



**UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA**

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**Deteccção de células imunes associadas ao tumor como
marcadores prognósticos no câncer colorretal**

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Câmpus de Botucatu, para obtenção do título de Doutor em Cirurgia e Medicina Translacional.

Orientadora: Profa. Dra. Sandra Aparecida Drigo Linde

**Botucatu
2023**

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Palavras-chave: Câncer colorretal; Immunoscore;
Imuno-histoquímica; Marcador tumoral.

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Resumo em português

O câncer colorretal (CCR) é doença heterogênea, com diferenças moleculares, de prognóstico e resposta a terapia de acordo com a localização anatômica do tumor. O infiltrado imune tem papel importante na evolução dos tumores CCR. O Immunoscore, baseado na densidade de linfócitos T CD3+ e CD8+ infiltrantes do tumor, mostrou-se como importante marcador prognóstico em CCR. O objetivo deste estudo foi avaliar as células imunes infiltrantes do tumor e identificar seu papel prognóstico em CCR de acordo com a localização anatômica. Foi realizada uma análise *in silico* utilizando o algoritmo xCell para estimar as células imunes infiltrantes do tumor para identificar populações imunes associadas ao prognóstico in CCR. As populações imunes infiltrantes foram identificadas a partir de dados de expressão gênica global de 542 amostras de CRC obtidas no banco de dados público Gene Expression Omnibus (GEO). A seguir, para validação experimental foram construídos microarranjos de tecidos (TMA) contendo regiões do centro do tumor (CT) e da margem de invasão (MI) de 206 casos de CCR de diferentes localizações anatômicas para detecção do Immunoscore e dos marcadores identificados na análise *in silico*. Para identificação do Immunoscore, as densidades dos marcadores CD3 e CD8 foram detectadas por imuno-histoquímica e semi-quantificadas nas regiões CT e MI por análise óptica manual. Duas categorias de Immunoscore Low e High foram consideradas nas análises. Vinte e sete células imunes detectadas pelo algoritmo xCell foram correlacionadas com sobrevida global em CCR. Identificamos quatro células imunes infiltrantes associadas ao prognóstico. Baixo infiltrado de neutrófilos foi associado a pior prognóstico em CCR ($p=0,033$), e particularmente em tumores do cólon direito ($p=0,020$). Além disso, células Th2 e células T CD4+ de memória foram associadas ao prognóstico em tumores do cólon direito ($p=0,006$ e $p=0,032$, respectivamente). Em tumores do lado esquerdo, células T CD8+ ($p=0,026$) e Th2 ($p=0,050$) foram associadas ao prognóstico. Destes, os neutrófilos foram selecionados para serem validados no grupo de validação. A análise do Immunoscore mostrou associação entre LOW Immunoscore e diminuição da sobrevida global em CCR, embora sem significância estatística ($p=0,065$). Em concordância com os resultados obtidos na análise *in silico*, pacientes com tumores do lado esquerdo e LOW Immunoscore

presentaram pior prognóstico ($p=0,034$), principalmente para pacientes com tumores retais ($p=0,020$). Além disso, foi observado baixo infiltrado de neutrófilos MPO+ nos tumores de cólon direito associado com diminuição da sobrevida nestes pacientes ($p=0,020$). Nossos resultados mostraram que o Immunoscore adaptado para análise não digital (óptico) foi associado ao prognóstico em nossa coorte de CCR, especialmente para tumores do lado esquerdo. Por outro lado, os neutrófilos foram indicados como um potencial marcador prognóstico em tumores do cólon direito, reforçando a importância do contexto das células imunes na progressão do CCR com diferenças na lateralidade do tumor. Além disso, a análise simplificada do Immunoscore aqui proposta pode oferecer uma abordagem facilmente aplicável na prática clínica, especialmente em instituições com recursos limitados.

Palavras-Chave: câncer colorretal, marcadores imunológicos, lateralidade tumoral, prognóstico, Immunoscore, imuno-histoquímica.

Resumo em inglês

Colorectal cancer (CRC) is a heterogeneous disease, with differences in molecular patterns, prognosis and response to therapy according to the tumor anatomical location. The tumor immune infiltrate plays an important role in CRC progression. The Immunoscore, based on the density of tumor infiltrating CD3+ and CD8+ T lymphocytes, has been shown to be an important prognostic marker in CRC. The aim of the present study was to evaluate tumor-associated immune cells as prognostic markers in CRC according to anatomical location. The aim of this study was to evaluate tumor-infiltrating immune cells and identify their prognostic role in CRC based on anatomical location. An *in silico* analysis using the xCell algorithm was performed to estimate tumor infiltrating immune cells to identify immune populations associated with prognosis in CRC. Infiltrating immune cells were identified from bulk gene expression data from 542 CRC samples obtained from the public Gene Expression Omnibus (GEO) database. Next, for experimental validation, tissue microarrays (TMA) were constructed containing regions of the tumor center (CT) and invasion margin (IM) of 206 cases of CRC from different anatomical locations to detect the Immunoscore and the markers identified in *in silico* analysis. To identify the Immunoscore, the densities of CD3 and CD8 markers were detected by immunohistochemistry and semi-quantified in the CT and IM regions by manual optical analysis. Two categories of Immunoscore Low and High were considered in the analyses. Twenty-seven immune cells detected by the xCell algorithm were correlated with overall survival in CRC. We identified four infiltrating immune cells associated with prognosis. Low neutrophil infiltration was associated with worse prognosis in CRC ($p=0.033$), particularly in right colon tumors ($p=0.020$). Furthermore, Th2 cells and memory CD4+ T cells were associated with prognosis in these tumors ($p=0.006$ and $p=0.032$, respectively). In left-sided tumors, CD8+ ($p=0.026$) and Th2 ($p=0.050$) T cells were associated with prognosis. Of these, neutrophils were selected to be validated in the validation group. Immunoscore analysis showed an association between LOW Immunoscore and decreased overall survival in CRC, although without statistical significance ($p=0.065$). In agreement with *in silico* analysis results, patients with left-sided tumors and LOW Immunoscore had a worse prognosis ($p=0.034$),

especially for patients with rectal tumors ($p=0.020$). Furthermore, a low infiltration of MPO+ neutrophils was observed in right-sided colon tumors associated with decreased survival in these patients ($p=0.020$). Our results showed that the Immunoscore adapted for non-digital (optical) analysis was associated with prognosis in our CRC cohort, especially for left-sided tumors. Conversely, neutrophils were indicated as a potential prognostic marker in right-sided colon tumors, reinforcing the importance of the immune cell context in the progression of CRC with differences in tumor laterality. Furthermore, the simplified Immunoscore analysis proposed here can offer an easily applicable approach in clinical practice, especially in institutions with limited resources.

Keywords: colorectal cancer, immunological markers, tumor laterality, prognosis, Immunoscore, immunohistochemistry.

Lista de abreviaturas e siglas

AJCC – *American Joint Committee on Cancer*

APCs – do inglês “*antigen-presenting cells*” – células apresentadoras de antígenos

CCD – carcinoma de cólon direito

CCE – carcinoma de cólon esquerdo

CCR - carcinoma colorretal

CR – carcinoma de reto

CT – centro do tumor

CTL – do inglês “*cytotoxic T lymphocyte*” – linfócito T citotóxico

DFS – do inglês “*disease free survival*” – tempo livre de doença

GEO - Gene Expression Omnibus

HR – do inglês “*hazard ratio*” – razão de risco

IFN – interferon

IL – interleucina

IS – Immunoscore

ISB – do inglês “*Immunoscore adapted to biopsies*” – Immunoscore quando adaptado a biópsias

MHC – do inglês “*major histocompatibility complex*” – complexo principal de histocompatibilidade

MI – margem de invasão

MMR – do inglês “*Mismatch repair*” – sistema de reparo de DNA

MMR deficiente ou dMMR – deficiência no sistema de reparo de DNA

MSI – do inglês “*microsatellite instability*” – instabilidade de microsatélites

MSI+ – instabilidade de microsatélites detectada

MSI-H – do inglês “*microsatellite instability high*” – alta instabilidade de microsatélites

MSS – do inglês “*microsatellite stability*” – estado estável de microsatélites

NK – células natural killer

NKT – células natural killer T

OS – do inglês *“overall survival”* – sobrevida global

TCR – do inglês *“T cell receptor”* – receptor de células T

TILs – do inglês *“Tumour-Infiltrating Lymphocytes”* – linfócitos infiltrantes de tumor

TMA – do inglês *“Tissue Microarrays”* – microarranjos de tecidos

TME – do inglês *“tumor microenvironment”* – microambiente tumoral

TNM – sistema TNM de estadiamento do câncer (T: tumor primário; N: linfonodos; M: metástase)

TTR – do inglês *“time to recurrence”* – tempo de recorrência

UICC – *Union for International Cancer Control* – União Internacional de Controle do Câncer

CAPÍTULO 1

Revisão da literatura

O câncer colorretal (CCR) é um problema de saúde pública mundial, sendo a 3ª neoplasia mais comum no mundo com 1,9 milhão (10%) de casos novos em 2020 (SUNG et al., 2021). No Brasil, a estimativa para o triênio 2023 a 2025 é que ocorrerão 72 mil (10,2%) casos novos de câncer de cólon e reto. Excluindo os cânceres de pele não melanoma, o CCR é o segundo mais incidente em homens e em mulheres, com 22 mil e 24 mil casos novos, respectivamente (SANTOS et al., 2023). Em 2020, ocorreram 9.889 óbitos por câncer de cólon e reto (8,4%) em homens e 10.356 (9,6%) em mulheres (SANTOS et al., 2023). Embora sua incidência geral esteja diminuindo em muitos países desenvolvidos, observa-se um aumento em adultos jovens (ARAGHI et al., 2019).

A doença metastática ainda é a maior causa de morte por câncer. Metade dos casos de CCR desenvolve metástases, sendo que 20% são metastáticos ao diagnóstico (TAMAS et al., 2015). Desde 2004, terapias alvo, administradas isoladamente ou em combinação com quimioterapia, têm sido utilizadas aumentando a sobrevida de pacientes CCR metastáticos (DEKKER et al., 2019; TRAN et al., 2015). No entanto, os tratamentos disponíveis ainda não são capazes de prevenir a progressão da doença ou diminuir substancialmente o número de mortes por CCR.

O câncer de cólon proximal e distal e o câncer de cólon esquerdo (CCE) e direito (CCD) são conceitos relacionados, porém não são sinônimos. De acordo com a lateralidade, os tumores podem ser agrupados como câncer de cólon direito (CCD), que inclui os tumores originados no ceco, cólon ascendente, flexura hepática e metade direita do cólon transversal; o câncer de cólon esquerdo (CCE) incluem os tumores localizados no metade esquerda do cólon transversal, flexura esplênica, cólon descendente e sigmoide; e câncer de reto (CR) (DEKKER et al., 2019; STINTZING et al., 2017). Os CCR podem ser agrupados de acordo com a localização anatômica do tumor nos três segmentos colorretais; sendo o câncer de cólon proximal que inclui os tumores localizados no ceco, cólon ascendente e cólon transversal; o câncer de cólon distal, incluindo aqueles localizados na flexura esplênica, cólon descendente ou cólon sigmoide; e o câncer de reto (CR) (KEUM; GIOVANNUCCI, 2019). Com relação à frequência, dados

populacionais de incidência de câncer nos Estados Unidos mostram uma frequência de 39% de tumores proximais, 24% distais e 30% CR, e 6% outros (SIEGEL et al., 2023). O CCD é mais frequente em mulheres e o CR em homens (TAMAS et al., 2015). Dados dos EUA mostram que embora na última década se observe uma queda na incidência de CCR como um todo, quando avaliados de acordo com a faixa etária se observa um aumento da incidência em indivíduos com menos de 50 anos, principalmente para o CR, com aumento de cerca de 2% ao ano, *versus* 0,5% e 1,6% ao ano para tumores no cólon proximal e distal, respectivamente (SIEGEL et al., 2023). Essa tendência de aumento de casos de CCR em indivíduos abaixo de 50 anos de idade é também observada em outros países desenvolvidos, como Austrália, Canadá, Nova Zelândia e Reino Unido (KEUM; GIOVANNUCCI, 2019). Com relação ao prognóstico, o CCD apresenta pior prognóstico que CCE independentemente do estadiamento, idade ou tratamento conforme reportado em uma meta-análise de 66 estudos (PETRELLI et al., 2017).

O prognóstico de pacientes com CCR ressecável baseia-se no estadiamento do tumor, seguindo a classificação do *American Joint Committee on Cancer* (AJCC) e *Union for International Cancer Control* (UICC), que considera o tamanho do tumor (T), presença de células neoplásicas nos linfonodos regionais (N) e evidência de metástase à distância (M). Embora seja uma ferramenta poderosa, a classificação AJCC/UICC-TNM falha ao fornecer uma informação prognóstica incompleta. O desfecho clínico pode variar significativamente entre pacientes dentro do mesmo estadio histológico do tumor (NAGTEGAAL; QUIRKE; SCHMOLL, 2011). Desta forma, vários marcadores baseados em vias moleculares, mutações e estratificação baseada no perfil de expressão gênica têm sido propostos para melhorar a estratificação de risco dos pacientes com CCR (TRAN et al., 2015).

A resposta imune no câncer

O sistema imunológico pode desempenhar um papel na prevenção da ocorrência, crescimento e difusão metastática do CCR. A infiltração de células imunes inatas como células natural killer (NK), natural killer T (NKT) e linfócitos T $\gamma\delta$ foi relatada como um fator de bom prognóstico. Ao contrário, os infiltrados de macrófagos

geralmente estão associados a um prognóstico ruim em carcinomas humanos (COCA et al., 1997; CORVAISIER et al., 2005; EDIN et al., 2013; SIEGEL et al., 2017; TACHIBANA et al., 2005).

A resposta antitumoral específica é gerada pelo sistema imune adaptativo e, em particular, pelos linfócitos T $\alpha\beta$. Resumidamente, após a lise de células tumorais por células do sistema imunológico inato, como células NK, células NKT e macrófagos, as células apresentadoras de antígenos, principalmente células dendríticas, podem capturar, processar e apresentar antígenos tumorais para linfócitos T CD4 através da classe de histocompatibilidade principal (MHC) II ou aos linfócitos T CD8 através do MHC I após um processo de maturação. A ativação das células T requer três sinais: (1) reconhecimento do peptídeo antigênico apresentado pela célula apresentadora de antígeno; (2) ativação de moléculas coestimuladoras (CD80/CD28, CD40/CD40L); e (3) recrutamento de citocinas (interleucina [IL]-1, IL-2, IL-6, IL-12, interferon [IFN] γ). As células T CD8+ ativadas podem então reconhecer e lisar células tumorais (MALKA et al., 2020).

As células T CD4+ ativadas podem modular a resposta imune antitumoral e podem se diferenciar em diferentes subgrupos de células. As células T CD4+ ativadas se diferenciam em várias classes funcionais com base no meio de citocinas, apresentação de antígenos e expressão de moléculas co-estimulatórias. Combinações de estímulos ambientais e produção de citocinas autócrinas levam à indução de várias vias de sinalização para regular a expressão de fatores de transcrição específicos de linhagem. As células T helper (Th) CD4+ são polarizadas para um dos vários tipos de células efetoras definidos pelos perfis de citocinas e funções imunológicas: Th1, Th2, Th17, Th9, T reguladora (Treg) e linfócitos T auxiliares foliculares (Tfh) (BASU et al., 2021).

Enquanto as células Th1, Th9 e Tfh estimulam a resposta imune antitumoral, as células Th2 e Treg induzem uma resposta pró-tumorigênica imunossupressora e um duplo papel das células Th17 contribui para a complexidade funcional dessa rede. As células dendríticas apresentam peptídeos antigênicos tumorais às células T CD4+ via molécula do MHC de classe II, que na presença de IL-12 leva à ativação da resposta imune Th1. O IFN- γ e o TNF- α secretados pelas células Th1 ativadas atuam diretamente nas células tumorais e induzem apoptose, senescência e parada da proliferação. Além

disso, as células Th1 podem regular positivamente a ativação das células NK e das células B para fortalecer ainda mais as respostas imunes antitumorais. As células Th1 ativadas secretam IL-2 que medeia a ativação direta de células T CD8⁺ que expressam IL-2R α e sua proliferação e sobrevivência. Em contraste, sabe-se que as células Treg, produtoras de IL-10, TGF- β , e IL-35; e as células Th2, produtoras de IL-4, IL-5, IL-10, IL-13 e IL-17, promovem um microambiente imunossupressor que permite a progressão do tumor (BASU et al., 2021).

O papel das células T CD8⁺ citotóxicas tem sido amplamente estudado no CCR e os linfócitos infiltrantes de tumor (*Tumour-infiltrating lymphocytes*; TILs) são os principais atores da resposta imune antitumoral. Os linfócitos T navegam e escaneiam efetivamente quase todas as partes do corpo em busca de material indesejado ou estranho; consequentemente, as células T naive e efetoras são migradores altamente habilidosos, centrais para a vigilância imunológica e o desenvolvimento da imunidade adaptativa contra infecções e câncer. Fatores de transcrição, citocinas, quimiocinas, integrinas e sinais metabólicos regulam estritamente a diferenciação e os fenótipos das células T, e as linhagens de células T são consideradas fixas e mutuamente exclusivas. As células T CD8⁺ citotóxicas são as principais exterminadoras de patógenos e células neoplásicas, com as células T CD4⁺ desempenhando papéis importantes na manutenção da resposta CD8⁺ e prevenção da exaustão (RASKOV et al., 2021).

