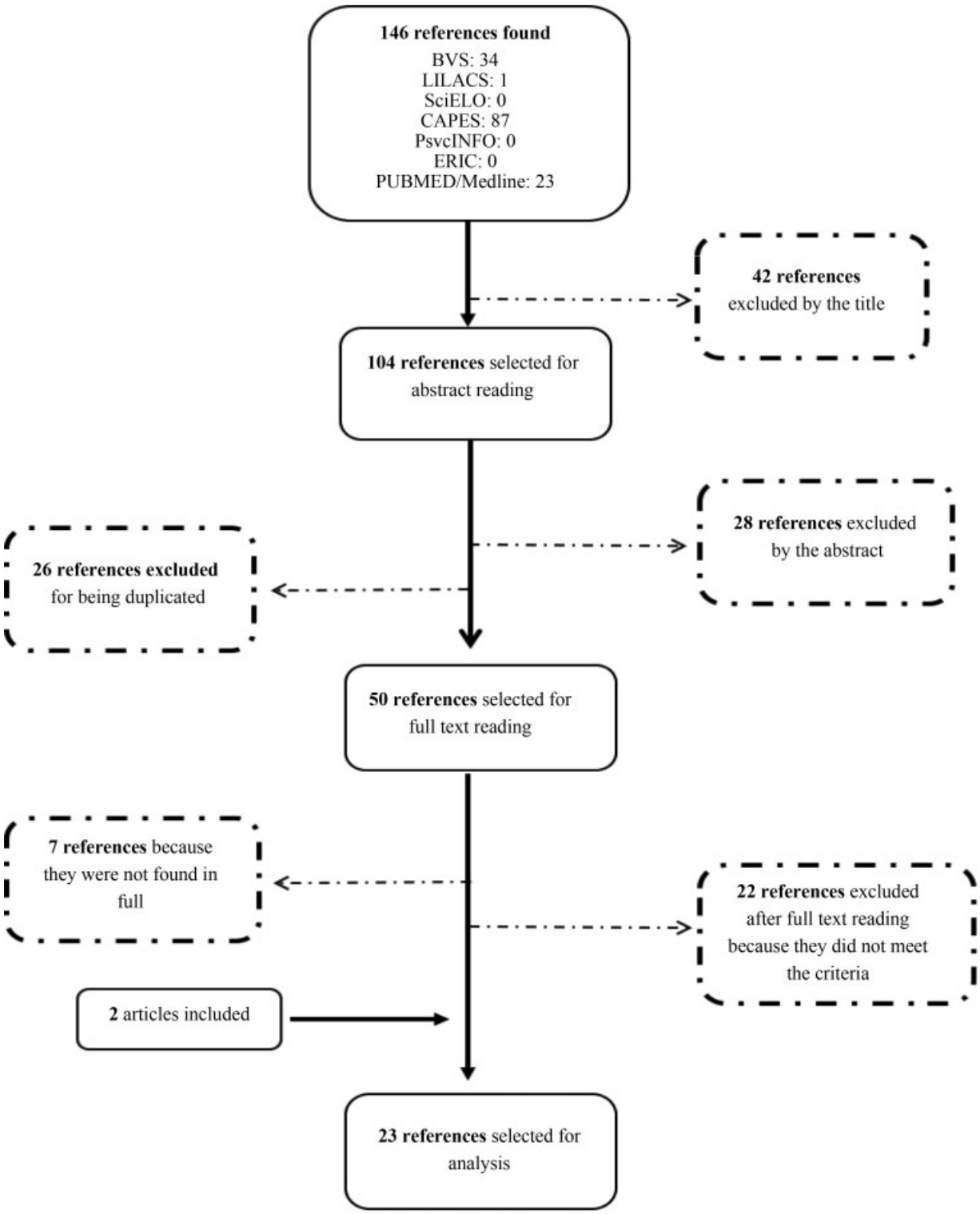

Fig. 1. Flowchart of the process of search and selection of studies.

Federal de São Carlos, Brazil, which supports the researcher in the systematic review.

Search strategy

The research question was formulated using the "PICO" framework, which means population (participants), intervention (or exposure, for observational studies), comparison, and "outcome", with some researchers preferring to add and "S" (for study design), therefore naming it PICOS.[12]

The search was carried out in the following electronic databases: Biblioteca Virtual de Saúde (BVS), Literatura Latino-Americana e do Caribe em Ciências da Saúde (LILACS), Scientific Eletronic Library Online (SciELO), Portal CAPES, PsycINFO, Education Resources Information Center (ERIC), PubMed/Medline, and in journals specialized in the field: *Revista Brasileira de Musicoterapia* ("Brazilian Journal of Music Therapy") and *Voices*.

It was delimited from January 2009 to June 2019, considering articles published in Portuguese, English and Spanish. We used descriptors in Health Sciences (DeCS), Medical Subject Headings (MeSH) and Thesaurus in Portuguese (*musicoterapia*, *gravidez*, *gestantes*, *revisão*), in English (*music therapy*, *pregnancy*, *pregnant women*, *review*) and in Spanish (*musicoterapia*, *embarazo*, *mujeres embarazadas*, *revisión*). Descriptors were combined using the Boolean operators "AND" and "OR".

The articles were selected and evaluated by peer review and organized in phases: in the first phase, an initial analysis of the titles of the manuscripts was carried out; in the second phase, an evaluation of the abstracts was performed. In the third phase, all selected articles were obtained in full, and were subsequently examined according to the established inclusion and exclusion criteria.

Inclusion and exclusion criteria

The inclusion criteria were: original articles published in journals; and studies published from January 2009 until June 2019. The exclusion criteria were: theses, dissertations, monographs and studies that did not reach a minimum score of 18 points in the Downs and Black[13] checklist. If differences occurred during the review of the articles, new discussions were held until both reviewers agreed with the review.

Data extraction

The following data were extracted from the articles included: title, year of publication, journal, author(s), database, country and date of collection, objective of the study, sample size, type of care, intervention, instruments used, results, and conclusion. The articles were organized in chronological order based on the year of publication.

Results

►**Figure 1** presents a flowchart of the search and selection process. In the analysis of the titles, 104 studies were selected and had their abstracts read; 50 studies were considered relevant, and their full texts were read. Out of these studies, 22 were excluded because they did not meet the eligibility criteria, and 7 references were excluded because they were not found in full. During the search, we included two articles found in the references of other articles. The electronic search generated 23 studies, with 8 articles on childbirth and postpartum, and 15 studies related to prenatal care (►**Figure 1**).

Discussion

Regarding the studies selected (►**Table 1**), 6 were from Turkey, and they reported decreased anxiety, FCF, PA, and postoperative pain;[5,14–18] 3 were from Brazil, with results regarding the reduction of pain;[1,19,20] 3 were from Taiwan, and they reported decreased stress and anxiety, improved quality of sleep, and decreased pain in the initial phase of labor[21–23]; 3 were from Spain, with results describing decreased anxiety, PAS, PAD and HR and fetal reactivity[4,7,24]; 1 was from Ireland, and the researchers achieved relaxation and increased bonding;[25] 1 was from the United Kingdom, and it reported decreased anxiety and prenatal depression;[26] 1 ws from China, and the researchers found decreased anxiety and physiological responses.[27] 1 was from the United States, reporting reduced suffering before delivery[28]; 1 was from India, and the researchers obtained fetal stimulation;[29] 1 was from South Korea, reporting decreased anxiety and FCF[30]; 1 was from Israel, reporting an increase in positive emotions and a decrease in negative emotions;[31] and 1 was from Iran, reporting lower pain scores.[32]

The articles regarding the prenatal period, delivery, and the postpartum period report relaxation, decreased levels of anxiety, psychosocial stress, depression and pain, increased maternal bond with the baby, improved quality of sleep, control of fetal heart rate and maternal blood pressure, and decreased drug intake in the postoperative period. According to Carvalho,[33] music stimulates action and emotional expression in individuals, and prompts them to control states of physical and psychological homeostasis, having effects on physiology, behavior, cognition, emotions, and social interaction.[33]

Regarding the prenatal period, 15 articles were analyzed, and 9 of them were relevant for the present review, for they dealt with anxiety in parturient women, and 4 out of these 9 studies were carried out during the nonstress test. According to Primo and Amorim,[34] during pregnancy women may experience anguish and anxiety due to the need to adapt to situations regarding maternity.