Utilizar o sistema imunológico do próprio paciente para combater o câncer não é uma ideia nova, e tem ganhado especial atenção nos últimos anos diante do sucesso da imunoterapia contra determinados tumores. A imunoterapia compreende diferentes abordagens, incluindo anticorpos monoclonais, drogas com moléculas pequenas, terapia celular adotiva, vírus oncolíticos e vacinas contra o câncer (ROY; SINGH; MISRA, 2022). A literatura recente enfatiza o papel das células T no tratamento do câncer, utilizando diferentes estratégias, como os inibidores de *checkpoint* imune, terapia celular adotiva e vacinas. A imunoterapia baseada em células Th CD4⁺ tem sido apontada com mais vantajosa em relação às estratégias focadas apenas em células T CD8⁺, por se tratar de células primordiais na resposta imune adaptativa, incluindo a ativação de células T CD8⁺ para uma resposta antitumoral amplificada. A exigência de reconhecimento de peptídeos específicos e correspondência de moléculas de HLA pode

limitar o sucesso terapêutico de células T CD8+ direcionadas a neoantígenos derivados principalmente de mutações pontuais, uma vez que essas mutações podem alterar significativamente a cinética de interação com células T CD8+ e diminuir a atividade de CTL. A natureza mais promíscua das células T CD4+ permite a interação com uma ampla variedade de neoantígenos e *drivers* oncogênicos. Simultaneamente, uma regulação recíproca de células Th CD4+ e células B tem se mostrado essencial para diferenciação e função de células Th1, Th2 e células B efectoras, o que indica o potencial terapêutico de estimular a resposta imune de Th CD4+ e aumentar indiretamente a secreção de anticorpos por B células para uma resposta antitumoral mais eficiente. No entanto, estudos em vários modelos pré-clínicos e ensaios clínicos que apontaram obstáculos potenciais, como um microambiente tumoral (TME) hostil, presença de populações de células T inibitórias e receptores de *checkpoint* imune sugerem que estimular uma única subpopulação de função Th CD4+ isoladamente pode não ser adequado para resposta antitumoral robusta. Combinações de terapias, tais como inibição direcionada de *drivers* oncogênicos por anticorpos bloqueadores/neutralizantes, inibidores de moléculas pequenas, inibidores de quinases reguladoras do ciclo celular, dentre outros, estão sendo testados em vários estágios de ensaios clínicos. Compreender o papel das células Th imunossupressoras, tais como TH2, Th17 e Treg, no microambiente tumoral (TME) é igualmente crítico para intervenção terapêutica. Uma imunoterapia de câncer bem-sucedida exigirá um equilíbrio cuidadoso do compartimento Th CD4+ para orquestrar esforços essenciais que mediem a regressão do tumor (ROY; SINGH; MISRA, 2022). Outras células do sistema imunológico, como células apresentadoras de antígenos (APC) e células natural killers (NK), além dos linfócitos T, são importantes componentes da resposta anti-tumor e têm sido estudados como potenciais imunoterapias no combate ao câncer. Os desafios para o desenvolvimento de imunoterapias efetivas ainda são diversos, incluindo o desenvolvimento de respostas imunológicas adequadas sem provocar toxicidades indesejadas.

Infiltrado imune como marcador prognóstico no CCR

Evidências crescentes demonstram que a evolução do câncer é fortemente dependente do complexo microambiente tumoral (TME) em que se desenvolve. O TME

compreende uma variedade de entidades celulares incluindo fibroblastos, células endoteliais, vasos sanguíneos, vasos linfáticos e células do sistema imunológico. A infiltração de células imunes adaptativas mostrou ter um valor prognóstico superior aos critérios clássicos de invasão tumoral, incluindo grau, estadio e status metastático em CCR (ANGELL et al., 2020; GALON et al., 2006; GALON; FRIDMAN; PAGÈS, 2007). Por outro lado, diferentemente do que se observa em CCR, estudos em diversos tumores examinaram o significado dos componentes imunes como fatores prognósticos, no entanto, nenhum deles superou o sistema de estadiamento TNM na análise multivariada (BRUNI; ANGELL; GALON, 2020; LI et al., 2016).

Uma potencial tradução clínica do contexto imune em um marcador prognóstico no CCR foi estabelecido e designado como “Immunoscore” (ANGELL; GALON, 2013; FRIDMAN et al., 2012; GALON; FRIDMAN; PAGÈS, 2007). O papel prognóstico do Immunoscore em CCR foi analisado pela primeira vez num estudo retrospectivo com uma grande coorte de CCR estadios I-IV ressecado há mais de 10 anos (GALON et al., 2006; PAGÈS et al., 2005). Pagès et al. (PAGÈS et al., 2005) encontraram em uma primeira coorte de 958 CCR estadios I-IV uma associação entre a presença de células T dentro do tumor e ausência de invasão metastática precoce (status VELIPI-negativo) e aumento da sobrevida. Usando a mesma coorte de pacientes, eles mostraram que o tipo, densidade e localização das células imunes dentro do tumor podiam influenciar o prognóstico clínico dos pacientes (PAGÈS et al., 2005).

A resposta imune adaptativa composta por células T (CD3+) com fenótipos citotóxicos (CD8+) e de memória (CD45RO+) avaliados no centro do tumor bem como na margem invasora foi avaliada em outro estudo do mesmo grupo e foi identificada como um parâmetro altamente significativo para predizer o tempo de recorrência e sobrevida global. Esses resultados iniciais foram validados em duas populações de pacientes com CCR (GALON et al., 2006). Após a padronização do método, a mesma equipe de pesquisadores mostrou a utilidade de um Immunoscore baseado na avaliação combinada de marcadores de memória e citotoxicidade para a predição de recorrência tumoral e sobrevida, especificamente em estádios iniciais (UICC/TNM estadios I e II) de CCR (PAGÈS et al., 2009). Assim, o *Immunoscore* quantifica por análise digital a densidade de linfócitos TCD3+ e CD8+ por imunomarcagem na região central do tumor

(CT) e na margem de invasão (IM) tumoral e varia de zero (I=0) a 4 (I=4) (Figura 1) (GALON et al., 2012).

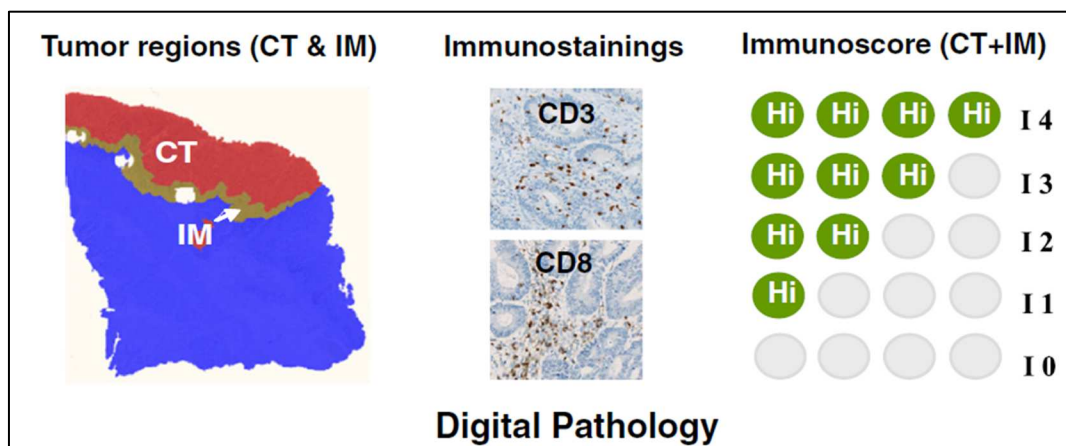


Figura 1. Definição e quantificação do Immunoscore. Os marcadores CD3 + e CD8 + são avaliados pelo método de imuno-histoquímica em cortes histológicos contendo duas regiões do tumor, região central do tumor (CT) e na margem de invasão (IM). A quantificação dos marcadores é feita por análise digital. O Immunoscore fornece uma pontuação que varia de zero (I0), quando são observadas densidades baixas dos dois marcadores em ambas as regiões (CT+IM), até quatro (I4), quando altas densidades são encontradas em ambas as regiões. (Figura retirada de GALON et al., 2012).

Outros estudos demonstraram a utilidade do Immunoscore na predição do prognóstico de CCR inicial e avançado (BERGHOF et al., 2016; MLECNIK et al., 2018, 2018; PAGÈS et al., 2009). Essa superioridade prognóstica mostrou-se estatisticamente significativo para CCR estadio I, II e III, na previsão de sobrevida livre de doença, sobrevida doença-específica e sobrevida global com progressão e invasão tumorais dependendo dos parâmetros imunológicos (BROUSSARD; DISIS, 2011; MLECNIK et al., 2011).

Mlecnik et al. (MLECNİK et al., 2011) investigaram a relação entre a extensão da densidade de células imunes (células T CD45RO+ e CD8+) no centro e margem invasora dos tumores, o grau de extensão do tumor (estádios pT e pN) e a frequência de recorrência tumoral em duas coortes independentes de 415 e 184 pacientes com CCR de estádios I-IV. No subgrupo de pacientes com CCR estádios I-III, eles mostraram um melhor prognóstico (ou seja, melhor sobrevida livre de doença e sobrevida global)

associado a uma forte densidade de células T CD45RO+ e CD8+ em CT e IM. Na análise multivariada, incluindo estadios pT e pN, idade, sexo, número total de linfonodos, grau tumoral, tipo mucinoso, oclusão e perfuração intestinal, apenas o Immunoscore e a perfuração intestinal permaneceram como marcadores prognósticos significativos para sobrevida livre de doença. Esses dados sugerem que o Immunoscore pode prever com precisão a recorrência além da classificação TNM. Curiosamente, em pacientes que não recidivaram, a densidade de infiltrados de CD8 foi inversamente correlacionada com o estadios T, enquanto em pacientes que recidivaram o número de células CD8 foi baixo independentemente do estadios T do tumor, sugerindo uma correlação inversa entre a resposta *in situ* e a progressão do tumor.

Berntsson et al. (BERNTSSON et al., 2017) avaliaram o valor prognóstico de linfócitos T CD3+, CD8+ e FoxP3+ (linfócitos T reguladores – Treg) em CCR de acordo com o sítio tumoral. Os autores verificaram que a alta densidade de linfócitos TCD8+ estava associada com melhor prognóstico em CCD independentemente da idade, sexo, estadios, grau e invasão vascular. Por outro lado, alta densidade de linfócitos Treg (FoxP3+) foi associada exclusivamente com melhor prognóstico em câncer de reto (CR), enquanto a densidade de linfócitos TCD3+ foi associada com melhor prognóstico tanto para CCD e CR, mas não para CCE. Estes resultados demonstram diferenças no padrão de infiltrado linfocitário de acordo com a localização do tumor e com prognóstico destes tumores. Estudo de Loree et al. mostrou que a classificação do CCR de acordo com a lateralidade, dividindo CCD, CCE e CR, sem levar em consideração a localização anatômica do tumor, não recapitula totalmente as variações da biologia do tumor (LOREE et al., 2018). Notadamente, o câncer em cólon transverso tem peculiaridades em termos de mutação e perfil transcricional em comparação com tumores em outros sítios do cólon direito, bem como a região retossigmoide nos tumores do lado esquerdo (LOREE et al., 2018).

Como o Immunoscore é uma medida direta da infiltração de células T em tumores, qualquer mecanismo que afete direta ou indiretamente esse processo também afetará a categoria do Immunoscore. No centro desse mecanismo está a presença de imunogenicidade tumoral “suficiente” (a capacidade de induzir uma resposta imune mediada por células T), que está relacionada à carga de neoantígenos e mais frequentemente encontrada em tumores com alta instabilidade de microssatélites

(MSI-H) ou pacientes com deficiência no sistema de reparo do DNA (dMMR). De fato, os CCRs associados à síndrome de Lynch (caracterizada por MSI-H) exibem maior infiltração de células imunes, incluindo CD3+ e CD8+ (WALKOWSKA et al., 2019).

Mlecnik et al. (MLECNIK et al., 2016a) demonstraram a partir da análise de duas coortes compreendendo mais de 900 pacientes com CCR, que os tumores com instabilidade de microssatélites (MSI+) e um subgrupo de tumores com estabilidade de microssatélites (MSS), são caracterizados por uma expressão gênica imune proeminente. Neste estudo, o Immunoscore, mas não os parâmetros MSI e TNM, foi significativamente preditivo de recorrência da doença do paciente e sobrevida na análise multivariada. Esses achados indicam que a avaliação do Immunoscore fornece um potente indicador de recorrência tumoral e pode ser um guia importante, além do estadiamento TNM e do status MSI, para quimioterapia adjuvante após a ressecção de CCR.

Um painel internacional independente de laboratórios especializados formando um consórcio mundial de Immunoscore com o apoio da *Society for Immunotherapy of Cancer* foi formado para investigar o Immunoscore bem como sua relação com o status MSI e o respectivo impacto prognóstico desses dois parâmetros em grandes coortes de CCR para promover o teste Immunoscore com marca registrada na prática clínica de rotina (GALON et al., 2012). Os principais objetivos deste consórcio foram demonstrar a viabilidade, reprodutibilidade, significância e robustez do Immunoscore em um estudo mundial. O consórcio identificou uma estratégia para demonstrar a viabilidade e reprodutibilidade do Immunoscore, validaram seu maior poder prognóstico e robustez no câncer de cólon estadios I/II/III e demonstraram a utilidade do Immunoscore para prever pacientes com câncer de cólon estadios II com alto risco de recorrência (GALON et al., 2012).

Os resultados do consórcio internacional foram publicados em 2018, por Pagès et al. (PAGÈS et al., 2018). A validação prospectiva do Immunoscore na prática clínica incluiu 2.681 tumores de cólon em estadios I-III ressecados de 14 centros em 13 países. Este estudo confirmou a boa reprodutibilidade intra e interobservador do Immunoscore e seu poderoso valor prognóstico, com uma taxa de recorrência de 8% em 5 anos em pacientes com Immunoscore alto em comparação com 19% e 32% em casos com Immunoscore intermediário e baixo, respectivamente (HR=0,20; $p < 0,0001$). Na análise

multivariada, o Immunoscore foi identificado como um fator prognóstico na sobrevida livre de doença, independentemente da idade, sexo, estadios T e N e status MSI. Esse valor prognóstico foi confirmado no subgrupo de 1.434 tumores de cólon estadios II (alto *versus* baixo, HR=20; $p < 0,0001$). O Immunoscore teve a maior contribuição relativa para o risco de recorrência entre todos os parâmetros clínico-patológicos. Esses resultados dão suporte à implementação do Immunoscore como um novo componente da classificação prognóstica do CCR (PAGÈS et al., 2018).

Dois outros grandes estudos, incluindo 843 e 768 pacientes, respectivamente, mostraram que os padrões de infiltrado imune tumoral são biomarcadores prognósticos significativos, mesmo após ajuste para estadios, envolvimento de linfonodos e marcadores moleculares tumorais prognósticos bem estabelecidos, incluindo status MSI ou mutação em *BRAF* (NOSHO et al., 2010; OGINO et al., 2009). Outros dois estudos populacionais de CCR sugeriram que, além dos TILs, alta inflamação linfóide difusa peritumoral (infiltrados de células linfóides no tecido estromal ao longo da margem invasiva) e reação linfóide semelhante à doença de Crohn (agregados linfóides correspondentes a estruturas linfóides terciárias), que são características frequentes de tumores MSI, estão significativamente associados a riscos reduzidos de sobrevida global e sobrevida câncer-específica em CCR, independentemente dos marcadores prognósticos tradicionais, incluindo o status MSI (HYNES et al., 2017; ROZEK et al., 2016).

Adicionalmente, mesmo em pacientes com CCR metastáticos, a alta densidade de linfócitos (I=4) foi associada com maior sobrevida (MLECNIK et al., 2016a). Mlecnik et al. (MLECNIK et al., 2016b) reportaram que o *Immunoscore* foi identificado como um marcador prognóstico superior comparado com status da estabilidade de microssatélites, TNM e invasão linfática e vascular em CCR, reforçando que o contexto imunológico do tumor tem papel importante na sua progressão (MLECNIK et al., 2016b). O mesmo grupo demonstrou que a quantificação das células imunes infiltrantes em tecidos metastáticos obtidos a partir das ressecções cirúrgicas de metástases hepáticas era capaz de prever a resposta ao tratamento e a sobrevida global de pacientes com CCR, reforçando a importância do contexto imune no comportamento clínico do CCR (MLECNIK et al., 2016b, 2018).

O Immunoscore demonstrou ter a maior contribuição relativa para o risco entre todos os parâmetros clínicos, incluindo o estadiamento AJCC/UICC-TNM (PAGÈS et al., 2018). A implementação do Immunoscore em ambientes clínicos e a introdução de um componente “imune” (I) para o estadiamento TNM (tornando-se TNM-I) têm sido defendidas. As vantagens imediatas desta abordagem são múltiplas, incluindo: melhor prognóstico de pacientes com câncer, uma melhor identificação de pacientes com alto risco de recorrência do tumor e uma melhor estratificação de pacientes que se beneficiarão de imunoterapias e terapias combinadas específicas (ANGELL; GALON, 2013; GALON; BRUNI, 2019).

Marliot et al. (MARLIOT et al., 2020) avaliaram a performance analítica do Immunoscore. A acurácia do Immunoscore foi estabelecida comparando contagens ópticas e automáticas. Foi observada uma alta concordância entre os dois métodos de contagem, as análises ópticas e automáticas de células CD3+ ou CD8+ foram fortemente correlacionadas ($r = 0,94$, $p < 0,001$ e $r = 0,92$, $p < 0,001$, respectivamente). A intensidade de coloração de CD3 e CD8 não foram alteradas pela idade do bloco tumoral durante um período de 30 anos. Nem a posição das seções de tecido testadas dentro de um bloco de tumor, nem a seleção dos blocos de tecido afetaram o desempenho do Immunoscore. A reprodutibilidade do Immunoscore não foi afetada por múltiplas variáveis, tais como: lotes de anticorpos, kits de revelação DAB (3,3'-Diaminobenzidina), automatizadores imuno-histoquímicos e operadores. A reprodutibilidade inter-ensaio e interlaboratorial do Immunoscore entre dois centros de testes atingiu 100% e 93%, respectivamente. A análise de alto desempenho confirmou a confiabilidade, reprodutibilidade e robustez do Immunoscore (MARLIOT et al., 2020). Como resultado desse longo processo, o Immunoscore é o primeiro biomarcador recomendado por instituições acadêmicas para quantificar o infiltrado imune tumoral para fins prognósticos [as diretrizes da ESMO 2020 (ARGILÉS et al., 2020) e a 5ª edição do WHO *Digestive System Tumors* (WHO, 2019)] (EL SISSY et al., 2021).

Vários estudos buscam entender o significado prognóstico do Immunoscore em outros tumores, incluindo câncer gástrico (JIANG et al., 2018, 2020), *non-small-cell lung cancer* (BOSCOLO et al., 2020), câncer de mama triplo negativo (RAPOPORT et al., 2022) e bexiga (ARIAFAR et al., 2022; NASSIF et al., 2021). O valor do Immunoscore vai além

de sua utilidade prognóstica. De fato, o Immunoscore pode constituir uma ferramenta poderosa para prever a resposta a novas abordagens terapêuticas, incluindo a imunoterapia, destacando assim seu potencial como biomarcador preditivo.

Infiltrado imune como marcador preditivo no CCR

O infiltrado inflamatório já avaliado quanto ao seu poder preditivo, ou seja, sua capacidade de prever com precisão as respostas à terapia no câncer de cólon. Emile et al. (EMILE et al., 2017, p. 20) investigaram 1.220 pacientes com câncer de cólon em estadio III tratados com FOLFOX adjuvante incluídos no estudo de fase III PETACC8 e avaliaram a infiltração de linfócitos usando um método de quantificação linear da densidade de CD3 da margem do tumor, método este previamente demonstrado como um fator independente prognóstico em um estudo de 119 cânceres de cólon estadios II-III (ALLARD et al., 2012). O objetivo principal do estudo foi comparar a porcentagem de pacientes vivos e sem recorrência em 2 anos de acordo com o status do infiltrado linfocitário (EMILE et al., 2017). Os autores mostraram que os pacientes com tumores com alta infiltração de linfócitos tiveram maior sobrevida livre de doença; a sobrevida em 3 anos foi de 81% versus 72% para tumores com baixa infiltração (taxa de risco [HR] = 0,69; $p = 0,01$); e maior sobrevida global, a sobrevida global em 5 anos foi de 89% versus 80%, HR = 0,58; $p = 0,0048$). O infiltrado de linfócitos juntamente com estadio do tumor, grau, status de mutação em *RAS* e *BRAF* mostrou-se como um fator prognóstico independente na análise multivariada (HR = 0,6; intervalo de confiança de 95%: 0,4-0,98; $p = 0,04$) (EMILE et al., 2017).

Mlecnik et al. (MLECNIK et al., 2020b) avaliaram o valor prognóstico do Immunoscore em pacientes com câncer de cólon estadio III e analisaram sua associação com o efeito da quimioterapia no tempo de recorrência. Foram investigados 763 pacientes com *American Joint Committee on Cancer/Union for International Cancer Control* TNM de 2 coortes: coorte 1 (Canadá/Estados Unidos) e coorte 2 (Europa/Ásia). O desfecho primário foi tempo para recorrência (TTR). Os desfechos secundários foram sobrevida global, sobrevida livre de doença, prognóstico de acordo com o status de estabilidade de microssatélites e valor preditivo da eficácia da quimioterapia. Pacientes

com Immunoscore alto apresentaram o menor risco de recorrência, em ambas as coortes. As taxas livres de recorrência em 3 anos foram de 56,9% (IC 95%= 50,3%-64,4%), 65,9% (IC 95%= 60,8%-71,4%) e 76,4% (IC 95%= 69,3%-84,3%) em pacientes com Imunoscoring baixo, intermediário e alto, respectivamente ([HR; high vs low], 0,48; IC 95%= 0,32-0,71; P = 0,0003). Pacientes com Immunoscore alto mostraram associação significativa com o maior tempo de recorrência, e sobrevida livre de doença e global prolongados (todos P < 0,001). Na análise multivariada estratificada por centro participante, a associação do Immunoscore com o tempo de recorrência foi independente do sexo do paciente, estadió T, estadió N, lateralidade, e status de instabilidade de microssatélites (HR [high v low], 0,41; IC 95%= 0,25-0,67; P = 0,0003). Associação significativa de um Immunoscore alto com tempo de recorrência prolongado também foi encontrada entre os pacientes com MSS (HR [high v low]=0,36; IC 95%=0,21-0,62; P = 0,0003). Em outra análise, os pacientes estadió III foram agrupados em baixo (T1-3 & N1) e alto (T4 ou N2) risco. Em ambos os grupos (alto e baixo risco) o Immunoscore alto foi associado a um tempo mais longo de sobrevida. A análise do benefício do tratamento quimioterápico adjuvante dependendo do Immunoscore identificou que o grupo de pacientes com tumores Immunoscore alto (I2-I4) houve uma significativa associação da quimioterapia com TTR (P = 0,009). Por outro lado, nos grupos de Immunoscore baixo (I0-I1), a quimioterapia não foi significativamente associada ao TTR. Este estudo mostrou que um Immunoscore alto se associou significativamente à sobrevida prolongada no estadió III do câncer de cólon e sugere que pacientes com alto Immunoscore se beneficiarão mais da quimioterapia em termos de risco de recorrência (MLECNIK et al., 2020b).

Anitei et al. (ANITEI et al., 2014) mostraram que o Immunoscore pode ser um marcador de prognóstico útil em pacientes com câncer de reto tratados primariamente com cirurgia. Num subgrupo de pacientes tratados com quimioradioterapia neoadjuvante, maior densidade de infiltrado linfocitário nas biópsias antes do tratamento foi associada com melhor resposta terapêutica, no entanto, o número de pacientes avaliados era de apenas 55 pacientes.

El Sissy et al. (EL SISSY et al., 2020) desenvolveram um Immunoscore adaptado a biópsia em amostras de câncer de reto localmente avançado para investigar a

capacidade do Immunoscore em prever a resposta ao tratamento neoadjuvante e auxiliar na definição dos pacientes elegíveis para estratégia de preservação de órgão (“Watch-and-Wait”). O Immunoscore alto identificou pacientes com menor risco de recidiva ou morte em comparação com Immunoscore baixo, resultado confirmado em um grupo de validação. Além disso, a combinação do Immunoscore com os resultados de imagem pós terapia neoadjuvante foi capaz de discriminar pacientes respondedores que poderiam se beneficiar da estratégia de preservação do órgão, sugerindo que o Immunoscore pode ser uma ferramenta importante na seleção de pacientes elegíveis para uma estratégia “Watch-and-Wait” (EL SISSY et al., 2020, 2020).

A relação entre os aspectos moleculares tumorais e o status imunológico dos CCRs tem ganhado especial atenção particularmente na seleção de pacientes para imunoterapia, uma modalidade terapêutica que tem ganhado destaque no tratamento de vários cânceres (RILEY et al., 2018). Resultados promissores têm sido obtidos com os inibidores de *checkpoint* imune, que são anticorpos direcionados para moléculas que dão sinais inibitórios para o sistema imune, impedindo a ação dos linfócitos T, tais como CTLA-4, e o PD-1 e seu ligante PD-L1. A interação entre PD-1/PD-L1 tem papel fundamental na supressão da resposta de linfócitos T *in vivo*, especialmente no microambiente tumoral.

Os inibidores anti-PD1, como nivolumab e pembrolizumab, têm mostrado respostas eficazes e duradouras no tratamento de pacientes com tumores com altas taxas de mutação, além de tumores com mutações nos genes de reparo do DNA (*mismatch repair*, MMR), como câncer de endométrio, estômago e CCR (LE et al., 2015; NISHINO et al., 2017). Foi demonstrado que a combinação de anti-PD-1 (nivolumab) e anti-CTLA4 (ipilimumab) promove resposta clínica duradoura e melhor sobrevida em CCR metastáticos deficientes MMR (MSI+) comparado com a monoterapia com anti-PD-1 (OVERMAN et al., 2018). Porém, os inibidores de *checkpoint* imune não têm demonstrado eficácia no tratamento dos CCRs sem defeitos nestes genes e que apresentam estabilidade de microssatélites (MSS), e que representam a maioria dos pacientes com CCR, incluindo quase a totalidade dos tumores de reto (DEKKER et al., 2019; MERLANO et al., 2017).

Um estudo de casos de 12 pacientes com CCR metastático com deficiência nos genes de reparo a danos do DNA (MMR-deficientes) e MSI+ e tratados com pembrolizumabe mostrou Immunoscore alto associado com resposta ao tratamento, tanto em termos de taxa de resposta objetiva quanto de duração do controle da doença. Os escores médios de densidade de marcadores de células T foram maiores em pacientes respondedores comparados aos não respondedores, bem como nos pacientes com controle da doença por mais de 1 ano comparado aos pacientes com controle da doença por tempo inferior a 1 ano. Os autores sugerem as diferenças de infiltrado de linfócitos CD3 e CD8 podem explicar, pelo menos em parte, as diferenças de resposta ao pembrolizumabe nos pacientes CCR metastáticos com deficiência de MMR (CHAKRABARTI et al., 2019).

Recentemente, um estudo avaliou o infiltrado de linfócitos tumorais para prever o benefício de inibidos de checkpoint imune no tratamento de pacientes com câncer colorretal metastático (MORETTO et al., 2023). Foi avaliada a expressão de linfócitos infiltrantes de tumor (TILs), imunomarcagem para o ligante de morte programada-1 (PD-L1), Immunoscore, e Immunoscore-IC em amostras de CCR. O Immunoscore-IC quantifica as densidades de células PD-L1+ e células T CD8+ citotóxicas no núcleo do tumor por patologia digital, bem como sua proximidade e agrupamento. Para análise foram avaliados cinco parâmetros medidos como valores lineares para gerar os escores Immunoscore-IC: densidade de CD8 total, densidade de CD8 livre (sem células PD-L1+ na proximidade), densidade de cluster CD8 (células CD8 na proximidade de menos de 20 micrômetros de outro CD8), densidade de células PD-L1+ e distância entre células CD8+ e células PD-L1+. O escore de risco foi calculado incorporando os cinco parâmetros e a seguir dicotomizado em grupo de baixo risco (valores elevados de marcadores Immunoscore-IC, definido como Immunoscore-IC-alto) e grupo de alto risco (baixos valores de densidade dos marcadores, definido como um Immunoscore-IC- baixo). Foram avaliadas amostras de 218 pacientes participantes do estudo AtezoTRIBE16 (NCT03721653), um ensaio clínico randomizado de fase II, multicêntrico aberto onde os pacientes com CCR metastático inicialmente irresssecável foram randomizados em uma proporção de 1:2 para receber FOLFOXIRI/bev (5-fluorouracil, leucovorin, oxaliplatina e irinotecan/bevacizumab) ou o mesmo regime mais o atezolizumabe (anti-PD-L1).

Pacientes com tumores com Immunoscore-IC alto apresentaram maior vantagem com a adição de atezolizumabe e prolongada sobrevida livre de doença. A mesma tendência foi observada em pacientes com tumores com Immunoscore alto com a adição de atezolizumabe ao FOLFOXIRI/bevacizumab. Este estudo mostrou que a densidade de células imunes no ambiente tumoral identificada principalmente pelo Immunoscore-IC foi capaz de identificar um subgrupo de pacientes com CRC metastático e com MMR proficiente que podem ser beneficiados pelo tratamento com o inibidor de checkpoint imune anti-PD-L1 ao tratamento inicial (MORETTO et al., 2023).

Identificação do perfil de infiltrado imune por ferramentas de bioinformática

A quantificação das diferentes populações de células imunes infiltrantes pode auxiliar na compreensão dos mecanismos que coordenam a resposta imune antitumor, com vistas na identificação de novos biomarcadores e auxiliar para o desenvolvimento de novas terapias. Os métodos tradicionais de quantificação do infiltrado imune são imunohistoquímica, imunofluorescência e citometria de fluxo (FINOTELLO; TRAJANOSKI, 2018). No entanto, com o crescente número de estudos transcritômicos em diferentes cânceres, ferramentas de bioinformática têm sido usadas para quantificar as subpopulações celulares do infiltrado inflamatório no tecido tumoral a partir de dados do transcrito (HACKL et al., 2016). Assim, algoritmos como CIBERSORT (NEWMAN et al., 2015), MCP-counter (BECHT et al., 2016), xCell (ARAN; HU; BUTTE, 2017) e EPIC (BOEGEL, 2020), entre outros, são capazes de quantificar indiretamente subpopulações de células imunes infiltrantes do tumor, auxiliando na compreensão do contexto imunológico dos tumores.

Mito et al. (MITO et al., 2021) utilizaram o algoritmo xCell para identificar o perfil imune em tumores de cabeça e pescoço associados com infecção por papilomavirus humano (HPV) com intuito de identificar novos biomarcadores que possam ser aplicados nestes tumores. Os autores avaliaram 33 células do sistema imune e identificaram escores mais altos de vários tipos de células imunes, incluindo células T CD4+, T CD8+, células B, células plasmáticas, basófilos, entre outras, associados com tumores HPV positivos. A assinatura linfocitária caracterizada por enriquecimento de linfócitos CD4,

CD8, células B e células plasmáticas foi também associada com maior sobrevida global nos pacientes HPV positivos (MITO et al., 2021). Em outro estudo, maiores escores de células Th1, NK, Treg e célula T CD4+ de memória foram associadas com maior sobrevida em pacientes com câncer gástrico utilizando a ferramenta xCell (REN et al., 2021). Uma assinatura de imunosscore constituída de 5 tipos de células imunes infiltrantes tumorais (células T CD8, células NK ativadas, neutrófilos, mastócitos ativados e macrófagos) foi identificada em câncer cervical utilizando a combinação das plataformas xCell e CIBERSORT (HE et al., 2021). As pacientes do grupo imunosscore alto mostraram pior sobrevida global comparadas às pacientes com imunosscore baixo (HR=3,0113; IC 95%=1,8746–4,8373; $p<0,0001$). A análise multivariada mostrou o imunosscore como um fator prognóstico independente (HE et al., 2021).

Em resumo, análise de células imunes infiltrantes tumorais, em particular o Immunosscore, pode servir como um biomarcador prognóstico, porém seu custo, benefício e viabilidade nos laboratórios de patologia ainda precisam ser testados, principalmente naqueles que não possuem sistema digital de análise de imagem. Além disso, os linfócitos T são populações celulares heterogêneas, além de outras células do sistema imune, como células natural killers (NK), linfócitos B, células dendríticas e macrófagos, também podem ter um papel relevante na progressão tumoral dos CCR. Assim mais estudos são necessários para melhor compreensão das interações tumor-sistema imune, bem como para sua aplicação clínica como biomarcadores. Apesar da crescente literatura indicando o Immunosscore como um marcador relevante em CCR, para o nosso conhecimento não há estudos investigando seu papel em pacientes Brasileiros.

Justificativa

O Immunoscore (IS) demonstrou valor prognóstico e preditivo no CCR e agora foi integrado ao guia de recomendações para pacientes (LANZI et al., 2020). Isso pode ajudar a selecionar pacientes que são mais prováveis de ter benefícios com quimioterapia adjuvante mais longa no estágio III e também, quando adaptado a biópsias (*Immunoscore adapted to biopsies* – ISB), para identificar pacientes com tumores retais que podem responder à terapia neoadjuvante e podem ser elegíveis a estratégias não invasivas (EL SISSY et al., 2021). Muitas imunoterapias estão sendo testadas e com resultados promissores. IS pode ter utilidade clínica importante em pacientes CCR para guiar de forma mais assertiva os tratamentos padrão além de auxiliar nos novos tratamentos.

Análise de células imunes infiltrantes tumorais, em particular o Immunoscore, pode servir como um biomarcador prognóstico, porém seu custo, benefício e viabilidade nos laboratórios de Patologia devem ser testados, principalmente naqueles que não possuem sistema digital de análise de imagem. Além disso, os linfócitos T são populações celulares heterogêneas, além de outras células do sistema imune, como células *natural killers* (NK), linfócitos B, células dendríticas e macrófagos, também podem ter papel relevante na progressão tumoral. Mais estudos são necessários para melhor compreensão das interações tumor-sistema imune, bem como para sua aplicabilidade clínica como biomarcadores.