As for childbirth, seven articles were analyzed; four of them were related to pain during labor, six dealt with anxiety, and two reported a significant reduction in blood pressure. In the study by Gayeski and Brüggemann,[35] the perception of mothers regarding non-pharmacological methods for pain relief, the feeling of well-being, an having emotional support were reported to facilitate the parturition process. The authors state that there is a need to expand information on these methods throughout pregnancy, and they point out that there are more investigative studies on the use of these non-pharmacological methods for pain relief in women in labor, which aim to improve humanized actions

Table 1 Summary of the articles selected for the systematic review

Title, author, year	Objective	Country, year of collection, and sample size	Type of care and intervention	Instruments used	Results	Conclusion
PRENATAL PERIOD						
Music therapy to relieve anxiety in pregnant women on bedrest: a randomized, controlled trial; Yang et al. (2009)[27]	To explore the effect of music therapy on anxiety relief for pregnant women on bedrest	China; 2007; 120 pregnant women	Group care Duration: 3 consecutive days. Description: the pregnant women in the experimental group received music therapy for 30 minutes on 3 consecutive days. Pregnant women who received routine care had a 30-minute rest on 3 consecutive days. The variables included anxiety and physiological responses	State-Trait Anxiety Inventory	Anxiety levels decreased and physiological responses improved significantly in the intervention group, which received music therapy during bedrest	Music carefully selected according to the preference of the pregnant woman is an inexpensive and effective method to reduce anxiety in women with high-risk pregnancies and who are on bedrest
Alleviating distress during antepartum hospitalization: a randomized controlled trial of music and recreation therapy; Bauer et al. (2010)[28]	To examine the effectiveness of a single music session or intervention with recreation therapy to reduce antepartum-related suffering among women with high-risk pregnancies who experience prolonged antepartum hospitalizations	USA/2009 61 pregnant women	Individual care. Duration: before and after, within 48 to 72 hours after delivery. Description: randomized, single-blinded study; participants received 1 hour of music or recreational therapy, or were placed in an attention control group. Suffering related to antepartum was measured by the Emotional Impact Inventory of Antepartum Rest, which was administered before and after the intervention and in a follow-up period of between 48 and 72 hours	Antepartum Bedrest Emotional Impact Inventory	Significant associations were found between the provision of music and recreational therapy and the reduction of suffering related to the antepartum in women hospitalized with high-risk pregnancies. These statistically significant reductions in suffering persisted over a period of 48 to 72 hours	Music interventions in a single recreational therapy session effectively alleviate antepartum-related suffering among high-risk women who undergo hospitalization before delivery, and should be considered as a complement to any comprehensive antepartum program
Novel method of feto-maternal monitoring using music therapy - a non-stress test; Kumar et al. (2011)[29]	To monitor fetal movements with and without music	India; 2010; 9 pregnant women	Individual care. Duration: not mentioned. Description: the music was set to be heard on a walkman and, the headphones were placed around the pregnant woman's abdomen. The volume of the music was kept at a moderate level of no more than 70 decibels. Fetal movements were measured by pressure sensors. The voltages obtained with and without music were amplified by the AD620 and fed to the NI 6015 for the purpose of monitoring and storage on the PC with a sampling time of 200 ms using the Labview environment	Not mentioned	With music, it increased from 146 bpm to 169 bpm. The test is reactive if there is a minimum 10-15 bpm increase in normal heart rate during fetal movements, otherwise the test is not reactive. This state was also verified by ultrasound. This test is reactive and good for the health of the fetus	Music can serve as a means of communication with the fetus through sounds and voices. Caressing the fetus through the belly, producing soft and melodic sounds, using lights and vibrations that are pleasant for the baby: these stimuli are in an organized and pleasant pattern
Effect of maternal anxiety and music on fetal movements and fetal heart rate patterns; Kafali et al. (2011)[14]	To investigate the effect of the non-stress test and music on maternal anxiety and the effect of maternal anxiety and music on fetal heart rate changes.	Turkey; 2009; 201 pregnant women. Group with music = 96; group without music = 105	Group and individual care Duration: not mentioned. Description: pregnant women who came for routine prenatal care were randomized to receive music (n = 96) or no music (n = 105) during the non-stress test. Before and after the test, these women were asked to complete the Spielberg State-Trait Anxiety Inventory in two interviews; the primary outcome was considered maternal state anxiety scores before and after the non-stress test. The secondary outcome was the baseline fetal heart rate, the number of fetal movements, major accelerations, dubious non-stress test, variable decelerations, and the minimum procedure time.	State-Trait Anxiety Inventory	Before the non-stress test, the average state-trait anxiety scores of the music and control groups were of 38.1 ± 8.8 and 38.08 ± 8.2 respectively. On the other hand, after the non-stress test, the average state-trait anxiety scores of the music and control groups were of 35.5 ± 8.2 and 40.2 ± 9.2 respectively. While in the control group the non-stress trest brought about a statistically significant increase in the state-trait anxiety scores, listening to music during the non-stress test resulted in a decrease in state-trait of anxiety scores in the study group; however it was not statistically significant. The baseline fetal heart rate of the music group was significantly higher than that of the control group	The non-stress test has anxiogenic effects on mothers and listening to music, and a positive impact on maternal and fetal parameters, but it is an open question whether maternal anxiety during pregnancy can affect fetal accelerations to the point of influencing clinical judgment
The Limerick Lullaby project: an intervention to relieve prenatal stress; Carolan et al. (2012)[25]	To explore the impact of lullaby singing during pregnancy	Ireland; 2009; 6 pregnant women	Group care. Duration: 4 sessions. Description: the pregnant women were recruited in childbirth classes at a maternity hospital. Six pregnant women participated and learned to sing three lullabies in four group sessions with musicians. In-depth qualitative views were taken approximately three months later to capture the experiences of the women.	Questionnaire with open questions	They suggest that learning to sing lullabies during pregnancy has benefited women in terms of relaxation, feeling closer to the fetus, connecting with other pregnant women, and providing an additional tool for communication at the beginning of the newborn period. Some women described a deep feeling of love	The main advantage of this intervention is that it is non-pharmacological and easy to implement. At the same time, it appears to be a pleasurable exercise for pregnant women, and it has an effect on reducing maternal stress and

(*Continued*)

Table 1 (*Continued*)