Desta forma, neste estudo propusemos avaliar as células imunes associadas ao tumor como marcadores prognósticos no câncer colorretal de acordo com a localização anatômica. A compreensão do contexto imune específico dos adenocarcinomas colorretais de acordo com o sítio anatômico é necessária para o desenvolvimento de estratégias terapêuticas que sejam mais eficazes para cada grupo de pacientes. Além disso, pacientes com tumores avançados ainda apresentam abordagens terapêuticas limitadas e prognóstico ruim. Desta forma, a investigação de marcadores prognósticos que melhorem a estratificação de risco dos pacientes é justificada. Além disso, não temos conhecimento de estudos semelhantes em pacientes brasileiros com CCR.

Hipótese

Há diferentes populações de células imunes infiltrantes do tumor de acordo com o sítio anatômico que podem ser utilizadas como marcadores prognósticos em CCR.

Objetivo

1.1 OBJETIVO GERAL

Avaliar as células imunes associadas ao tumor como marcadores prognósticos no câncer colorretal de acordo com a localização anatômica.

1.2 OBJETIVOS ESPECÍFICOS

1. Detectar os marcadores CD3+ e CD8+ por imuno-histoquímica em amostras de CCR de diferentes localizações anatômicas em microarranjos de tecidos (TMA) e calcular o *Immunoscore*.
2. Correlacionar os resultados do *Immunoscore* com dados clínicos, histopatológicos e de sobrevida dos pacientes.
3. Selecionar outros marcadores imunes associados com evolução da doença utilizando dados de transcrito de CCR de diferentes localizações utilizando algoritmos específicos para estimar as populações de leucócitos infiltrantes.
4. Detectar os marcadores imunes candidatos nos tecidos colorretais (TMAs) para confirmar seu valor prognóstico.

Material e Métodos

Delineamento do estudo e casuística.

1. SELEÇÃO DOS CASOS

Foram obtidos tumores primários de câncer colorretal consecutivos registrados entre janeiro de 2010 e janeiro de 2015, no Serviço de Patologia do Hospital das Clínicas da Faculdade de Medicina de Botucatu (HCFMB), UNESP. Foram identificados 236 registros de tumores primários de câncer colorretal.

Realizou-se revisão dos prontuários eletrônicos e laudos anatomopatológicos para coleta de informações epidemiológicas e clínico-evolutivas, além da revisão morfológica de todos os casos. Esse estudo foi aprovado pelo Comitê de Ética em Pesquisa (CEP) da FMB – UNESP (CAAE 13241819.1.10000.5411, parecer 3.383.175).

Critérios de inclusão: Pacientes adultos, maiores de 18 anos, com tumores CCR primários de tipo histológico tubular (usual) ou mucinoso, de qualquer estadiamento e grau histológico obtidos por ressecção cirúrgica e não tratados com quimioterapia e/ou radioterapia anteriormente à coleta das amostras.

Critérios de exclusão: Pacientes com histórico de tumores prévios ou sincrônicos e pacientes com ausência de blocos de parafina.

Os casos incluídos foram reavaliados para confirmação diagnóstica de carcinoma colorretal de acordo com checklist do Colégio Americano de Patologistas (WASHINGTON et al., 2009a) e critérios definidos pela Organização Mundial de Saúde (OMS/WHO). De cada caso foram coletadas informações como: localização anatômica do tumor, grau de diferenciação, tipo histológico, presença de invasão angiolinfática e invasão perineural, estadiamento TMN (8ª edição) (BRIERLEY, 2017) e aspectos macroscópicos. Foram obtidas informações de seguimento clínico, como diagnóstico de recorrência da doença (local ou a distância) e óbito por câncer, quando disponível. A razão de linfonodos comprometidos (*lymph node ratio* – LNR) foi definida como número de linfonodos comprometidos dividido pelo total de linfonodos examinados (PETRELLI; BORGONOVO; BARNI, 2011a).

A análise de sobrevida global foi documentada a partir da data da cirurgia até o período final de seguimento pelas anotações preenchidas nos prontuários. Os pacientes considerados como perda de seguimento foram aqueles que não retornaram nas datas agendadas e não foram encontrados após tentativas de manter contato pelo serviço social do hospital. Para as análises de sobrevida foram excluídos 30 pacientes que foram apenas operados no serviço e não seguiram com o tratamento no HCFMB.

Nesse trabalho classificamos os CCR de acordo com a localização anatômica em: câncer de cólon direito, que inclui os tumores localizados no ceco, cólon ascendente e cólon transverso; e câncer colorretal esquerdo, incluindo aqueles localizados na flexura esplênica, cólon descendente, sigmoide reto.

2 CONSTRUÇÃO DO MICROARRANJO DE TECIDO (TMA)

Ao total foram construídos 12 TMAs (do inglês *Tissue Microarrays*) contendo amostras em parafina de CCR de diferentes localizações anatômicas, designando regiões específicas do fronte de invasão (IM) e do centro do tumor (CT). Utilizando agulha de 2,0mm de diâmetro, áreas representativas das duas regiões tumorais (IM e CT) (de cada localização anatômica) identificadas pelo patologista foram incluídas no bloco doador usando um *Tissue Microarrayer* (Pathology Devices Inc., Beecher Instruments, Sun Prairie, WI, USA). A Figura 1 mostra um exemplo de montagem de dois blocos de TMA, contendo em um bloco amostras do fronte (IM) e no outro amostras do centro do tumor (CT). Seções seriadas de 4 µm foram cortadas dos blocos de TMA e processadas para imuno-histoquímica.



Figura 1. Exemplos de 2 blocos de parafina “receptor” (bloco de TMA).

3 ANÁLISE *IN SILICO* PARA SELEÇÃO DE MARCADORES IMUNES CANDIDATOS

3.1 Seleção dos estudos para análise de estimativa de populações imunes infiltrantes do tumor.

Dados públicos do transcrito de tumores primários de CCR foram obtidos do banco de dados Gene Expression Omnibus (GEO) (<https://www.ncbi.nlm.nih.gov/gds>), utilizando como ferramenta de busca as palavras “colorectal cancer” e utilizando a ferramenta de filtro “expression. profiling”. A busca resultou em inúmeros estudos, e destes selecionamos três estudos que atendiam aos seguintes critérios: informação da localização anatômica dos tumores, >100 amostras de tumores primários, informação de seguimento (sobrevida global) e outras informações clínicas/histopatológicas relevantes. A tabela 1 detalha os estudos selecionados.

Estas análises de bioinformática foram realizadas em colaboração com o Dr Fabio A. Marchi, especialista em bioinformática.

Tabela 1. Estudos selecionados no banco de dados GEO para análise in silico das populações imunes infiltrantes associadas com prognóstico em CCR.

IDENTIFICAÇÃO GEO	DESCRIÇÃO CASOS	PLATAFORMA	REFERENCIA
GSE28814	125 tumores colorretais, inclui informações de localização anatômica, informações clínicas e de seguimento.	Rosetta custom human 23K array	(LOBODA et al., 2011)
GSE41258	182 amostras de tumores primários. Tem informação de grupos com e sem metástase, inclui informação de localização anatômica.e de seguimento	Affymetrix Human Genome U133A Array	(SHEFFER et al., 2009)
GSE103479	156 amostras CCR com informações de localização anatômica e de seguimento.	Almac Diagnostics Custom Xcel array	(ALLEN et al., 2018)

Ao final, foram incluídos nas análises 452 casos de CRC, sendo 210 casos de câncer de cólon direito, 189 de câncer de cólon esquerdo e 53 casos de câncer de reto.

3.2 Estimativa de populações imunes infiltrantes utilizando o algoritmo xCell.

Foi utilizada a plataforma xCell (<http://xCell.ucsf.edu/>) que permite estimar a presença de 64 tipos de células imunes e estromais, incluindo células de resposta imune adaptativa e inata, células hematopoiéticas, células epiteliais e células da matriz extracelular (ARAN; HU; BUTTE, 2017). Para determinar o escore de abundância de cada tipo celular nas amostras, o xCell parte de um compêndio de 489 conjuntos de genes extraídos de diferentes estudos de larga escala; o escore de abundância é determinado em quatro passos: (1) utilização da análise de enriquecimento de grupos de genes de amostra única (ssGSEA) para cada uma das assinaturas gênicas, (2) cálculo da média dos escores para cada tipo celular (o resultado é uma matriz de 64 linhas e N colunas), (3) transformação de cada elemento da matriz como descrito em Aran et al. (ARAN; HU; BUTTE, 2017)e (4) compensação de “spillover” utilizando o método dos quadrados mínimos para evitar que o ES de uma célula interfira com o de células semelhantes.

Dos 64 tipos celulares identificados pelo xCell foram analisados 27 tipos celulares: basófilos, eosinófilos, mastócitos, neutrófilos, monócitos, macrófagos, macrófagos M1, macrófagos M2, células dendríticas (DC), DC ativadas (aDC), DC convencionais (cDC), DC plasmocitóides (pDC), células B, células B de memória,

plasmócitos, células natural killer (NK), células NKT, células T gama-delta, células TCD4, Th1, Th2, células T CD4 de memória, células T CD4 de memória central, células T CD4 de memória efetora, células CD8, CD8 memória central e células T reguladoras (Treg). Além disso, foram calculados os escores imune (Immunoscore), score de stroma (Stromascore) e de microambiente (Microenvironmentscore).

Cada estudo foi submetido individualmente a análise na plataforma, e os valores de enriquecimento de cada tipo celular foram obtidos para cada amostra. Os escores gerados para cada célula imune foram dicotimizados em alto e baixo de acordo com mediana dos valores de cada célula imune no conjunto de amostras tumorais de cada estudo separadamente. Valores acima da mediana foram considerados como alto (high) e valores iguais ou abaixo da mediana foram considerados como baixo (low).

4 ESTUDO IMUNO-HISTOQUÍMICO (DETECÇÃO DE CÉLULAS IMUNES INFILTRANTES)

4.1 Identificação do Immunoscore (I):

Foi utilizado o método de imuno-histoquímica (IHQ) nos TMAs para detecção dos marcadores: CD3 (pan-T) e CD8 (T citotóxico). Os protocolos de imunomarcacão seguiram as recomendações de Pagès et al. (2018) com modificações. Resumidamente, o estudo imuno-histoquímico, para detecção de linfócitos CD3 e CD8, foi realizado em secções histológicas do bloco de TMA, utilizando o polímero *EnVision*® (Dako, California, USA) como sistema de detecção. O método de recuperação antigênica foi realizado de acordo com a necessidade específica de cada anticorpo, utilizando módulo de pré-tratamento *PTLink*® (Dako, California, USA). Anticorpos primários, diluições, fontes, métodos de recuperação de antígenos e sistemas de detecção estão listados na Tabela 2.

Imagem representativa de um core do TMA corado para CD3 está apresentado na Figura 2.

Tabela 2. Especificações dos anticorpos primários, diluição e fonte

Anticorpo	Clone	Diluição	Fonte	Tipo	Recuperação antigênica	Sistema de detecção
CD3	565	ready to use	DAKO	Polyclonal rabbit	pH alto	EnVision [®]
CD8	C8/144B	ready to use	DAKO	Monoclonal Mouse	pH alto	EnVision [®]

A média e distribuição das intensidades de coloração foram monitoradas para obter um controle de qualidade interno de cada lâmina.

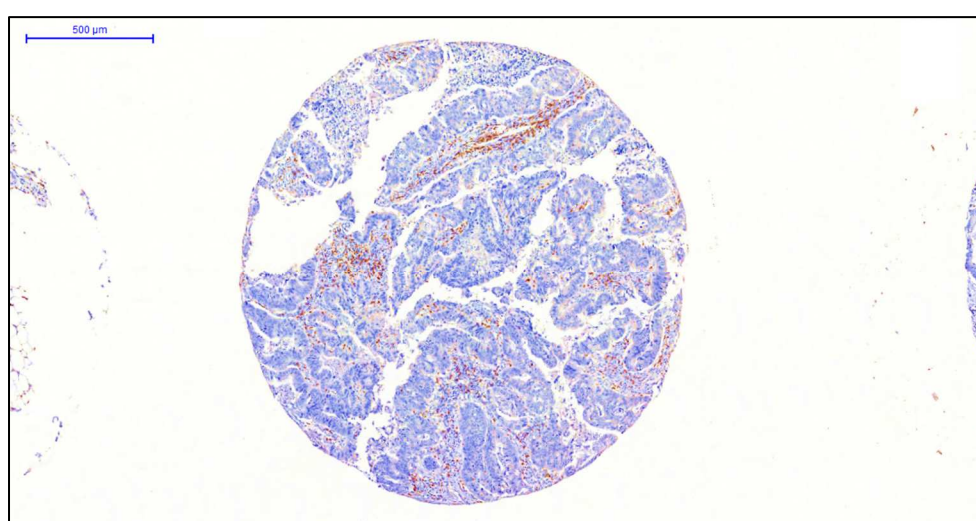


Figura 2. Imagem representativa de core imunocorado para CD3.

4.2 Cálculo do Immunoscore (IM)

De acordo com os critérios estabelecidos por Galon et al. (GALON et al., 2014), o cálculo *Immunoscore* é realizado quantificando os marcadores imunocorados (CD3 + CD8 +) em duas regiões tumorais (centro e margem de invasão). A quantificação objetiva dos marcadores é feita por análise digital, utilizando cinco percentis ([1] 0-10% ou Immunoscore classe I0; [2] >10-25% ou Immunoscore classe I1; [3] >25-70% ou Immunoscore classe I2; [4] >70-95% ou Immunoscore classe I3; [5] >95-100% ou Immunoscore classe I4).

Com intuito de simplificar e adaptar o cálculo à microscopia convencional (não digital), optamos por modificação similar à descrita por Mlecnik et al. (MLECNIK et al.,

2020a). Quantificamos os mesmos marcadores imunocorados (CD3 + CD8 +) nas mesmas regiões tumorais (centro e margem de invasão), mas utilizamos dois percentis: <25% e >25%.

Para semi-quantificação imuno-histoquímica foram construídas pranchas histológicas comparativas, representando densidades linfocitárias maiores que 25% e menores que 25% (Figuras 3 e 4).

Os pacientes que apresentaram densidade celular <25% para os 2 marcadores e nas 2 regiões ou apenas uma densidade >25% em uma única região foram categorizados como grupo BAIXO. Todos os demais foram categorizados como grupo ALTO, conforme apresentado no Quadro 1.

Quadro 1. Cálculo do Immunoscore.

CD3 (centro)	CD8 (centro)	CD3 (margem)	CD8 (margem)	Classes	GRUPOS DE IMMUNOSCORE
<25%	<25%	<25%	<25%	I0	BAIXO (low)
>25%	<25%	<25%	<25%	I1	
>25%	>25%	<25%	<25%	I2	ALTO (High)
>25%	>25%	>25%	<25%	I3	
>25%	>25%	>25%	>25%	I4	

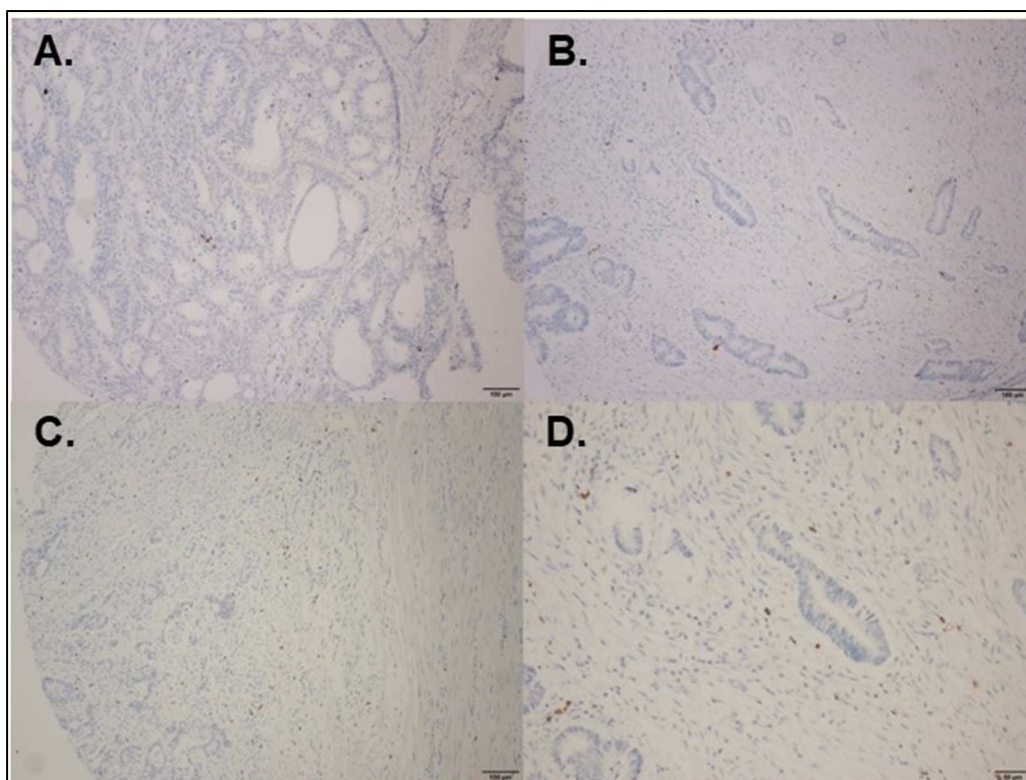


Figura 3: Imagens representativas da análise imuno-histoquímica para linfócitos infiltrantes CD8. Fotos (A), (B), (C) e (D) ilustrativas de imunocoloração para CD8 ilustrando densidade linfocitária <25% determinada como BAIXA (aumento de 20x).

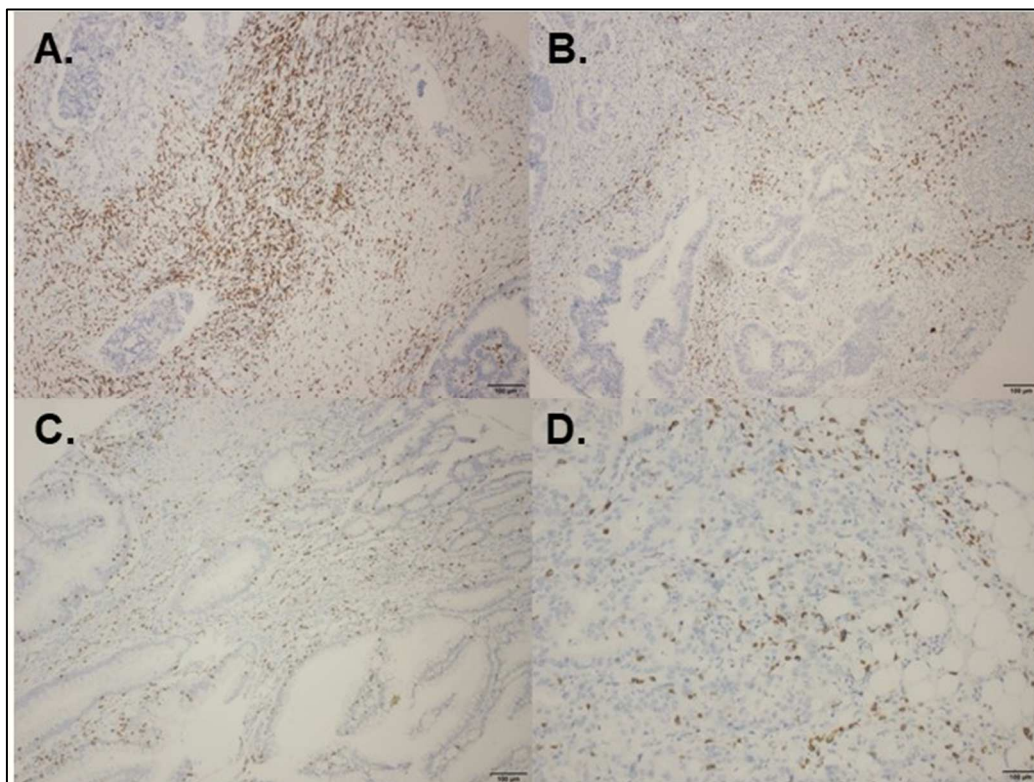


Figura 4: Imagens representativas da análise imuno-histoquímica para linfócitos infiltrantes CD8. Fotos (A), (B), (C) e (D) ilustrativas de imunocoloração para CD8 ilustrando densidade linfocitária >25% determinada como ALTA (aumento de 20).

4.3 Imunoistoquímica do marcador selecionado a partir da análise *in silico*:

Foi utilizado o método de imuno-histoquímica (IHQ) nos TMAs para detecção de neutrófilos. Foi utilizado a mieloperoxidase (MPO) como marcador dessas células nessas análises. O estudo imuno-histoquímico foi realizado em secções histológicas do bloco de TMA, utilizando anticorpo anti-MPO policlonal de coelho (Dako, ready to use). O método de recuperação antigênica foi realizado em pH alto, utilizando módulo de pré-tratamento *PTLink*[®] (Dako, California, USAOs). O polímero *EnVision*[®] (Dako, California, USA) como sistema de detecção em sistema automatizado (Ventana Benchmark XT). A média e distribuição das intensidades de coloração foram monitoradas para obter um controle de qualidade interno de cada lâmina. Quantificamos o marcador imunocorado MPO nos tumores utilizamos apenas dois percentis: <25% (classificados como BAIXO) e >25% (classificados como ALTO).

Imagens representativas da imunomarcação para MPO com escores Baixo e Alto estão apresentadas na Figura 5.

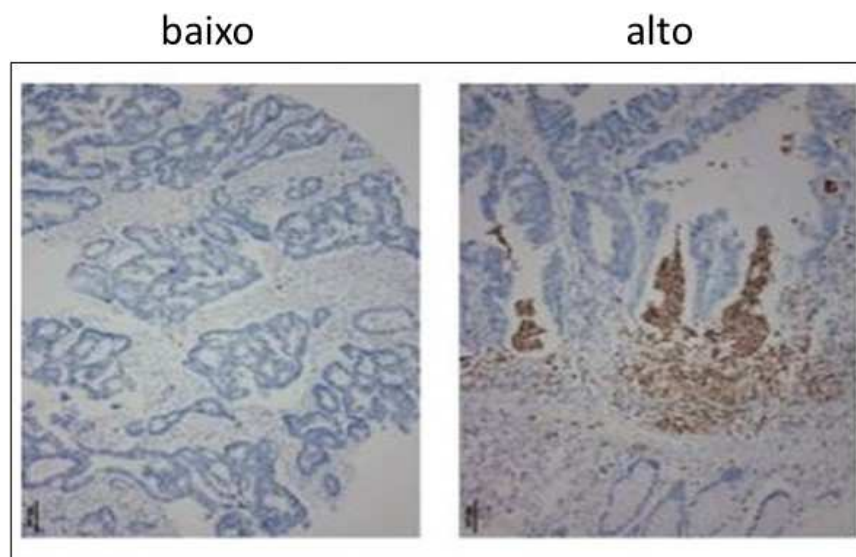


Figura 5: Imagens representativas da imuno-histoquímica da Imunocoloração para MPO ilustrando as densidades determinadas como ALTO e BAIXO.

5 ANÁLISE ESTATÍSTICA

Os dados obtidos das populações imunes pelo xCell foram avaliadas quanto ao seu valor prognóstico utilizando dados clínicos dos estudos do GEO. Curvas de sobrevida câncer-específicas foram construídas para cada uma das 27 populações agrupando os dados dos três estudos selecionados, totalizando assim 452 casos de CCR. Foram selecionadas as populações associadas com prognóstico nos CCR. O Immunoscore e o marcador selecionado na análise *in silico* foram analisados em relação a dados clínicos e histopatológicos das pacientes. Curvas de sobrevida de acordo com o Immunoscore e o marcador selecionado foram construídas pelo método de Kaplan-Meier. Foram utilizados os programas GraphPad Prism v.8 (GraphPad Software Inc., La Jolla, CA) e SPSS v.25 (SPSS; Chicago, IL). Foram considerados resultados estatisticamente significativos com $P < 0,05$.

CAPÍTULO 2

Manuscript draft - Article in preparation

Immune cell infiltrates and prognosis in colorectal cancer: a single center retrospective analysis.

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Keywords: colorectal cancer, tumor inflammatory cell infiltrate, prognosis.

Abstract:

Colorectal cancer (CRC) is a heterogeneous disease, exhibiting differences in prognosis and therapy response based on the anatomical location of the tumor. The tumor immune response has been proven critical to prognosis in CRC. In this study, infiltrating immune cells were assessed and associated with prognosis in CRC based on anatomical location. *In silico* analysis was conducted to identify immune cell populations associated with CRC prognosis. Twenty-seven immune cells detected by the xCell algorithm in 542 CRC samples obtained from GEO studies were correlated with differences in overall survival. We identified four infiltrating immune cells associated with prognosis. Low neutrophils were associated with worse prognosis in all CRC samples and in right-sided colon tumors. Additionally, Th2 cells, and memory T CD4⁺ cells were associated with prognosis in right-sided colon tumors. In left-sided CRC, Th2 cells and T CD8⁺ were associated with prognosis. Of these, neutrophils (MPO) were selected to be validated in tissue microarrays (TMAs). Immunohistochemical-based TMA analyses including regions from the tumor center (CT) and invasive margin (IM) of 206 CRC cases with different anatomical locations were performed to detect Immunoscore (CD3 and CD8), and MPO (neutrophil) markers. Two categories of Immunoscore, Low and High, were used in the analyses. Low Immunoscore was associated with decreased overall survival in CRC, albeit without statistical significance ($p=0.065$). In agreement with xCell *in silico* analysis, patients with left-sided CRC and low Immunoscore exhibited a worse prognosis ($p=0.034$), particularly for patients with rectal tumors ($p=0.020$). In addition, low MPO staining and decreased survival in patients with right-sided colon tumors was observed ($p=0.020$). Our results showed that optical adapted Immunoscore was associated with prognosis in our CRC cohort, especially for left-sided tumors. Conversely, neutrophils were indicated as a potential prognostic marker in right-sided colon tumors, reinforcing the importance of immune cell context in CRC progression with differences in tumor sidedness. Moreover, the simplified Immunoscore analysis proposed here can offer an approach easily applicable in clinical practice, especially in institutions with limited resources.

INTRODUCTION

Increasing evidence demonstrates that cancer progression is strongly associated with its complex tumor microenvironment (TME). The TME comprises a variety of cellular entities including fibroblasts, endothelial cells, blood vessels, lymphatic vessels, and immune cells. Adaptive immune cell infiltration has been shown to have a prognostic value superior to classical tumor invasion criteria, including grade, stage and metastatic status in colorectal cancer (CRC) (ANGELL et al., 2020; GALON et al., 2006; GALON; FRIDMAN; PAGÈS, 2007). A potential clinical translation of the immune context into a prognostic marker in CRC has been established and designated as “Immunoscore” (ANGELL; GALON, 2013; FRIDMAN et al., 2012; GALON; FRIDMAN; PAGÈS, 2007).

The Immunoscore was initially described in 2006 as a scoring system that reflected the density of CD3+ and CD8+ T cells within the tumor and at its invasive margin (GALON et al., 2006). In this study, tumors from patients without recurrence had higher densities of immune cells in the center of the tumor (CT) and at the invasive margin (IM) than those patients whose tumors had recurrence (GALON et al., 2006). Subsequent studies demonstrated the usefulness of the Immunoscore in predicting the prognosis of early and advanced CRC (BERGHOF et al., 2016; MLECNIK et al., 2018; PAGÈS et al., 2009, 2018a). This prognostic superiority proved to be statistically significant for CRC stages I, II and III, in predicting disease-free survival, disease-specific survival and overall survival with tumor progression and invasion depending on immunological parameters (BROUSSARD; DISIS, 2011; MLECNIK et al., 2011). A global consortium supported by the Cancer Immunotherapy Society (GALON et al., 2012b) demonstrated the feasibility and reproducibility of the Immunoscore and validated its greater prognostic power and robustness in stage I/II/III colon cancer and the usefulness of the Immunoscore to predict patients with stage II colon cancer at high risk of recurrence (GALON et al., 2012b).

High-performance analysis confirmed the reliability, reproducibility and robustness of Immunoscore (MARLIOT et al., 2020). Optical and automatic analyzes of CD3+ or CD8+ cells were strongly correlated ($r = 0,94$, $p < 0,001$ and $r = 0,92$, $p < 0,001$, respectively). Immunoscore reproducibility was not affected by multiple variables, such as antibody batches, DAB development kits, immunohistochemistry automators, and

operators. Immunoscore inter-assay repeatability and inter-laboratory reproducibility between two testing centers reached 100% and 93%, respectively. As a result of this long process, Immunoscore is the first biomarker recommended by academic institutions to quantify tumor immune infiltrate for prognostic purposes (ARGILÉS et al., 2020; BOARD, [s.d.]; EL SISSY et al., 2021). Immunoscore has been shown to have the greatest relative contribution to risk among all clinical parameters, including staging AJCC/UICC-TNM (PAGÈS et al., 2018a). The implementation of Immunoscore in clinical settings and the introduction of an “immune” (I) component to TNM staging (becoming TNM-I) have been defended (ANGELL; GALON, 2013; GALON; BRUNI, 2019). In addition, the Immunoscore may play a role as a predictive marker to predict response to therapies (ANGELL; GALON, 2013; GALON et al., 2012a, 2012b, 2013).

The analysis of other tumor infiltrating immune cells in addition to the Immunoscore can have a prognostic relevance in CRC. However, particularly for Immunoscore, its cost-effectiveness and feasibility in pathology laboratories, especially those lacking digital image analysis systems, still require testing. Furthermore, T lymphocytes represent heterogeneous cellular populations, and other immune system cells such as natural killer (NK) cells, neutrophils, CD4+ cells, B lymphocytes and dendritic cells, may play a significant role in CCR progression. In addition, understanding the specific immune context of colorectal adenocarcinomas according to their anatomical site is essential for the development of therapeutic strategies that are more tailored to each patient subgroup. Additionally, patients with advanced tumors still face limited therapeutic options and a poor prognosis. Therefore, the investigation of prognostic markers that enhance patient risk stratification is still warranted.

In this study we assessed tumor-associated immune cells as prognostic markers in CRC based on tumor sidedness. First, we screened potential immune cells associated with prognosis using transcriptome data from a large cohort of CCR samples from different anatomic locations. Next, the candidate immune markers together with Immunoscore markers (CD3 and CD8) were experimentally validated in IM and CT tumor regions arranged in tissue microarrays (TMAs) from an independent cohort of 183 CRC patients to confirm their prognostic value. In addition, we proposed a modification in

Immunoscore analysis, aiming to simplify and offer a readily applicable approach in resource-constrained laboratory settings.

PATIENTS AND METHODS

1. *In silico* estimation of infiltrating immune populations in CRC

CRC dataset

For *in silico* analysis, mRNA expression data and relevant clinical information from patients with CRC were downloaded from Gene Expression Omnibus (GEO) datasets (GSE28814, GSE41258 and GSE103479). A total of 452 CRC cases with tumor anatomic location, stage and survival information was included. The median follow-up was 64 months (1-205 months). A flowchart summarizing the study design is shown in Figure 1.

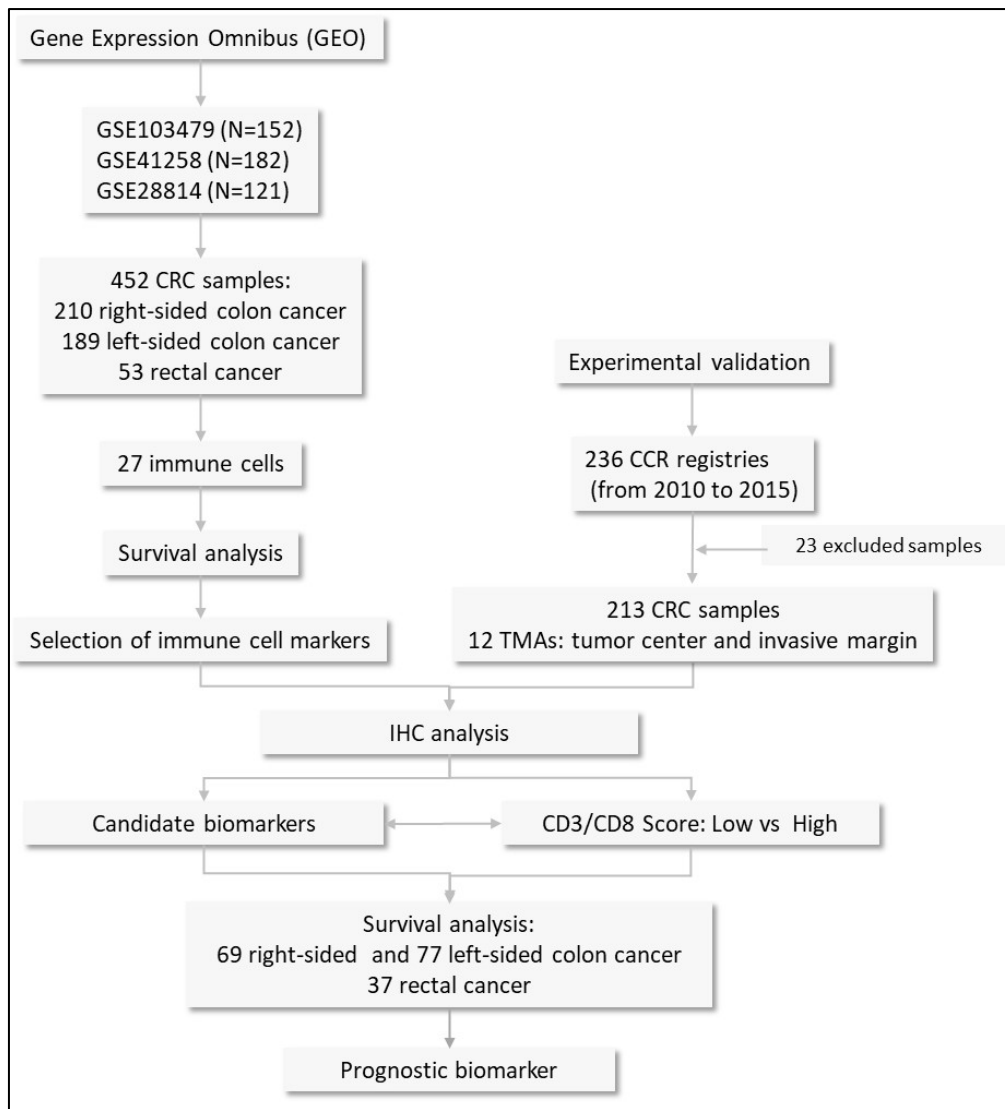


Figure 1. Flowchart of the study design.