					and connection with the fetus while singing the lullabies	encouraging infant attachment
Effects of music therapy on parturient anxiety; Lima et al. (2014)[1]	To evaluate the effectiveness of music therapy in reducing anxiety during the first clinical period of childbirth using the methodology of clinical, controlled and randomized teaching	Brazil; 29 pregnant women; study group = 15; control group = 14	Care was not mentioned. duration: not mentioned. description: not mentioned.	State-Trait Anxiety Inventory	All pregnant women in the study group reported that undergoing music therapy was easy, and that they would use music therapy in the next labor. After the intervention, the researchers observed a decrease in the grade of anxiety from high to medium in 2 patients, and from high to low in 1 patient	When submitted to the intervention with music therapy, there was a reduction of anxiety in 3 patients in the study group. The parturients in the control group maintained the degree of anxiety throughout delivery
The effects of music listening on psychosocial stress and maternal-fetal attachment during pregnancy Chang et al. (2015)[21]	To examine the effects of listening to music on psychosocial stress and maternal-fetal attachment during pregnancy	Taiwan; 2009-2010; 296 pregnant women; study group = 145; control group = 151	Individual care. Duration: 30 minutes for 2 weeks. Description: the study group received routine prenatal care and listened to music. The control group received only routine prenatal care	Pregnancy Stress Rating Scale; Perceived Stress Scale; and Maternal Fetal Attachment Scale	The results of the posttest identified a significantly lower level of psychosocial stress in the study group compared with the controls, particularly regarding the stresses related to baby care, the change in family relationships, and the identification of the maternal role	The findings support the effectiveness of listening to music in helping pregnant women cope with stress, especially pregnancy-related stress. Although this study found no effect on musical hearing on perceived general life stress or maternal-fetal attachment, the evidence indicates that music is an effective non-invasive pregnancy-related intervention for women which has minimal or no side effects and is economical and convenient
Effects of music listening on stress, anxiety, and sleep quality for sleep-disturbed pregnant women; Liu et al. (2016)[22]	To examine the effects of listening to music on stress, anxiety and quality of sleep in pregnant women with sleeping disorders	Taiwan; 2014; 121 pregnant women; study group = 61; control group = 60	Individual care. Duration: 30 minutes for 2 weeks. Description: the control group received only the usual prenatal care. The study group was instructed to listen to at least 1 record (30 minutes) of the five pre-recorded CDs compiled by the researcher, or a minimum of 30 minutes of their favorite music per day at bedtime for two weeks	Pittsburgh Sleep Quality Index, Perceived Stress Scale, and State-Trait Anxiety Inventory	No statistically significant differences were identified among the 60 pregnant women with sleeping disorders in their demographic and clinical characteristics or the scores on the scales prior to the administration of musical intervention. The analysis confirmed that the posttest scores on the scales reflected significant differences from their initial scores. With all other variables controlled, the women in the study group had statistically lower scores on the scales than the controls	This study pointed out that two-week music-listening interventions can reduce stress, anxiety and improve the quality of sleep of pregnant women with sleeping disorders. The analysis of participants' diaries also suggested that mothers' choices about musical genres may be more correlated with perceived prenatal benefits or with the desire to interact with the fetus
Effect of music intervention on maternal anxiety and fetal heart rate pattern during non-stress test; Oh et al. (2016)[30]	To examine the effects of musical intervention on maternal anxiety, fetal heart rate pattern, and test time during non-stress test for prenatal fetal assessment	South Korea;2013-2014; 60 pregnant women; study group = 30; control group = 30	Individual care. Duration: 20 minutes; the number of days was not mentioned. Description: the prepared songs had a time of 60 to 80 beats, and were based on the pregnant woman's heart count. The songs were divided into 5 genres, such as hymns or contemporary Christian music, classics, pop, and, with the help of music experts, a total of 25 CDs were made (5 songs of each genre). The isolated space was used to block out the noise. In the study group, after the non-stress test, the State-Trait Anxiety Inventory was applied, blood pressure, pulse and temperature were recorded, while the songs selected by the pregnant woman were played for 20 minutes. In the control group, the non-stress test was applied, but there was no music while collecting the data	State-Trait Anxiety Inventory	The study group had significantly lower scores on the anxiety scale than the controls. There were no significant differences in systolic blood pressure and pulse rate between the two groups. The baseline fetal heart rate was significantly lower in the study group than in the controls. Acceleration frequency in fetal heart rate was significantly increased in the study group compared to the controls. There were no significant differences in fetal movement and test time for reactive non-stress test between the groups	Musical intervention can be effective for anxiety during the non-stress test
Effects of prenatal music stimulation on state/trait anxiety in full-term pregnancy and its influence onchildbirth: a	To investigate the effect of music on maternal anxiety, before and after the non-stress test, and	Spain; 2013-2014; 409 pregnant women; study group = 204; control group = 205	Individual care. Duration: 40 minutes per session; listening to music for 14 sessions, three times a week, at the same time of day. Description: the 409 pregnant women who went for	State-Trait Anxiety Inventory	Before the non-stress test, term pregnant women who received musical intervention had a state-trait-anxiety score similar to those of the control group. After the test, the average anxiety	Prenatal music intervention can be a useful and effective tool to reduce anxiety in pregnant women at term during the non-

Table 1 (*Continued*)

randomized controlled trial; García González et al. (2018)[24]	the effect of music on delivery		routine prenatal care were randomized in the third trimester to receive music (n = 204) or no music (n = 205) stimulation during the non-stress test. The study group intervention were informed about how to listen to the music at home and received music recorded on CDs		score score of each group was recorded; study group: 30.58 ± 13.2; control group: 43.11 ($p < 0.001$).	stress test and improves the delivery process by reducing the first stage of labor
Effects of prenatal music stimulation on fetal cardiac state, newborn anthropometric measurements and vital signs of pregnant women: A randomized controlled trial; García González et al. (2017)[4]	To identify the effects of prenatal musical stimulation on the vital signs of pregnant women at term, on the modification of the fetal cardiac state during the fetal monitoring cardiotocograph and on the anthropometric measurements of newborns after birth	Spain; 2013-2014; 409 pregnant women; study group = 204; control group = 205	Individual care. Duration: 40 minutes per session; listening to music for 14 sessions, 3 times a week, at the same time of day. Description: The 409 pregnant women who went for routine prenatal care were randomized in the third trimester to receive music (n = 204) or no music (n = 205) stimulation during the non-stress test. The study group were informed about how to listen to the music at home and received music recorded on CDs	Fetal cardiac status, maternal vital signs, anthropometric measurements of the fetus	The graphs showed a significant increase in FCFB and greater fetal reactivity, with accelerations of fetal heart rate in pregnant women with musical stimulation. After the fetal monitoring cardiotocograph, there was a statistically significant decrease in systolic and diastolic blood Pressure and heart rate in women in the study group	Music can be used as a tool that improves the vital signs of pregnant women during the third trimester, and can influence the fetus, increasing fetal heart rate and fetal reactivity
Effect of Turkish classical music on prenatal anxiety and satisfaction: a randomized controlled trial in pregnant women with pre-eclampsia; Toker and Kömürcü (2017)[15]	To evaluate the effect of music therapy on anxiety and satisfaction in pregnant women with pre-eclampsia	Turkey; 2012-2014; 70 pregnant women; study group = 35; control group = 35	Individual care. Duration: 30 minutes, every day, for 7 days. Description: the pregnant women in the study group were subjected to a 30-minute classical Turkish music session every day for a period of 7 days (5 days before and 2 days after delivery) while the controls received routine care and were also assigned 30 minutes bedrest per day	Personal Information Form, State-Trait Anxiety Inventory, systolic and diastolic blood pressure, pulse and respiratory rate, non-stress test, fetal movements, fetal heart rate (for the first 5 days)	The differences in anxiety scores were not statistically significant ($p > 0.05$). On the other hand, the Newcastle Satisfaction scores of the study group were higher than those of the controls ($p < 0.01$). Finally, when considering fetal movement counts, a significant increase was determined in the study group, while music therapy reduced the fetal heart rate and blood pressure ($p < 0.05$)	It can be suggested that nurses and midwives can use music therapy in the care and monitoring of pregnant women with pre-eclampsia in obstetric units
Prenatal singing – sound alchemy for pregnant women; Martins (2017)[19]	To broaden awareness of women's body wisdom, empowerment, expression of the pregnant woman's feelings, affective communication between the pregnant woman and the baby in the womb	Brazil; 2016; 12 pregnant women	Group care. Duration: weekly, 2 hours long. Description: the methodology of the prenatal singing classes involved female songs and games of musical and vocal improvisation; sound meditations with creative visualizations; sound bath; circle singing; body breathing; and vocal exercises to prepare for childbirth; and sound improvisations with musical instruments	Not mentioned	Not mentioned	Vocal exercises were keys that opened the doors for connection with the nature of the female body. They had as objectives to release the voice and to express sensations and feelings vocally to unveil the relations among the voice, the pelvic floor and breathing, and to send affective sonic vibrations to the fetus in the womb. The experiences emphasized the affective dimension in the act of singing: the vibrational communication that the pregnant woman established in the communication with her unborn child
Prenatal listening to songs composed for pregnancy and symptoms of anxiety and depression: a pilot study; Nwebube et al. (2017)[26]	To determine whether listening to specially-composed music would be an effective intervention to reduce symptoms of prenatal anxiety and depression	United Kingdom; 2014-2015; 111 pregnant women	Individual care. Duration: 20 minutes, for 12 weeks. Description: the study group listened to specially-composed songs daily, and the control group did daily relaxation. Composer Jennie Muskett wrote the songs specifically for use during pregnancy. The songs were composed using specific times, musical forms and phrases designed to induce a calm state	State-Trait Anxiety Inventory, Edinburgh Postnatal Depression Scale	The study group showed lower values of trait anxiety ($p = 0.0001$) (effect size: 0.80), state anxiety ($p = 0.02$) (effect size: 0.64) and Edinburgh Postnatal Depression Scale ($p = 0.002$) at week 12 in relation to the baseline by the paired *t*-test. There were no such changes in the control group	Although this pilot study showed high levels of friction, the results suggest that listening to relaxing music regularly should be further explored as an effective non-pharmacological means to reduce anxiety and prenatal depression
State-trait anxiety levels during pregnancy and fetal parameters following intervention with music therapy; Garcia-Gonzalez et al. (2018)[7]	To investigate the effect of music therapy on maternal anxiety, before and after the non-stress test, and the effect of maternal anxiety on the process of childbirth and birth	Spain; 2013-2014; 409 pregnant women; study group = 204; control group = 205	Individual care. Duration: 40 minutes per session; listening to music for 14 sessions, 3 times a week, at the same time of day. Description: the 409 pregnant women who went for routine prenatal care were randomized in the third trimester to receive music (n = 204) or no music (n = 205) stimulation during the non-stress test. The study	STAI	After the non-stress test, the study group had significantly lower scores on state anxiety as well as trait anxiety than the controls. In addition, the study group had lower levels of trait anxiety than the controls in relation to the variables of the birth process, and greater weight at birth and breast	The intervention of music therapy during pregnancy can reduce high levels of trait anxiety during the third trimester. Further research on the influence of music therapy as an intervention on maternal anxiety and on the