Evaluation of infiltrating immune populations

The xCell platform (<http://xCell.ucsf.edu/>) was used to assess the cellular heterogeneity within the colorectal cancer microenvironment from bulk gene expression data (ARAN; HU; BUTTE, 2017). xCell platform can estimate the presence of 64 types of immune and stromal cells, of which 27 immune cells were analyzed.

The GEO studies was individually subjected to analysis on the platform, and enrichment values for each cell type were obtained for each sample. The scores generated for each immune cell were dichotomized into high and low according to the median values in the set of tumor samples from each study separately. Values above the

median were considered high and values equal to or below the median were considered low. Cancer-specific survival curves were constructed for each of the 27 populations by grouping data from the three selected studies (N=452). Cell populations associated with CRC prognosis were selected for further validation (Figure 1).

2. Experimental validation of immune cells in CRC tissue samples

Patients and sample collection

From January 2010 and January 2015, 236 records of primary colorectal cancer tumors were identified. Formalin-fixed, paraffin-embedded (FFPE) primary CRC samples were obtained from Department of Pathology, Botucatu Medical School (UNESP). The inclusion criteria included: patients >18 years old with primary CRC tumors of tubular (usual) or mucinous histological type, of any stage and histological grade obtained by surgical resection and not treated with chemotherapy and/or radiotherapy prior to sample collection. The exclusion criteria included patients with a history of previous or synchronous tumors and patients with no paraffin blocks or without clinical data.

For this study, 177 (78%) of the 236 CRC cases with follow-up data were selected for analysis. Among the patients, 82 were females and 101 males. The mean age at diagnosis was 64 ± 13 years with 86% of patients diagnosed above the age of 50 years. The median follow-up of 63 months (1–143 months). From 183 cases, 69 cases were right-sided colon tumors (22 cecum, 35 ascending colon, 4 hepatic angle and 8 transverse colon); 77 left-sided colon tumors (6 splenic angle, 15 descending colon and 56 sigmoid) and 37 rectal cancers.

The included cases were reevaluated to confirm the diagnosis of colorectal carcinoma according to the College of American Pathologists checklist (WASHINGTON et al., 2009b) and criteria defined by the World Health Organization (WHO). Clinical data including patient demographics, disease characteristics, and treatment history were collected from electronic medical records. TNM staging followed the AJCC staging system (8th edition). The lymph node ratio (LNR) was defined as the number of affected lymph nodes divided by the total number of lymph nodes examined (PETRELLI; BORGONOVO; BARNI, 2011b).

Tissue microarray construction, staining and analysis.

In total, 12 TMAs were constructed containing FFPE CRC tissue samples from different anatomical locations, designating specific regions of the invasion margin (IM) and the tumor center (CT). Using a 2.0 mm diameter needle, representative areas of the two tumor regions (IM and CT) from each tumor specimen were identified by the pathologist and included in the donor block using a *Tissue Microarrayer* (Pathology Devices Inc., Beecher Instruments, Sun Prairie, WI, USA).

The immunohistochemistry (IHC) method was used on TMAs to detect the Immunoscore using the CD3 and CD8 markers. The immunostaining protocols followed the recommendations of Pagès et al. (PAGÈS et al., 2018b) with modifications. Briefly, detection of CD3 and CD8 lymphocytes was performed on histological sections of the TMA block, using the EnVision[®] polymer (Dako, California, USA) as a detection system. The antigen retrieval method was carried out according to the specific needs of each antibody, using a pre-treatment module *PTLink[®]* (Dako, California, USAOs).

The mean and distribution of staining intensities were monitored to obtain internal quality control for each slide. In order to simplify and adapt the calculation of Immunoscore to manually counting (non-digital) microscopy, we opted for a modification similar to that described by Mlecnik et al. (MLECNIK et al., 2020). Aiming to have a Immunoscore that can be easily applied in clinical practice, the final Immunoscore was categorized into 2 groups. Cases with cell density <25% for the 2 markers (CD3 and CD8) in both CT and IM regions was categorized as LOW Immunoscore, and tumors presenting >25% of positivity for both markers in CT and IM regions were categorized as High Immunoscore.

The IHC method was used in TMAs to detect the selected immune cell, neutrophil in our cohort. Myeloperoxidase (MPO) was used as a neutrophil marker. The immunohistochemical study was performed on histological sections of the TMA blocks, using rabbit polyclonal anti-MPO antibody (Dako, ready to use). The antigen retrieval method was performed at high pH, using *PTLink[®]* pretreatment module (Dako, California, USAOs). EnVision[®] polymer (Dako, California, USA) was used as a detection system in an automated system (Ventana Benchmark XT).

The mean and distribution of staining intensities were monitored to obtain internal quality control for each slide. Immunostaining of MPO in CRC was quantified in two categories: <25% (classified as LOW) and >25% (classified as HIGH).

3. Statistical analysis

Data obtained from immune populations by xCell were evaluated for their prognostic value. Cancer-specific survival curves were constructed for each of cell population in all cases from the GEO selected studies. The Immunoscore and MPO marker were analyzed in relation to the patients' clinical and histopathological data. Cancer-specific survival curves according to Immunoscore and MPO scores were constructed using the Kaplan-Meier method. The programs GraphPad Prism v.8 (GraphPad Software Inc., La Jolla, CA) and SPSS v.25 (SPSS; Chicago, IL) were used. Statistically significant results were considered at $P < 0.05$.

RESULTS

Profile of immune infiltration in CRC

By using xCELL algorithm, we investigated the prognostic value of 27 subpopulations of immune cells in 452 CRC tissues (GSE28814, GSE41258 and GSE103479). It was found that five enriched infiltrating immune cells were associated with survival in CRC: We identified four enriched infiltrating cellular populations associated with prognosis: neutrophils, CD8 cells, Th2 and memory T CD4 cells (Figure 2).

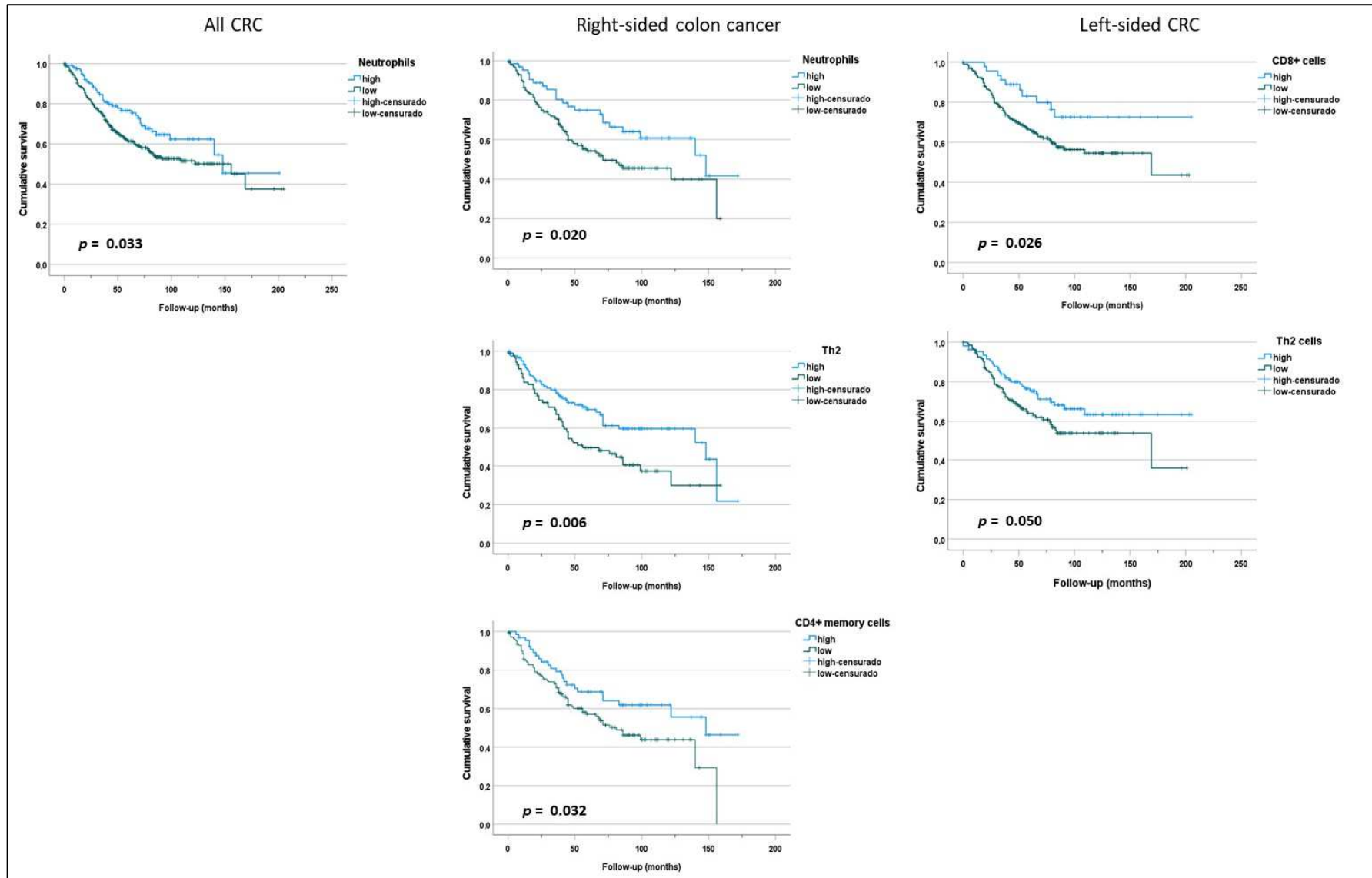


Figure 2. Cancer-specific survival curves based on the infiltrating immune cells in CRC using xCell algorithm. Kaplan–Meier survival curves with high (light blue) and low (dark blue) infiltrated immune cells in all CRC samples (N=452); in right-sided colon cancer samples (N=210) and in left-sided CRC samples (N=242, including rectal cancer). *log-rank P-value*.

Low tumor infiltration of neutrophils was associated with worse prognosis in all CRC samples (N=452, $p=0.033$, Figure 2). No association with prognosis was observed for all other immune cells evaluated. Next, we evaluated the prognostic value of infiltrating immune cells in tumor according to sidedness. We found that lower infiltration of neutrophils ($p=0.020$), Th2 cells ($p=0.006$) and memory T CD4 cells ($p=0.032$) were associated with decreased survival in patients with right-sided colon tumors (N=210) compared with patients with high tumor infiltration of these cells (Figure 2). In left-sided CRC (including left-sided colon cancer and rectal cancer), low T CD8 infiltration was significantly associated with shorter survival ($p=0.026$). A trend for association of low infiltration of Th2 cells and worse prognosis was observed in these tumors ($p=0.050$, Figure 2).

Of these, neutrophils were selected for further experimental validation in an independent cohort.

Experimental validation of immune cells in TMA

We next interrogated if adapted Immunoscore and neutrophils (MPO) were associated with prognosis in our cohort of CRC patients. Illustrative images of CD3 and CD8 and MPO immunostaining in CT and IM are shown in Figure 3A.

Reduced survival was associated with low Immunoscore in CRC samples, although with no statistical significance ($p=0.065$) (Figure 3B). The 5-year relative survival rates were 68% and 53% for high and low Immunoscore, respectively.

We further evaluated the Immunoscore prognostic value in CRC according to sidedness. Patients with low Immunoscore in tumors located in the left side (including colon and rectal cancer) showed a decreased survival time compared to patients with tumors with high Immunoscore ($P=0.027$; Figure 3B). The 5-year relative survival rates were 69% and 49% for high and low Immunoscore, respectively. Particularly, low immunescore was associated with worse prognosis in rectal tumors ($p=0.020$). Regarding cases with right-sided tumors, no statistically significant association of survival and Immunoscore was observed (data not shown).

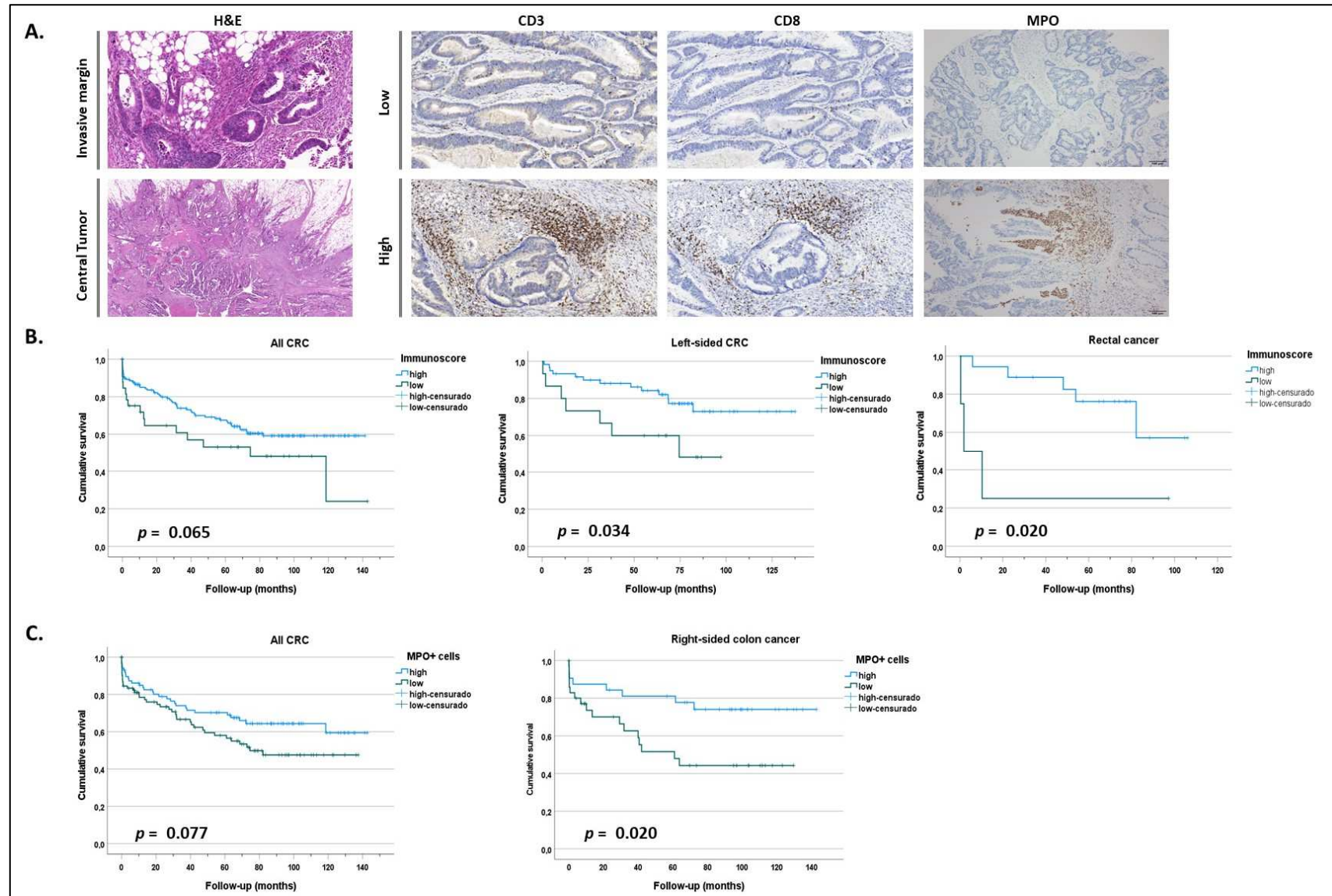


Figure 3. Prognostic analysis of Immunoscore and neutrophils (MPO) in CRC. **A.** Illustrative microscopic images of Hematoxylin & Eosin (H&E) stained colorectal tissues arranged in tissue microarrays (TMA) showing the tumor invasive margin and central tumor region. Representative images of immunohistochemical staining of immune cell markers, CD3, CD8 and MPO in colorectal cancer tissues showing low and high densities of infiltrating CD3+ cells, T CD8+ cells and neutrophils (MPO+). **B.** Cancer-specific survival curves according to Immunoscore (high vs low) in all patients with CRC, patients with left-sided CRC and rectal cancer. **C.** Cancer-specific survival curves according to MPO score (high vs low) in all cases of CRC and in right-sided colon cancer. *Log-rank test*. Light blue curves – high score and dark blue – low score for each marker.

Decreased survival associated with low MPO scores in CRC cases was observed, but it was not statistically significant (Figure 3C). In agreement with xCell analysis, significant association of low infiltration of neutrophils (MPO+ cells) and decreased survival was observed for patients with right-sided colon tumors ($p=0.020$).

DISCUSSION

In the present study, we combined *in silico* approach and experimental validation to identify immune cells as prognostic markers in colorectal tumors from different anatomical sites. We assessed bulk gene expression in GEO public database to estimate the infiltration of immune cells with interest in CRC using xCell platform. We detected different immune cell populations associated with prognosis in CRC according to tumor sidedness, such as CD8+ cells, neutrophils, memory T CD4+ and Th2 cells. In the next step, we analyzed the infiltration of neutrophils and evaluated the adapted Immunoscore method in an independent cohort of patients followed at our institution to identify the prognostic value of these markers in our patients. We found a good correlation between the results obtained in the *in silico analysis* and the immunohistochemical study, identifying the Immunoscore as an important prognostic marker, mainly in left-sided colorectal tumors, while neutrophils showed greater prognostic value for right-sided colon tumors.

Despite the wide variety of inflammatory cells capable of histological identification in the tumor environment, such as macrophages, plasma cells, neutrophils and eosinophils; the role of intratumoral infiltrating lymphocytes (TIL) as an anti-tumor immune response has become evident (DIEDERICHSEN et al., 2003; DUNN; DUNN; CURRY, 2007). The presence of inflammatory infiltrate in tumors, mainly cytotoxic T lymphocytes (TCD8+) has been associated with a better prognosis in several neoplasms, including CRC (CHRAA et al., 2019).

Since the establishment of Immunoscore as a prognostic marker in CRC (Pages et al.), many studies have been conducted to confirm its prognostic value, including an international Immunoscore consortium involving 14 centers of 13 countries with 3539 TNM stage I–III colon cancer patients. This consortium concluded that the Immunoscore

could be used as a new component of a TNM-immune classification of colon cancer. In fact, the first step towards a new classification of colorectal carcinoma took place, in mid-2020, with the publication of the latest (5th) edition of the WHO book Tumors of the digestive system, pioneeringly introducing the assessment of the immune response as a criterion essential and desirable diagnosis for colorectal cancer (CRC) (MLECNIK et al., 2020).

In order to simplify and adapt the Immunoscore analysis to conventional (non-digital) microscopy, we quantified the immunostained markers CD3+ and CD8+ in the same tumor regions (center and margin of invasion), using two-category scoring system: <25% (low) and >25% (high), as previously described by Mlecnik et al. (MLECNIK et al., 2018, 2020a) with modifications. Our results with Immunoscore in 2 categories corroborate those already reported by other authors, who also used Immunoscore in two categories (high vs low) (MLECNIK et al., 2020; PAGÈS et al., 2018a). Furthermore, the validation of a non-digital semi-quantitative method, readily applicable in resource-constrained laboratory settings, assumes paramount significance within the framework of public health initiatives in developing countries. The adoption of a simplified reading approach has the potential to enhance result reproducibility among pathologists and across different center facilities. Using this scoring system, we observed that low Immunoscore was associated with decreased survival in CRC, albeit it was not statistically significant. The 5-year relative survival rates were 68% and 53% for high and low Immunoscore, respectively. However, significant association was observed between low Immunoscore and worse prognosis in patients with stage I-III left-sided tumors, showing 5-year relative survival rates of 69% and 49% for high and low Immunoscore, respectively. These data are in agreement with our *in silico* results, that showed low infiltration of CD8 cells associated with reduced survival in left-sided CRC cases. Evaluating cancer-specific survival in patients with different anatomical locations, we did not identify significant differences in the survival curves according to the Immunoscore for patients with tumors of the cecum, ascending colon, transverse colon and hepatic flexure, splenic flexure, descending colon, and sigmoid tumors (data not shown). These results may reflect the small number of tumors in each group. However, we found a significant correlation between low Immunoscore and decreased survival in rectal

tumors (Stage I to III). The prognostic value Immunoscore in rectal tumors was previously reported (ANITEI et al., 2014). Low densities of CD3+ and CD8+ densities translated to low Immunoscore in rectal tumors was associated with worse prognosis from patients who did not receive neoadjuvant chemotherapy. In addition, low CD3 and CD8 densities in tumor biopsies were associated with non-response to neoadjuvant therapy in a series of 48 patients (ANITEI et al., 2014). In contrast, no significant difference in survival was observed according to the Immunoscore in right-sided colon tumors. It is worth noting the difference in the number of samples evaluated between the distal and proximal colon, which may influence the statistical power of the analyses. While for the distal colon, 114 cases were evaluated with complete follow-up data, for the proximal colon we analyzed 69 cases, which represents 38% of the total CRC cases in the study. Therefore, we cannot rule out the possibility that the Immunoscore is a prognostic marker in CRC, regardless of tumor laterality.

Based on *in silico* analysis results, we next interrogated if infiltrating neutrophils in colorectal tumors could be associated with prognosis in our cohort. We observed that low levels of neutrophil infiltration, here detected by MPO immunostaining, were associated with decreased survival time in CRC, although it was not statistically significant. In contrast, low MPO score was correlated with a poorer prognosis in right-sided colon tumors. Droeser et al. (DROESER et al., 2013) previously reported that lower neutrophil infiltrates were associated with a worse prognosis in CCR. The CD15 and MPO markers were analyzed in 1491 CRC samples (609 training set and 583 validation set). Although tumor infiltrated MPO+ and CD15+ cells were significantly correlated, only the MPO+ cells were associated with significantly improved survival in training ($p=0.038$) and validation ($p=0.002$) sets. Multivariate analysis revealed that high score MPO+ infiltration was an independent prognostic marker in CRC, after adjusting for other factors such as age, sex, T stage, N stage, tumor grade, vascular invasion, tumor border configuration and microsatellite stability. However, in this report the association of neutrophil infiltration and tumor sidedness was not investigated (DROESER et al., 2013). An association between low levels of neutrophil infiltration and a poorer prognosis in CCR, in a study involving 448 stage II and III tumor samples distributed in tissue microarrays (TMAs) and using CD66b as the marker was also reported (WIKBERG et al.,

2017). In this report, CD66b+ cells were investigated in 448 CRC samples, evaluating tumor invasive front, tumor center, and within the tumor epithelium. Low tumor infiltration of CD66b+ cells correlated with worse prognosis in CRC. CD66b infiltration was detected as an independent prognostic marker in stage I-II CRC cases. Similar CD66b positivity patterns were observed in right-sided and left-sided colon tumors, the survival analysis according to tumor sidedness was not reported in this study. Positive correlation was observed for CD8+ and CD66+ cells. Interestingly, when survival analysis was conducted combining CD8 score (high vs low) and CD66b score (high vs low), only CD8 score was associated with cancer-specific survival of colon cancers (WIKBERG et al., 2017).

The strength of our study lies in the investigation of infiltrating immune cells in CRC based on tumor laterality using two different methodologies and in diverse patient cohorts from various countries. Consistent results were observed between the *in silico* analysis and the immunohistochemical study, thereby enhancing the robustness of our findings. Furthermore, a noteworthy aspect of our case series is the extended follow-up of patients, with a median duration of 5 years in both cohorts evaluated by the two analytical approaches. These factors are particularly significant within the context of prognostic biomarker analysis, underscoring the importance of our study. Additionally, the adapted Immunoscore analysis reported here can be easily implemented in other pathology laboratories, irrespective of their resources, which is relevant in the context of disseminating the method across different countries.

In summary, the prognostic value of the adapted Immunoscore and infiltrating neutrophils in CRC, with differences according colon tumor sidedness and can be potentially implemented in other pathology laboratories.

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Conclusão

Resultados consistentes do papel prognóstico de células imunes infiltrantes nos tumores colorretais foram observados tanto na análise *in silico* incluindo uma grande coorte de pacientes com CRC quanto no estudo imuno-histoquímico dos marcadores imunes na casuística da nossa Instituição

O Immunoscore adaptado, baseado na leitura não digital da densidade de linfócitos T CD3+ e CD8+ infiltrantes do tumor detectados por imuno-histoquímica no centro do tumor e na margem de invasão mostrou valor prognóstico em CCR de acordo com a lateralidade dos tumores colorretais da nossa casuística.

Pacientes com tumores colorretais do lado esquerdo e Immunoscore baixo, particularmente os casos com câncer de reto, mostraram associação com pior prognóstico comparado aos pacientes com tumores com Immunoscore alto, corroborando o papel do infiltrado imune na evolução dos tumores colorretais.

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CAPÍTULO 3

Outros trabalhos publicados durante o desenvolvimento do Doutorado

Anexo 1

Santaella FJ, Hamamoto Filho PT, Poliseli GB, de Oliveira VA, Ducati LG, **Moraes MPT**, Zanini MA. **Giant Thoracolumbar Dumbbell Ganglioneuroma. Pediatr Neurosurg.** 2018;53(4):288-289. doi: 10.1159/000488499. Epub 2018 Apr 26. PMID: 29698954.

Giant Thoracolumbar Dumbbell Ganglioneuroma

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A 6-year-old girl presented with a history of 6 months of abdominal pain. On physical examination, she exhibited a large abdominal mass. No neurological abnormalities were observed. Magnetic resonance imaging (MRI) showed a large dumbbell contrast-enhancing mass from the right retroperitoneum to the spine, extending from T10 to L3. The tumor had eroded the vertebral bodies of L1 and L2 and enlarged multiple intervertebral foramens,

as well as the spinal canal itself, displacing the spine to the left (Fig. 1). A 2-step resection was performed, first through a T11-L3 laminectomy for removal of the spinal component, then via laparotomy for resection of the retroperitoneal component. The patient had an uneventful postoperative recovery. Pathological analysis confirmed a ganglioneuroma (Fig. 2).

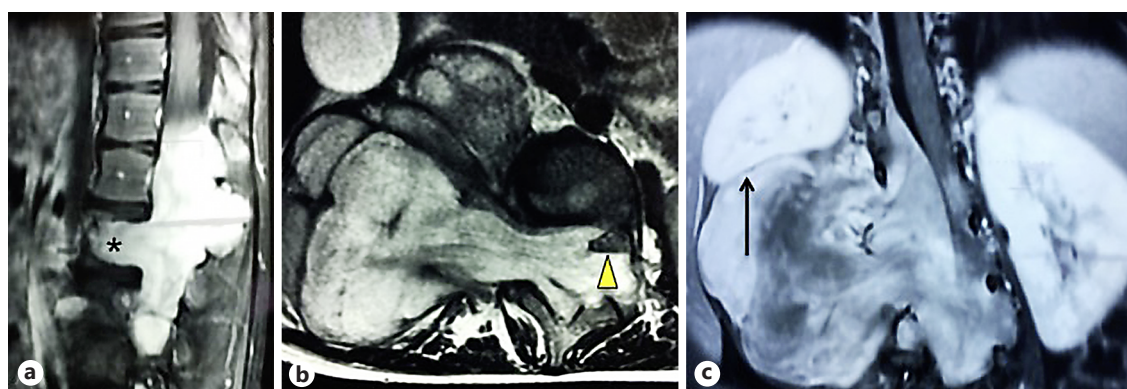
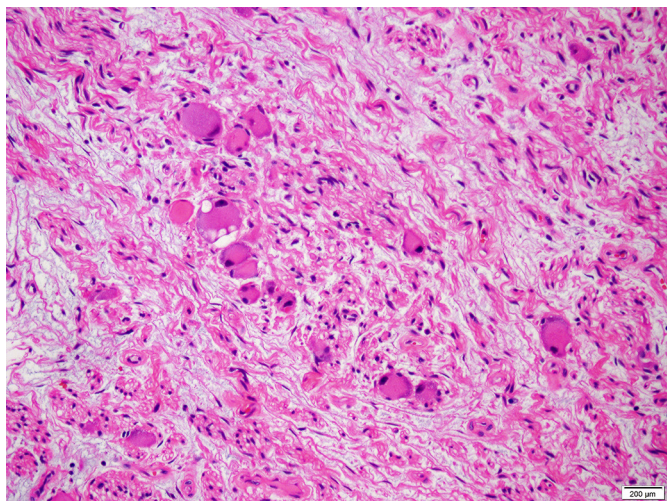


Fig. 1. MRI in sagittal (a), axial (b), and coronal (c) views. The tumor had eroded the vertebral body of L1 (*), had a dumbbell shape, and occupied a large portion of the spinal canal, displacing the spine to the left (arrow-head). In the retroperitoneum, the tumor displaced the right kidney upwards (arrow).



Color version available online

Fig. 2. Histologic analysis revealed fusiform cells with round nuclei and mature ganglion cells.

Ganglioneuromas are uncommon, benign, neoplastic tumors that originate from the primitive neural crest cells that differentiate into the sympathetic nervous system.

They belong to the group of peripheral neuroblastic tumors, and generally have a good prognosis, in contrast to neuroblastomas [1–3]. Most ganglioneuromas are located within the mediastinum and abdominal cavity. Ganglioneuromas involving the spinal canal comprise <10% of cases [2]. The clinical presentation may vary from asymptomatic to back pain or neurological deficits [4]. On MRI, they typically exhibit hypointensity on T1 and hyperintensity on T2. As a benign tumor, complete surgical resection enables patients to be cured of this tumor, although risky adhesions to great vessels often lead to subtotal resection, which is acceptable since this tumor exhibits a very low rate of growth.

Statement of Ethics

Informed consent to publish these data was obtained from those legally responsible for the patient.

Disclosure Statement

The authors declare no conflicts of interest.

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Anexo 2

Martins D, Tonon CR, Pacca RL, Matchil NL, Junior LAJ, Queiroz DS, Pereira FWL, Silva AM, Padovese V, **Padovani de Toledo Moraes M**, Luiz da Silva D, Cardoso Nóbrega V, Curcelli EC, Okoshi MP. **Gout Storm**. Am J Case Rep. 2021 Sep 20;22:e932683. doi: 10.12659/AJCR.932683. PMID: 34855717; PMCID: PMC8475732.

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Gout Storm

Authors' Contribution:

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Statistical Analysis C
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Conflict of interest: None declared

Patient: Male, 55-year-old
Final Diagnosis: Gout • spinal gout
Symptoms: Back pain • fever • incontinence • pain • paresthesia of extremities • tachycardia
Medication: —
Clinical Procedure: Antibiotics • arthrocentesis • biopsy • CT scan • surgery • ultrasonography
Specialty: General and Internal Medicine

Objective: Unusual clinical course

Background: Gout is a chronic disease characterized by deposition of monosodium urate crystals, typically manifesting as arthritis. Clinical presentation of gout usually results from activation of local inflammatory response. Despite being one of the oldest diseases in the world, gout pathophysiology is incompletely understood and clinical features are still surprising. Recent reports describe unusual manifestations including atypical joints involvement, tenosynovitis, panniculitis, and multinodular inguinal swelling. Another atypical feature is the acute polyarticular gout with severe systemic inflammatory response.

Case Report: We report the case of a 55-year-old man presenting with fever, tachycardia, cauda equina syndrome, left-knee arthritis, and systemic inflammatory manifestations. Lumbar spine magnetic resonance imaging showed a 4.0×1.3×2.2 cm calcified mass inside the vertebral canal at the L4-L5 level, causing stenosis of the dural space and intervertebral foramen. Clinical diagnoses were septic knee arthritis and lumbar spine meningioma. Despite antibiotic therapy and left-knee surgical drainage, fever and increased C-reactive protein persisted, and arthritis developed in the elbows and right knee. As cultures were negatives, we then diagnosed gout flare in the affected joints accompanied by a severe systemic inflammatory reaction. A few days after starting colchicine and anti-inflammatory drugs, symptoms and inflammatory markers subsided. It was such a severe attack that we called it a "gout storm".

Conclusions: The report highlights the difficulty in diagnosing acute polyarticular gout affecting atypical joints, particularly when faced with a severe systemic inflammatory reaction.

Keywords: Cauda Equina Syndrome • Diagnosis • Gout

Full-text PDF: <https://www.amjcaserep.com/abstract/index/idArt/932683>



Background

Gout is characterized by acute and chronic joint inflammation in response to monosodium urate crystal deposition, most commonly affecting the first metatarsophalangeal joint, knees, ankles, wrists, and hand joints [1,2]. Gout is one of oldest diseases in the world, first recognized by the Egyptians in 2640 BC [3]. Despite this long knowledge, gout pathophysiology is incompletely understood and clinical features are still surprising [4-8]. Acute manifestations are caused by immune reaction to the monosodium urate crystals that damage tissues surrounding the joints. Innate immune cells such as monocytes, macrophages, mast cells, and neutrophils are involved in the pathogenesis of acute gout inflammation [9]. Recent case reports in the literature have described unusual gout manifestations including tenosynovitis, panniculitis, multinodular inguinal swelling, and involvement of atypical joints [4-6,8,10-12]. We report the case of a patient presenting with a polyarticular gout attack affecting the knees, elbows, and lumbar spine, with intense systemic inflammatory manifestations simulating infection with systemic inflammatory response syndrome.

In this case, as in several reports from literature [8,12,13], the patient was primarily managed for infection. Therefore, we would like to highlight to internist physicians the possibility of this clinical presentation of acute gout, which reminded us of a “gout storm”.

Case Report

A 55-year-old man presented to our hospital with a 5-day history of low back pain with lower-limbs paresthesia, gait impairment, and fecal and urinary incontinence. In the last 2 days, he also had fever and left-knee arthritis. His medical history included hypertension, diabetes, dyslipidemia, obesity, and gout, characterized by inflammation crises involving his first right metatarsophalangeal joint and both knees since 2013. The patient had been taking enalapril, metformin, simvastatin, and insulin. Allopurinol was interrupted by the patient some months ago. There was no history of recent trauma, infection, or neoplastic disease.