(*Continued*)

Table 1 (*Continued*)

			group were informed about how to listen to the music at home and receive music recorded on CDs		circumference in the newborn, respectively	birth process and birth size are needed during pregnancy
DELIVERY						
Effect of music on labor and on the newborn; Tabarro et al. (2010)[20]	To verify and describe the effects of music in the labor of women assisted in five maternities; to verify the baby's behavior and reactions, when submitted to the melodies listened to by their mothers during pregnancy and labor, through the mothers' speeches, obtained in the first three months after delivery	Brazil; 2008; initially, 87 pregnant women, but only 27 fulfilled the criteria for inclusion	Group care. Duration: from prenatal care to the postpartum period. Description: musical awareness through a portable tape player, a series of 8 to 10 melodies was made available, selected especially for the study, in an intensity compatible with the acceptance of the group. The period for this experiment ranged from 35 to 45 minutes. In each session, a different series of melodies was listened to by the same group. The groups ranged from two to nine women. The information recorded on the sheets of each pregnant woman was used to record an individualized CD that was then delivered to each future mother with the recommendation to take it to the maternity ward at the time of delivery. During the time of observation of labor, every 2 hours, the music was suppressed for a period of 30 minutes. At the end of each of these periods, the elements of control of the evolution of labor were recorded on an observation sheet	Not mentioned	Only 12 parturients had their labor accompanied by the melodies of their choice, and they were interviewed in the postpartum period. As for the effect of music on the newborns, 20 mothers were interviewed; 1 of the 12 accompanied in labor did not have her stereo during the puerperium, and could not perform the observation with her baby	Effects such as pain relief during contractions, help in reducing tension and fear, environmentalization of the parturient in the hospital, encouragement to prayer and spirituality have been reported.
Effects of music therapy on labor pain and anxiety in Taiwanese first-time mothers; Liu et al. (2010)[23]	To investigate the effects of music on the reaction to pain and anxiety during labor	Taiwan; 2009; 60 pregnant women; study group = 30; control group = 30	Individual care. Duration: during delivery. Description: the study group received routine care and music therapy, while the controls received only routine care. A visual analog self-report scale for pain and a nurse assessed the behavioral intensity present to measure labor pain. Anxiety was measured with a visual analog scale for anxiety and finger temperature. Pain and anxiety between groups were compared during the latent phase (2-4 cm of cervical dilation) and active phase (5-7 cm) separately	Visual Analog Scale for Pain, Present Behavioral Intensity, Visual Analog Scale for Anxiety and Finger Temperature	In comparison with the controls, the study group presented significantly lower pain, anxiety and finger temperature during the latent phase of labor. However, no significant differences were found between the two groups in any of the outcome measures during the active phase	This study provides evidence for the use of music as an intervention for pregnant women having labor pains and anxiety during the latent phase of labor. The results confirm that listening to music is an acceptable and non-medical coping strategy for pregnant women, especially for the reduction of pain and anxiety in the initial phase of labor
Comparison between massage and music therapies to relieve the severity of labor pain; Taghinejad et al. (2010)[32]	To compare the effects of massage and music therapy on the severity of labor pain	Iran; 2007; 101 pregnant women; massage group = 51; music therapy group = 50	Individual care. Duration: during the latent phase of labor. Description: pregnant women hospitalized for normal delivery were randomly divided into two groups. Pain was measured using the visual analog scale, and the two groups were compared in terms of pain intensity before and after the interventions. As soon as the cervix was dilated by up to 3-4 cm, women in the massage therapy group were asked to close their eyes and breathe rhythmically and deeply During contractions of the uterus, they were asked to breathe more deeply and more calmly, concentrating on the massage. All patients in this group received a 30-minute massage. The women in the music therapy group were asked to listen to soft traditional music (1 of 5 optional types) without lyrics, using headphones for 30 minutes, starting early in the active phase of labor	Visual Analog Scale	Mothers in the massage therapy group had a lower level of pain compared to those in the music therapy group ($p = 0.009$). A significant difference was observed between the two groups in terms of severity of pain after the intervention ($p = 0.01$). Labor pain was significantly relieved after therapeutic massage ($p = 0.001$)	Massage therapy has proven to be an effective method for reducing and relieving labor pain compared to music therapy, and can be clinically recommended as an alternative. It is a safe and affordable method of pain relief, in which the use of pharmacological or non-pharmacological methods are optional
Effect of music on labor pain relief, anxiety level and postpartum analgesic	To evaluate the effect of music on labor pain and anxiety, maternal	Turkey; 2012; 156 pregnant women; study group = 77; control group = 79	Individual care. Duration: during labor. Description: the study group listened to music during labor. Pain intensity and anxiety	Visual Analog Scale	The study group had a lower level of pain and anxiety compared to the controls at all stages of labor. A significant	Listening to music during labor has a positive impact on labor pain and anxiety,

Table 1 (*Continued*)

requirement: a randomized controlled clinical trial; Simavli et al. (2014)[16]	hemodynamics, fetal-neonatal parameters, and the need for analgesics in the postpartum period in pregnant women		levels were measured using the Visual Analog Scale. The two groups were compared in terms of pain severity, anxiety level, maternal hemodynamics, fetal-neonatal parameters, and need for analgesics in the postpartum period		difference was observed between the two groups in terms of maternal hemodynamics and fetal heart rate after the intervention. Postpartum analgesic requirement decreased significantly in the study group	maternal-fetal parameters and the need for analgesics
Effect of music therapy during vaginal delivery on postpartum pain relief and mental health; Simavli et al. (2014)[5]	To evaluate the effects of music therapy on postpartum pain, anxiety level, satisfaction, and rate of early postpartum depression	Turkey; 2012; 161 pregnant women; study group = 80; control group = 81	Individual care. Duration: during labor. Description: The study group listened to self-selected songs during labor. Postpartum pain intensity, anxiety level and satisfaction rates were measured using the Visual Analog Scale, and the postpartum depression rate was assessed using the Edinburgh Postpartum Depression Scale in postpartum days one and eight	Visual Analog Scale and Edinburgh Postnatal Depression Scale	The study group had a lower level of postpartum pain and anxiety than the controls, and this was statistically significant at all time intervals. A significant difference was observed between the two groups in terms of satisfaction rate ($p < 0.001$) and the rate of postpartum depression on days 1 and 8	The use of music therapy during labor reduced anxiety and postpartum pain, increased satisfaction with the child's birth and reduced the rate of early postpartum depression. Music therapy can be clinically recommended as an analogous, safe, easy and pleasurable non-pharmacological method for postpartum well-being
Effects of music during multiple cesarean section delivery; Handan et al. (2018)[17]	To evaluate the effects of nursing intervention using music therapy to relieve anxiety levels in pregnant women with multiple cesarean sections	Turkey; 2015-2016; 60 pregnant women; study group = 30; control group = 30	Individual care. Duration: during the c-section. Description: a list of their favorite songs was selected to be played during the c-section. They were reproduced at the desired volume of each patient throughout the surgery, using a stereo player. Physiological parameters and anxiety levels in the form of the questionnaire were recorded on the suture too. The data from the questionnaire were collected from women in the control group through interviews; their vital findings were recorded before and after anesthesia procedures, without intervention during the entire surgery	Structured questionnaire and Visual Analog Scale	The physiological indicators of anxiety and blood pressure were reduced regarding the initial values in the study group when compared to the control group	Music therapy reduces the physiological and cognitive responses of anxiety in patients undergoing multiple c-sections, and can be used in the clinical practice
Coping with preoperative anxiety in cesarean section: physiological, cognitive, and emotional effects of listening to favorite music; Kushnir et al. (2012)[31]	To assess the effects of listening to music while waiting for a c-section: emotional, cognitive and stress-related physiological reactions	Israel; 2005; 60 pregnant women; study group = 28; control group = 32	Individual care. Duration: 40 minutes before c-section. Description: a list of songs of their choice was selected. The study group listened to selected songs using a headset 40 minutes before the c-section.	Mood State Scale; Perceived Threat of surgery scale; vital signs	The study group experienced a significant increase in positive emotions and a significant decline in negative emotions and perceived threat of the situation when compared to the controls, who exhibited a decline in positive emotions, an increase in perceived threat of the situation, and no change in negative emotions. The study group also exhibited a significant decrease in systolic blood pressure compared to a significant increase in sdisatolic blood pressure and RF in the controls	Listening to your favorite music just before a c-section can be an economic and emotionally-focused coping strategy
POSTPARTUM PERIOD						
The efficiency and duration of the analgesic effects of musical therapy on postoperative pain; Sen et al. (2010)[18]	First, to discover the effect of music therapy on postoperative analgesia, and, secondly, to determine the duration of its effect.	Turkey; 2009; 70 pregnant women; group 1 with music = 35; group 2 with no music = 35	Individual care. Duration: 1 hour after surgery. Description: pregnant women who underwent c-sections were included and randomly allocated to two groups as follows: in group 1, pregnant women listened to music through a headset for an hour after surgery, while in group 2, they did not listen to any music during the same period. In the postanesthetic care unit, pregnant women were connected to a patient-controlled analgesia device (tramadol 3 mg/ml), which was adjusted to deliver a 20 mg bolus, with a 15 min blocking interval and a maximum 4-hour dose of 150mg. Postoperative pain was assessed using the visual analog scale and tramadol consumption was recorded at 4, 8, 12, 16, 20 and 24 hours	Visual Analog Scale	There was a significant decrease in group 1 in relation to the frequency of analgesic delivery in the 4th postoperative hour. Regarding the consumption of tramadol in the postoperative period, the values measured in the fourth hour were significantly lower in group 1. The total amount of tramadol consumption and additional analgesic use in the 24-hour postoperative period were again lower in group 1 when compared to group 2. All scores on the Visual Analog Scale were lower in group 1 when compared to those of group 2	Music therapy provided after surgery reduces postoperative pain in the first 24 hours and analgesic consumption in the first 4 hours

in assisting parturient women, resstablishing the autonomy of women regarding labor and birth.[35]

As for the results found, we could not perform an in-depth analysis of the methodology, since some studies were inaccurate, omitted data, and/or presented vague information. In addition to the incomplete methodology, some of the studies selected do not inform if they were conducted by music therapists, and most of them were performed by other health professionals. As a result, these studies did not have a theoretical framework for music therapy and did not follow a validated protocol. Low methodological quality was a common finding among systematic reviews that examine music-based interventions, with variations between the number of interventions and the duration of each session, which can interfere with the results, limiting the benefits that the pregnant woman and her fetus could obtain; therefore, it is necessary to think about comprehensive interventions that cover the prenatal, delivery and postpartum periods.[8]

Carvalho[33] states that, in the practice of music therapy, music is not therapeutic, and is not used as an end in itself, but becomes a mediator of therapeutic individual or group relationship guided by a qualified and certified music therapist. It is important to remember that music therapy is included among the services provided by the Brazilian Unified Health System. In addition to maintaining its autonomy, it develops a practice consistent with the principles, seeking the necessary transformations, and without restricting its vision. The conviction regarding the contribution of studies on music therapy for the medical field brings another level of scientific knowledge necessary for the development of music therapy, thus answering existing questions. The need for the area to discuss some concepts is understood, contributing to clinical practice in different contexts.[36]

Another aspect to be observed was that most of the studies found were conducted outside Brazil (only three Brazilian publications were found), which suggests that further studies in this area should be carried out. Based on the results of previous studies, Brandalise[37] states that there are few music therapists who publish articles and books reporting their findings and professional experiences, suggesting that there should be an incentive and preparation for the professional to engage in research.

Conclusion

From the results obtained, we can concluded that the performance of music therapy during the prenatal, delivery and postpartum periods can provide several benefits to the pregnant woman and the fetus, thus justifying its importance in this field. There is a demand in the job market for more professional music therapists, as well as for more studies on this subject performed by these professionals.

Conflict of Interests

The authors have no conflict of interests to declare.

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Lipschütz Ulcer: An Unusual Diagnosis that Should Not be Neglected

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Abstract

The diagnosis of genital ulcers remains a challenge in clinical practice. Lipschütz ulcer is a non-sexually transmitted rare and, probably, underdiagnosed condition, characterized by the sudden onset of vulvar edema along with painful necrotic ulcerations. Despite its unknown incidence, this seems to be an uncommon entity, with sparse cases reported in the literature. We report the case of an 11-year-old girl who presented at the emergency department with vulvar ulcers. She denied any sexual intercourse. The investigation excluded sexually transmitted infections, so, knowledge of different etiologies of non-venereal ulcers became essential. The differential diagnoses are extensive and include inflammatory processes, drug reactions, trauma, and malignant tumors. Lipschütz ulcer is a diagnosis of exclusion. With the presentation of this case report, the authors aim to describe the etiology, clinical course, and outcomes of this rare disease, to allow differential diagnosis of genital ulceration.

Keywords

- vulvar ulcer
- adolescent
- non-venereal lesions

Introduction

The differential diagnosis of genital ulcers is challenging in clinical practice. Overall, the etiology of vulvar ulcers is commonly infectious; nonetheless, they may be a presentation of a wide variety of pathologies, such as autoimmune disorders, inflammatory processes, drug reactions, trauma, or malignant tumors.

Vulvar ulcers are unusual in non-sexually active adolescent girls. When diagnosed in young girls, parents and clinicians should inicially suspect sexually transmitted infection due to sexual contact or abuse. After excluding this scenario, knowledge of different etiologies of non-venereal ulcers becomes essential, considering the pathological characteristics, related symptoms, and clinical manifestations.

In 1913, the Austrian dermatologist Benjamin Lipschütz[1] first described acute genital ulcers in adolescent girls without any evidence of sexually transmitted infections. These ulcers, named Lipschütz ulcers (LUs), are also known as acute genital ulcers, reactive non-sexually related acute genital ulcers, ulcus vulvae acutum, acute vulval ulcers, or primary aphthous ulcers.

The correct incidence of this disease cannot be estimated because it continues being poorly understood and underdiagnosed. The absence of established diagnostic criteria turns this entity into a diagnosis of exclusion.

The aim of the present case report is to describe the etiology, clinical course, and outcomes of LUs allowing the performance of a correct differential diagnosis of vulvar ulcerations.

Case Report

An 11-year-old girl, healthy and without any comorbidities, presented at our emergency department with painful vulvar lesions, vulvar edema, and burning sensation on urination. The symptoms started 6 days before and were preceded by a sudden onset of fever, malaise, and odynophagie. She denied

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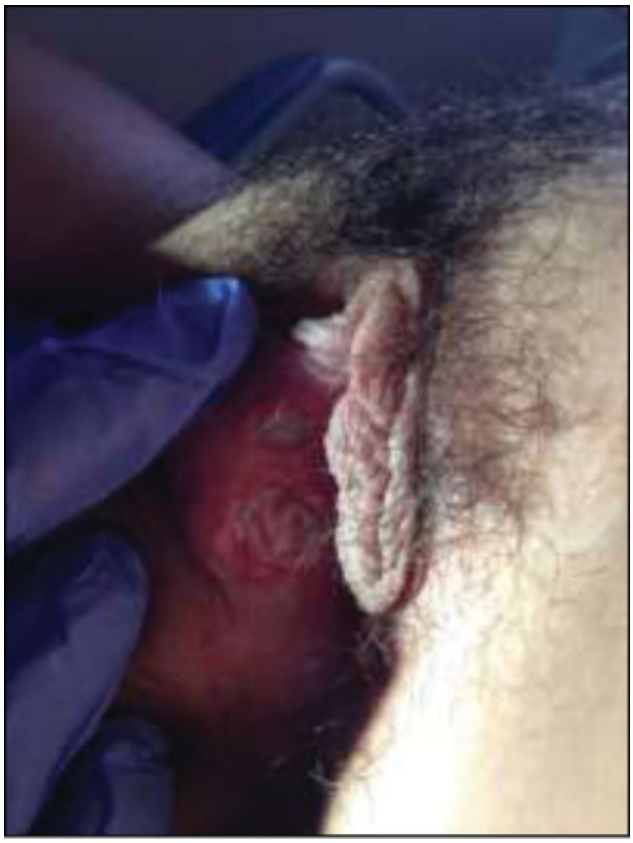

Fig. 1 Day 6.

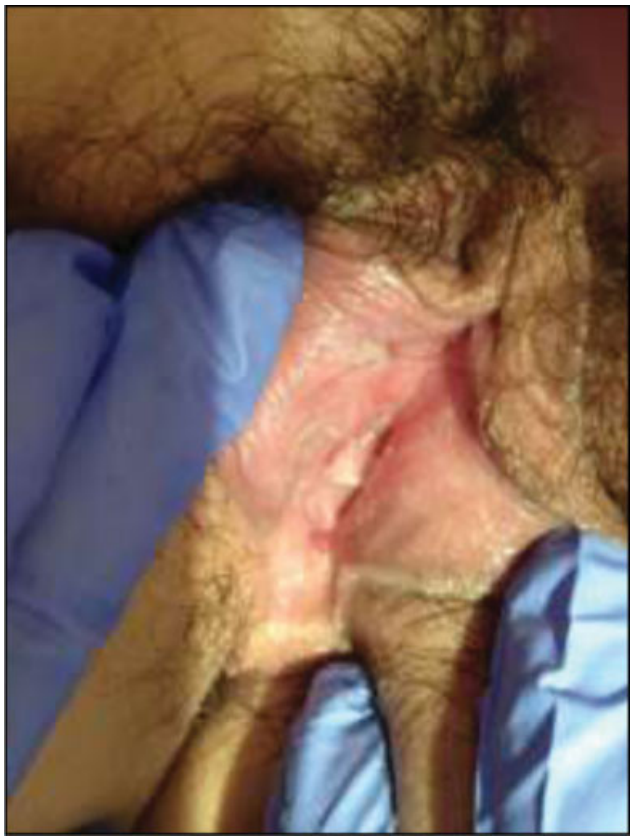

Fig. 2 Day 14.

any sexual intercourse for the time being, trauma, oral ulcerations, drugs intake, recent travels, or similar previous episodes.

Physical examination revealed three painful ulcerated and necrotic lesions on the medial face of the right labia minora, the largest with 15 mm in diameter, with regular and well delimited margins, an overlying grey exudate surrounded by a purpuric halo (►**Figure 1**). No enlarged lymph nodes were detected, neither other skin or mucous membrane lesions.

In the laboratory evaluation, despite the total leukocyte count being normal, there was mild lymphocytosis (50%). C-reactive protein and liver enzymes where within the normal range. Herpes simplex virus, cytomegalovirus, treponema pallidum, hepatitis C virus, clamydia trachomatis, toxoplasmosis, mycoplasma, and human immunodeficiency virus serologies were all negative. There was evidence of prior Epstein-Barr virus infection (IgG positive, IgM negative).

The patient was treated with topical cinchocaine 10 mg/g, for pain relief, and comfort measures (sitz baths and voiding in the tub to minimize external dysuria), with progressive health improvement (►**Figure 2**). Complete healing occurred in about 3 weeks, with no scarring and no recurrence to date.

Discussion

Lipschütz ulcers are a rare cause of acute vulvar ulcerations of nonvenereal origin and in most cases affect young people without previous history of sexual contact. This condition presents with sudden onset of a painful vulvar ulcer, that is usually preceded by influenza or mononucleosis-like symptoms, such as malaise, fever, asthenia, myalgia, pharyngotonsilitis, lymphadenopathy, and headache.

The exact incidence of LU is unknown, and the average age at diagnosis reported in a large series of patients by Farhi et al.[2] was 16.6 years.

The etiology remains unclear, although infectious or idiopathic causes seem to be associated. Its onset is associated with an exacerbated immune response to viral diseases, such as Epstein-Barr virus, cytomegalovirus, influenza virus, paratyphoid fever, toxoplasmosis, *mycoplasma pneumoniae*, and mumps. However, in most cases, the association with an infection could not be confirmed. Furthemore, these ulcers may as well be caused by drugs.

The exact mechanism involved in the formation of ulcers distant to the primary infection site is poorly understood. It has been suggested the theory of a hypersensitivity reaction to a viral or bacterial infection, leading to deposition of immune complexes in the dermal vessels, which subsequently activates the complement system, resulting in microthrombi formation and consequent tissue necrosis.[2]

The ulcers are deep, with red borders and a necrotic centre covered by grey exudate or grey-black eschar, presenting in a mirror pattern. They primarily affect the medial face of the labia minora and vestibule, with variable size, with lesions $>$ 1 cm having been described. Secondary erythema and edema may be impressive.

There is no clear consensus in the literature regarding the precise diagnosis of this pathology. Fahri et al.[2] attemped to establish some diagnostic criteria (major and minor), suggesting that the diagnosis could be achieved if all major and at least one minor criteria were present. The proposed major criteria are age < 20 years, ulcer with sudden onset and acute evolution, first and unique episode, no sexual contact in the 3 months prior to complaint, and absence of immunodeficiency. Moreover, the minor criteria are one or more ulcers with necrotic or fibrinous core, with a well delimited, painful, and symmetrical pattern. Nevertheless, in a retrospective study conducted by Vieira-Baptista et al.,[3] the authors concluded that the diagnostic criteria for Lipschutz ulcer should be less strict.[4]

Lipschutz ulcer is a diagnosis of exclusion, and it is reached only after precluding other causes of genital ulcer. The differential diagnoses are extensive and include inflammatory processes, drug reactions, trauma, and malignant tumors. Histologic examination is not of diagnostic value because findings are nonspecific.[5–10]

The treatment is mainly symptomatic. Pain relief is the fundamental aim of supportive care (topical anaesthetics/oral analgesics), even tough, for multiple, large, or deep necrotic ulcerations, topical or a short course of systemic corticosteroids may be considered if the patient fails to respond to topical agents.[11]

It is important to emphasize the non-sexual transmission of the disease, and patients should also be informed about the self-limiting nature of the syndrome.

Generally, the natural course is benign, with spontaneous regression within a few weeks.

Conclusion

The presence of an erosion or ulcer in genitalia usually leads us to think of sexually transmitted infections. However, it is important to have a broader approach and consider non-venereal causes as well. Lipschütz ulcers have been considered an uncommon and probably underdiagnosed entity. Therefore, it is crucial to recognise and include them in the differential diagnosis of vulvar ulcerations; otherwise, patients with this type of lesions will continue to be treated for herpes simplex and other disorders, without any benefit. Besides, this would create unnecessary distress and concern in patients and parents, due to suspected sexual abuse.

Conflict of Interests
The authors have no conflict of interests to declare.

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Federação Brasileira das Associações de Ginecologia e Obstetrícia

FEBRASGO POSITION STATEMENT

Management of hypoactive sexual desire disorder in women in the gynecological setting

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The National Specialty Commission for Sexology of the Brazilian Federation of Gynecology and Obstetrics Associations (FEBRASGO) endorses to this document. The content production is based on scientific studies on a thematic proposal and the findings presented contribute to clinical practice.

Key-points

- Hypoactive Sexual Desire Disorder (HSDD) is the most prevalent sexual dysfunction among women.
- Psychological, biological, behavioral, relational and environmental factors are the main causes of HSDD and should be assessed during HSDD investigation.
- Gynecologists should assess women's sexual problems during routine gynecological consultations.
- Sexual education is the start point of managing HSDD, secondary to psychological, biological, behavioral, relational and environmental factors.
- The multidisciplinary teamwork approach achieves the best results to deal with HSDD.

Recommendations

- Allow women to talk about sexual concerns in the gynecological setting.
- Offer general sexual education measures (information on physiology of sexual response, genital anatomy, erogenous zones, sexual fantasies, masturbation, bibliotherapy) to improve sexual repertoire. Inform women that sexual desire is an individual dimension that varies from person to person.
- Suggest shared activities and communication techniques for couples in order to bring sex into their everyday routine.
- Suggest a non-hormonal method (copper IUD) for those complaining of HSDD after hormonal contraceptive use. Prescribe topical hormonal therapy, vaginal moisturizers, and lubricants for climacteric women.
- Assess mental health (anxiety, depression) and refer to a competent professional.
- Do not recommend self-masturbation for women victims of sexual abuse as this may arise intrusive thoughts of the sexual abuse.
- For victims of sexual violence, use strategy to redirect their feeling of guilt. Inform that children are victims of the perpetrator as they are unable to give permission or refuse sexual practices.
- Discuss sexual rights and pleasure with women victims of sexual violence. Tell them about their right to have a healthy and pleasurable sexual life just like any other woman, and inform them it was not their fault; it was the perpetrator's crime.
- Counsel for the adoption of a healthy lifestyle, including physical activity, a healthy diet and avoiding smoking, which may contribute to their wellbeing and more receptivity to sexual stimuli.
- Refer to psychotherapy or sex therapy when appropriate.

Background

Hypoactive Sexual Desire Disorder (HSDD) is defined as the absence or marked reduction of desire or motivation to engage in sexual activity, as manifested by any of the following criteria: *i)* reduced or absent spontaneous desire (sexual thoughts or fantasies); *ii)* reduced or absent responsive desire to erotic cues and stimulation; or *iii)* inability to sustain desire or interest during sexual activity over a period of at least six months that causes distress.[1] A study conducted in 29 countries involving 14,000 women aged 40 to 80 years showed that 26 to 43% of them complained about HSDD.[2] In Brazil, a systematic review showed a prevalence of 11 to 75% of HSDD.[3-7]

There are several psychological, biological, behavioral, relational and environmental factors that interfere with sexual desire.[3] The treatment of HSDD requires knowledge about factors that may be associated with the diagnosis of HSDD (Chart 1).

Chart 1. Factors associated to sexual complaints in women.

Use of medications and health problems
Current or past psychiatric disorders
History of sexual, physical or emotional abuse
Beliefs and attitudes towards sex Body image disturbances
Alcohol, drug and substance use disorders
Work-related stress
Relational conflicts

There are personal characteristics, such as motivation for a healthy sex life, level of self-awareness about one's sexual response, knowledge of sexuality, adjustment of interpersonal and dyadic relationships, optimistic personality, among others that define one's ability to modulate the effects of these factors on the sexual function.[4]

What is the scenario regarding the management of HSDD in women in the gynecological setting?

Assessment of the sexual function is often missed in general practitioner settings mostly because physicians have limited knowledge about sexuality and limited academic training in sexual health issues.[5] Women are less likely to report spontaneously their sexual problems to their physicians. However, about 27% of second-year gynecology residents from a tertiary service in human reproduction did not assess the sexual function of their female patients. Moreover, they tend to categorize the sexual response as normal or abnormal according to their own beliefs.[6]

What is the role of the gynecologist in the assessment of HSDD in women?

Gynecologists (Gyn) may provide women with strategies for improvement of the HSDD related to biological conditions, habits, lack of information of sexual health, poor sexual repertoire, and pain penetration disorders. Education measures may help women's understanding of biological and psychological aspects of female sexual response and important aspects of sexual rights and sexual pleasure, as the anatomy of genitalia, erogenous zones, and sexual repertoire.[7] However, when conditions such as sexual violence, dyadic problems, sexual repression and poor mental health are the basis for HSDD, the woman should be referred to psychotherapy and/or a sex therapy (ST).

What are the most common biologic factors related to HSDD in women in gynecologic routine care?

Several pathological condition may interfere with the female sexual response. We listed some common factors in gynecological routine care that can impair sexual desire in women.

Arterial hypertension, diabetes, obesity, and metabolic syndrome

Metabolic syndrome and its components like diabetes mellitus, dyslipidemia, obesity, and arterial hypertension may be associated to HSDD and orgasm disturbances, although there is still controversy on this matter.[8,9] The physiopathology of these diseases is related to endothelial dysfunction and frequently associated with anxiety and depression disorders that culminate in high (68.2%) rates of HSDD in women.[10] In postmenopausal women suffering from arterial hypertension, obesity and diabetes some conditions such as the aging process, depression, and the lack of sexual attractiveness are additional risks for sexual dysfunctions.[11]

The management of HSDD in women with metabolic syndrome and correlated pathologies of arterial hypertension, diabetes and obesity may comprehend drug prescription[12] and education measures. Personalized sexual pharmacotherapy on-demand such as testosterone, psychoactive agent (bupropion, buspirone), PDE-5 inhibitor (sildenafil) are used to enhance the neuroendocrine balance between sexual excitation and sexual inhibition.

Psychiatric disorders: anxiety, depression, stress and schizophrenia

Generalized anxiety disorder, panic disorder and stressful experiences offer high risk for HSDD.[13] Chronic stress may increase circulating cortisol levels, which may alter hypothalamus-hypophysis axis (HHA) function, leading to alteration in sexual steroids synthesis. Emotional stress may alter cognition and concentration on sexual stimuli during sexual relations.[13] The odds for women presenting sexual dysfunction increases 4.11 times with depression.[14] Symptomatology associated may be apathy, lack of interest or irritability, restlessness, and sleep disorders, affecting the sex drive. Unsatisfactory sexual activity, in turn, may lead to depression and/or anxiety and relational conflicts.[15]

The management of HSDD due to anxiety and depression symptoms involves the use of antidepressants (ADs) that, in turn, may have negative impact on the sexual function, especially serotonin.

Schizophrenia is associated to HSDD in women, and antipsychotic medication and mood stabilizers increase prolactin (PRL) levels, which contribute even more for HSDD in these women.[16,17] The management of HSDD in women with schizophrenia involves referral to a psychiatrist, who may reduce the dosage, change the drug, or add aripiprazole[18] to improve sexual desire.

Selective Reuptake Inhibitors and Serotonin-Noradrenaline Reuptake Inhibitors

The intensity of the adverse effect in sexual response of women using selective reuptake inhibitors (SSRIs) and

serotonin-noradrenaline reuptake Inhibitors (SNRIs) is underestimated and variable. [19] In a population of women with major depressive disorder in remission, HSDD was diagnosed in 64.3% of those using fluoxetine compared to 37.5% of those using escitalopram.[20]

Reduction of sex drive induced by antidepressants occurs through a number of mechanisms, including anticholinergic effects, blockade of the 5-HT2C receptor, increase of serotonergic activity, inhibition of nitric oxide production, D2 blockade, testosterone, FSH and LH reduction, sedation, increase in prolactin concentration and through action of neurotransmitters in the parasympathetic system and central nervous system.[15] The use of antidepressants such as bupropion, mirtazapine, trazodone and vilazodone was associated to less negative impact on the sexual function, since they are used as "antidotes" in the treatment of sexual dysfunction induced by SSRIs and SNRIs.[21] Multimodal antidepressants such as vortioxetine at a dosage of 10-20 mg/day have less impact on sexual function, sleep and weight, which favors treatment adherence.[22] Follow-up with a psychiatrist together with a gynecologist is highly recommended.[23] The management of sexual dysfunction induced by serotonin reuptake inhibitors may include discussing the possibility of adding/changing medication by the psychiatrist (Chart 2).

Chart 2. Add-back therapy used in sexual dysfunction induced by selective serotonin reuptake inhibitors and selective noradrenergic inhibitor

- Trazodone at a dose of 200-400 mg/day may enhance sexual desire
- Bupropion at a dose of 150-30 mg/day may enhance sexual desire, arousal and orgasm
- Buspirone at a dose of 30-60 mg/day may enhance sexual desire and orgasm
- Mirtazapine at a dose of 30-60 mg/day may enhance orgasm
- Refer to psychotherapy and, if necessary, to sex therapy and couples therapy

Hormonal contraceptives use

Combined oral contraceptives (COC) may reduce sex drive, arousal and sexual pleasure, but do not interfere with sexual satisfaction.[24] Combined oral contraceptives inhibit the HHA interfering with sexual steroids synthesis, which may reduce sexual motivation.[13] In addition, COCs increase the synthesis of the sex hormone binding globulin (SHBG) that binds to testosterone (T), promoting the reduction of circulating testosterone (T), and this may lead to HSDD. Compared to women who use a copper intrauterine device (IUD), those who use medroxyprogesterone acetate quarterly, the vaginal ring or etonogestrel implant are, respectively, at 2.62, 2.53 and 1.60 times more risk of HSDD.[25] Decreased frequency of sexual thoughts and arousability may lead to contraceptive discontinuation.[26] The management of HSDD due to hormonal contraceptives use implies identifying possible confounding factors to assume COC as the real cause of HSDD. Counseling for the adoption of a healthy lifestyle, including physical activity may contribute to the wellbeing and more receptivity to sexual stimuli.[27] A non-hormonal method (copper IUD) may improve HSDD in these cases.

What are the most common sociocultural and psychological factors related to HSDD in women in the gynecologic routine care?

Several sociocultural and psychological factors such as myths, beliefs, taboos, repression, dyadic problems, sexual violence, and long-term relationship may interfere with the female sexual response. Gyn should assess briefly these conditions in the gynecological routine care.

Myths, beliefs, taboos, repression

Many women find the educational source of their sexual behavior and practice in religious teachings and the media, although these are not always reliable sources of scientific information. In some cultures, according to religious precepts and morals, sex is a religious duty for women and virginity is a feminine virtue to preserve, women should have sex with their husbands even if they do not wish to do so, they have no right to reach sexual pleasure and should always remain passive when having sex.[28] Thus, some sexual practices that are not aligned with their religious values and beliefs may promote guilt, anxiety, depressive symptoms and relationship difficulties, as well as psychological discomfort, potentially leading to sexual dysfunctions.[29]

Unhealthy lifestyle habits and exposure to continuous stressors such as work and caring for small children cause cognitive distraction and negative emotions, which affect sexual desire and arousal.[30] In these cases, managing sexual dysfunctions involves adapting the therapeutic guidance to the religious cultural context and to the patient's belief system.[31] So far, there is no consensus on the management of HSDD resulting from sociocultural factors, mainly secondary to religious beliefs. However, based on expert opinion, it is important to validate the importance of faith and discuss with women about their right to sexual pleasure and the risk for general and sexual health implied in neglecting female sexual pleasure.[7] Techniques such as physical activity[27] and focused attention exercises for stress management decrease cognitive distraction and improve sexual desire/arousal[30] and may contribute to wellbeing and more receptivity to sexual stimuli. Also, prescribe bibliotherapy and erotic movies to improve sexual repertoire and offer general sexual education

measures (erogenous zones, sexual fantasies, clitoral manipulation to achieve orgasm).

Dyadic factors

Interpersonal aspects such as infidelity, violence (sexual, domestic, psychological and physical), the partner's sexual dysfunction (erectile dysfunction, premature ejaculation), unemployment, poor education, post-traumatic stress, sexually transmitted infections (STIs), low sexual satisfaction, sexual intercourse focused on penetration, and limited sexual repertoire are commonly interrelated with sexual dysfunction.[3] Overall satisfaction with the sex life influences the quality and maintenance of the desire, while rejection produces a negative influence.[32] The complaint of HSDD is common in women whose partners have sexual dysfunctions such as erectile dysfunction (ED) and premature ejaculation.[33] Likewise, when the woman has HSDD, the male partner has more difficulties with his sexual performance, forming a vicious cycle that exacerbates the couple's sexual difficulties.[34] Limited sexual communication between partners leads to both limitation of sexual repertoire and sexual dissatisfaction.[35]

Long-term relationships

Sex drive in long-term relationships may be affected by individual, interpersonal and social factors (Chart 3).[32]

Chart 3. Factors associated to sexual dysfunctions in long-term relationships

Aspects	Variables that interfere with sexual function
Individual	General and sexual expectations toward the partner, attractiveness, autonomy, affection, commitment, self-esteem, stress, fatigue
Interpersonal	Responsiveness to partner, emotional intimacy with the partner, communication between partners, professional status, sexual compatibility, sexual satisfaction, relationship over time, routine, monotony
Social	Gender expectations, equality, power war, restrictive sexual attitudes, sexual repression

HSDD as a consequence of a long-term relationship is frequently linked to unrealistic expectations, relational routine and sexual discrepancies between couples.[36] Facilitators of sexual function include attractiveness between partners, prioritizing the relationship, maintaining one's individuality, having good communication, emotional intimacy, self-esteem and control over external stressors.[32] Suggest shared activities and communication techniques to the partner aiming to restore the sexual interest and bring sex into everyday routine.[27] Inform women that sexual desire may decline in a long-term relationship associated with the sex routine and aging process.[36]

Marital infidelity

Marital infidelity is defined as a breach of the exclusivity contract established by the couple regarding psycho-emotional and sexual aspects.[37] It refers to the emotional and/or sexual involvement with a person that is not the primary partner in the relationship. Studies on the prevalence of infidelity are long-standing, revealing 20 to 25% of occurrence among married peoples.[38] Infidelity can be caused by several factors especially cultural issues, the couple's sexual dysfunctions, dissatisfaction with the relationship, family patterns, self-esteem issues, sexual abuse in the childhood, and pathological states like hypersexuality.[37] Infidelity may have negative impact on sexual desire in women.

The management of HSDD due to infidelity may include counseling measures such as providing information to women on infidelity rates among couples with the aim to motivate discussion on this issue. In addition, the Gyn may list some factors related to infidelity, like poor quality of the marital relationship, lack of communication and intimacy of couples. Explain that a break in the sexual routine enhances intimacy and that the social interaction of the couple may enhance the dyadic relationship and improve the sexual response.[37] Inform of strategies to improve the sexual repertoire through bibliotherapy and sex aids (toys, literature and erotic movies), stimulate individual or shared sexual fantasies, and innovate the atmosphere for sexual relations. Alternatively refer the woman to a psychotherapist, couple's therapist, and/or sex therapy.

Sexual violence (sexual abuse and rape)

Sexual assault affects women's general health and may result in HSDD and avoidance of intimate relationships due to lack of confidence, emotional blunting and fear of touching the body related to the traumatic experience.[39] In this case, the woman should be treated by multidisciplinary team, as it is essential to deal with mental health issues. In order to assess sexual problems in these women, the Gyn should ask them to talk about their experience by using open-ended question (would you like to talk about the assault you suffered?). In addition, the Gyn may validate her sexual complaint related to the assault, and may use strategy to redirect possible feelings of guilt (it is a crime for an adult to sexually molest a child. The child is not aware that the family member or close person is having inappropriate conduct. The child is not capable of consenting or refusing sexual practices. The genital zone has nerve endings that trigger pleasure when touched. The perpetrator of abuse usually attracts the child with gifts, promises and good behavior, but later on, threatens

the child, causing fear). Moreover, it should be interesting to inform women on the prevalence of sexual abuse (unfortunately, sexual abuse is very common mainly among girls and is usually perpetrated by a family member or person related to the child). This approach may help women to feel more confident to share their experience related to the trauma. Finally, the Gyn should refer the woman to a psychiatrist or psychotherapy, depending on the level of distress, and later, to a sex therapist.

How physiological hormones affect sexual desire in women?

Pre- and post-menopause period

During menopausal transition, endocrine alterations and the genitourinary syndrome of menopause (GSM) may affect the quality of life and cause HSDD.[40] Social conditions or stressors are also important in the decline of sexual desire and should be primarily observed when dealing with menopausal women.[41] Dyspareunia due to hypoestrogenism may cause sexual activity avoidance.[42] In these cases, local therapy may be suggested.[43] In postmenopausal women, the use of transdermal testosterone may improve HSDD.[44] Oral or injected testosterone is not recommended and the use of dehydroepiandrosterone (DHEA) to treat HSDD does not seem effective. In women with genitourinary syndrome, topical estrogen is recommended according to eligibility criteria.[41] Alternatively, the use of vaginal moisturizers and lubricants is safe to improve sexual pain in these women.

Pregnancy-puerperal period

In this period, the changes in the female sexual response affect 40 to 70% of pregnant women.[45] A reduction in the sexual frequency and satisfaction has been shown as a consequence of fear of hurting the fetus, impaired body image, dissatisfaction with the partner and mood problems in this phase,[46] as well as because of changes in the focus of attention and also due to the woman's tiredness.[47] The second quarter of pregnancy is the most favorable period for the female sexual function.[48] In the puerperal period, 58.3% of Brazilian nursing mothers complained of a reduction in sexual frequency and poor communication with the partner.[49] Dyspareunia may affect 41% of women after vaginal delivery and 2% after cesarean delivery[50], which may cause discomfort and HSDD. The management of HSDD in the pregnancy-puerperal period is based mainly in counseling measures[7,51], the prescription of sexual educational strategies for women's management of HSDD (erogenous zones, sexual fantasies, clitoral manipulation to achieve orgasm), and the use of lubricants and moisturizers to improve pain during penis-vagina penetration.

Final considerations

HSDD is prevalent in women and there are several psychological, biological and sociocultural factors related to this condition. Gynecologists should receive training to manage this problem in the gynecologic setting. Several factors related to HSDD cannot be addressed by the Gyn and women should be referred to a specialist for an adequate management of HSDD. However, the Gyn may promote sexual health through counseling, education strategy and specific measures to treat endocrine problems related to HSDD in women.

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