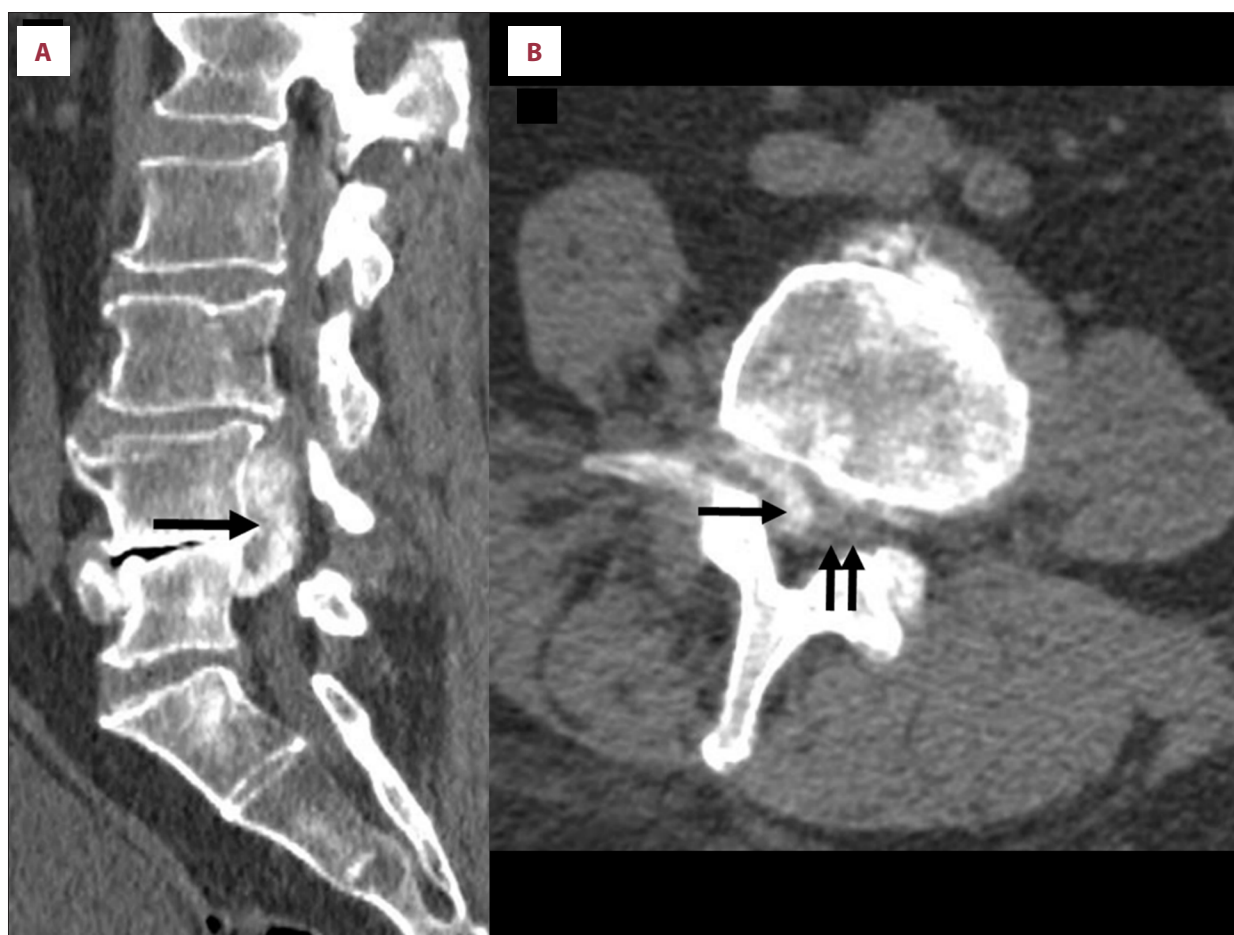


Figure 1. Non-contrast computed tomography scan sagittal (A) and axial (B) views showing a heterogeneous calcified epidural mass (arrow in A) compressing and displacing posterolaterally the dural sac (arrows in B).

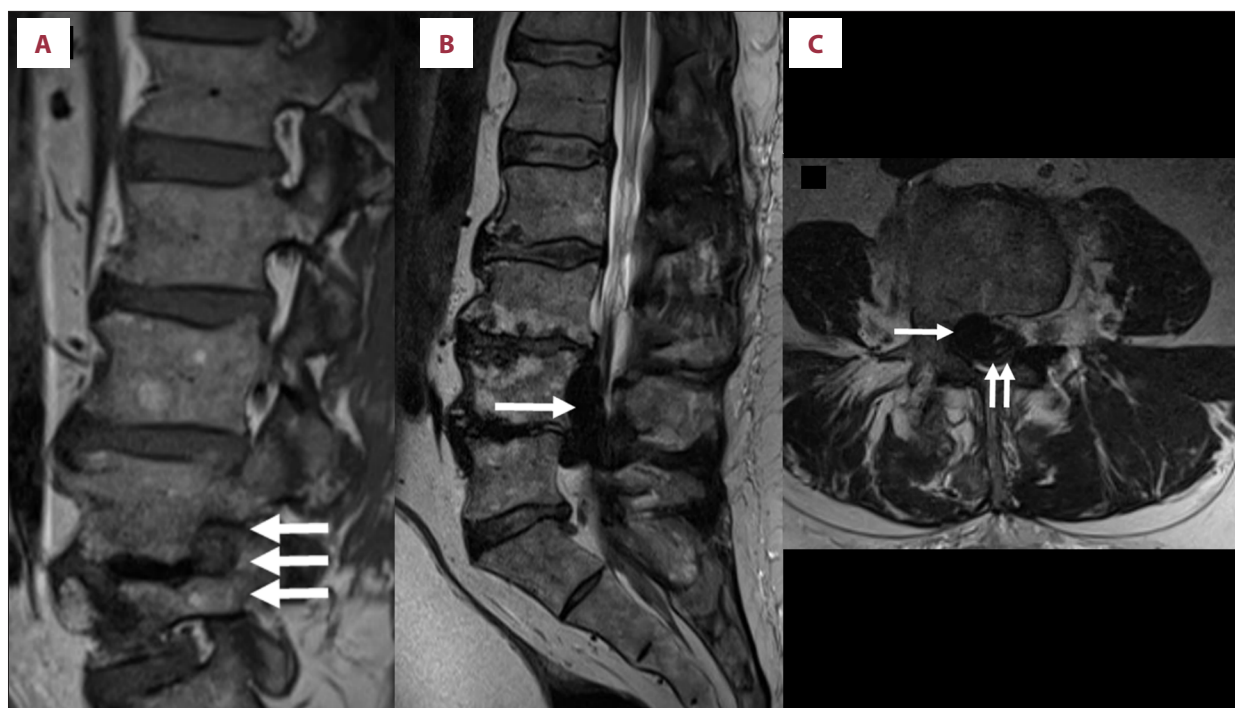


Figure 2. Magnetic resonance imaging in T1-weighted sagittal (A) and T2-weighted sagittal (B) and axial (C) images showing a heterogeneous hypointense epidural mass (arrow) compressing and displacing posterolaterally the dural sac (double arrow), and causing right foraminal stenosis at the L4-L5 level (triple arrow). As a consequence, the descending and emerging roots were compromised.

Physical examination showed a temperature of 38°C, heart rate 118 bpm, left-knee arthritis, grade IV reduction in left leg muscle strength, anesthesia in the left foot, and hypoesthesia in both lower limbs. No gout tophus was observed in physical examination. The remainder of physical examination was unremarkable. Laboratory evaluation revealed hemoglobin 13.5 g/dL (reference range for men 13.5-17.5 g/dL), white cell count 14 100/mm³ (10 039/mm³ neutrophils, 1424/mm³ monocytes, and 2200/mm³ lymphocytes), and creatinine 1.1 mg/dL (reference range 0.8-1.5 mg/dL), with an estimated glomerular filtration rate of 75 mL/min/1.73 m² according to the CKD-EPI formula, erythrocyte sedimentation rate (ESR) 65 mm/h (reference < 15 mm/h), C-reactive protein (CRP) 19.8 mg/L (reference < 1 mg/dL), and uric acid 5.0 mg/dL (reference 3.5-8.5 mg/dL). His medical record showed a serum uric acid concentration dated 2016 of 11.5 mg/dL.

A non-contrasted computed tomography (CT) scan showed a heterogeneous calcified epidural mass compressing and displacing posterolaterally the dural sac (Figure 1). Lumbar spinal magnetic resonance imaging (MRI) showed an extensively calcified lesion measuring 4.0×1.3×2.2 cm located inside the vertebral canal at the L4-L5 level, causing stenosis of the dural space and intervertebral foramen and compressing the receptive descending and emerging roots (Figure 2). Ultrasonography of the left knee revealed thick fluid and echogenic debris with

an estimated volume of 16 cm³ located at the left suprapatellar bursa extending to the lateral and medial surfaces of the knee.

Initially, the main clinical diagnoses were septic knee arthritis and lumbar spine meningioma. The patient received intravenous ciprofloxacin and clindamycin and was subjected to left-knee surgical drainage, producing a thick brown liquid. Cultures from blood and knee fluid collected before antibiotic therapy and knee tissue fragment were negative.

In spite of receiving antibiotics, the patient persisted with fever, fatigue, malaise, and elevated inflammatory markers. A few days later, arthritis developed in both elbows and the right knee. Left elbow arthrocentesis revealed a thick yellow synovial fluid with 1700 leukocytes/mm³ and 96% segmented cells. Crystals were observed at microscopic evaluation; unfortunately, it was not possible to examine arthrocentesis fluid under polarized light. Right elbow arthrocentesis showed citrine yellow synovial fluid with no leukocytes. Both synovial fluid cultures were negative. Ultrasonography of the right knee revealed a heterogeneous image next to the patellar tendon measuring 2.6×1.2 cm, which might correspond to a gouty tophus.

After observing synovial crystals and excluding infection, antibiotics were discontinued and gout treatment was initiated with colchicine, 0.5 mg twice a day, anti-inflammatory drugs, and finally the xanthine oxidase inhibitor allopurinol. Fever,

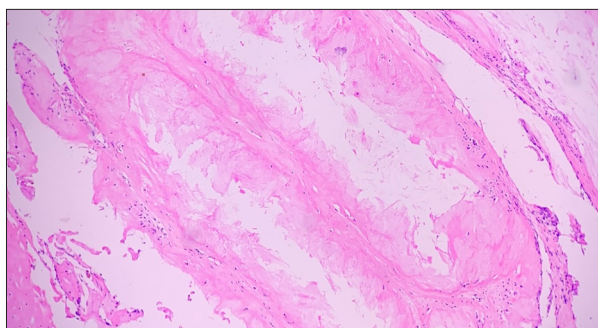


Figure 3. Histopathology section of spine mass showing a thin layer of mononuclear inflammatory infiltrate with giant cells surrounding the amorphous crystalline material, typical of gout tophi. Hematoxylin-eosin staining, ×100.

general health status, and CRP levels improved significantly a few days later, and the polyarthritis quickly resolved; neurological changes persisted. One month after hospitalization, laboratory tests revealed leukocytes of 9300/mm³, CRP 3.7 mg/dL, and creatinine 0.7 mg/dL.

The patient was then subjected to incisional removal of the spinal mass and lumbar spine arthrodesis. The spine mass had a hard consistency, which at this point reinforced our diagnosis of gout tophus. Histopathological examination showed tissue fragments with mononuclear inflammatory infiltrate and partially calcified acellular material with giant cells surrounding the amorphous crystalline material, compatible with a gout tophus (Figure 3). Unfortunately, pathology analysis could not show uric acid crystals as the surgical specimen was fixed in formalin, which causes dissolution of uric acid crystals [14,15]. The patient was discharged after improvements in lower-limb paresthesia and left-limb muscle force and remission of fecal and urinary incontinence. Twelve days after surgery, he returned to our hospital presenting mental confusion, surgical site infection, and suture dehiscence. He underwent surgical debridement and wound washing. Surgical site and blood cultures revealed *Klebsiella pneumoniae*; surgical tissue fragment and wound secretion growth cultures revealed *Staphylococcus aureus*. Despite clinical treatment, the patient developed refractory septic shock and died 1 week later.

Discussion

This patient had 2 important clinical features: the polyarticular involvement of joints, including an atypical joint, the spine, and a severe systemic inflammatory reaction, similar to that seen in sepsis. Despite his previous gout, the current history and physical examination pointed to left-knee infectious arthritis in a patient with a spinal tumor. Only after the involvement of both elbows and the right knee was gout flare diagnosed. The fact that a very severe attack of gout involving an

atypical joint occurred in a patient with no gout tophus makes this a rare case. Although not previously used in the literature, the term “gout storm” appeared appropriate to define these serious clinical and laboratorial manifestations. Some reports from the literature reinforce the dilemma in diagnosing acute polyarticular gout or spinal gout to explain the whole clinical picture at the time of presentation [6-8,12].

Although gout physiopathology is not completely understood, it is recognized that monosodium urate crystals deposition in the synovium induce direct cytotoxicity, inflammation, and cell necrosis. Furthermore, synovium release of reactive oxygen species and reactive nitrogen species enhances the inflammatory process [16]. Activated immune cells produce pro-inflammatory cytokines, tumor necrosis factor, and cyclo-oxygenase [9]. Monosodium urate crystals also activate the NLRP3 inflammasome, which leads to caspase-1-dependent cleavage of pro-interleukin (IL)-1 β , thereby triggering mature IL-1 β release and enhancing the inflammatory response [16]. We assume that, in this case, the inflammatory response hyperactivation was triggered by the polyarticular involvement.

Several triggers for gout attacks have been described [1]. The only precipitating factor we could find in this case was a slight renal function impairment. However, it was not possible to establish whether worsening renal function was a precipitating factor or followed an acute attack, which could have impaired oral ingestion and led to dehydration.

This case emphasizes the importance of cytological examination of synovial fluid, which is often mistaken for a purulent exudate of septic arthritis. Even though fever, neutrophilia, and raised inflammatory markers are common in acute gout [8,11,17-19], their presence guides physicians toward a diagnosis of bacterial infection. CRP, ESR, and procalcitonin at high levels as observed in infectious disease have been described during acute gout attacks and chronic gout [17,18,20,21]. A severe systemic inflammatory response syndrome simulating sepsis has also been observed in acute polyarticular gout [22] and immediately after spinal surgery for axial gout [14]. On left-knee ultrasound, our findings of thick fluid and echogenic debris have low specificity and accuracy for diagnosing gout, but a double contour sign and articular tophus have a high specificity for gout diagnosis [23].

Spinal tophaceous gout is not a common finding. In most cases in the literature, a diagnosis of spinal tophi was not initially suspected [7,17,18,24,25]. The most common clinical presentation was back and neck pain, followed by neurological impairment. The main anatomical site was the lumbar spine [17]. The imaging tests most often performed are MRI and CT. In a review on spine gout, 96.6% of CT scans were abnormal, with 18.6% of scans interpreted as a high probability of tophaceous

gout [18]. MRI is the most commonly used method to analyze spinal lesions; in a review on spine gout, 98.9% of the cases had abnormal MRI. However, scans were read as a gouty or tophaceous lesion only in 21% of the patients [18]. As in our case, gouty tophi usually appear as a hypointense mass on T1- and/or T2-weight MRI [24].

The treatment of gout involves drugs to reduce inflammation in the acute phase, such as nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, and colchicine, and drugs to lower uric acid levels and prevent acute flare. NSAIDs inhibit production of cyclo-oxygenase; glucocorticoids inhibit transcription of inflammatory genes, and colchicine blocks activity of the NLRP3 inflammasome [26]. Recent studies have shown that IL-1 antagonists can be used in gout flare. The xanthine oxidase inhibitor allopurinol is the first line treatment and uricosuric agents are the second-line treatment for chronic gout [27]. The urate oxidase pegloticase can be used in refractory cases [27]. Treatment for spinal tophi depends on the clinical picture [19]. Although medical treatment may be sufficient, neurological impairment warrants surgical intervention [7,17,18]. In many cases, surgery was performed to assess suspected epidural abscess or spinal mass [13]. As drugs failed to decrease spinal symptoms, our patient was subjected to spinal decompression, which was initially successful. Unfortunately, post-operative surgical site infection led to patient death.

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Conclusions

This report highlights the difficulty in diagnosing acute polyarticular gout affecting an atypical joint, particularly when faced with a severe systemic inflammatory reaction. Furthermore, it emphasizes the importance of properly controlling serum uric acid concentration to prevent acute gout flare. Treatment should also include avoidance of risk factors for gout development, flare management, and healthy lifestyle.

Patient Consent

The patient's wife provided written consent for reporting this case.

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Declaration of Figures Authenticity

All figures submitted have been created by the authors who confirm that the images are original with no duplication and have not been previously published in whole or in part.

Anexo 3

Vasconcelos MM, Pupo da Silveira CFDSM, Felício ML, **de Toledo Moraes MP**, Teixeira R, Trivellato SA, Pinheiro Modolo G, Bazan R, Zanati Bazan SG. **Mitral Valve Papillary Fibroelastoma as the Cause of Ischemic Stroke in a Young Patient: A Case Report.** Am J Case Rep. 2021 Mar 13;22:e930561. doi: 10.12659/AJCR.930561. PMID: 33713402; PMCID: PMC7970527.

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Mitral Valve Papillary Fibroelastoma as the Cause of Ischemic Stroke in a Young Patient: A Case Report

Authors' Contribution:

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Conflict of interest: None declared

Patient: Female, 27-year-old
Final Diagnosis: Ischemic stroke • papillary fibroelastoma
Symptoms: Motor aphasia and hemiparesis in the right dimidium
Medication: —
Clinical Procedure: —
Specialty: Cardiology • Neurology

Objective: Rare disease
Background: Primary cardiac tumors represent less than 5% of total cardiac tumors. Fibroelastoma is a rare benign cardiac tumor that is usually asymptomatic but is acknowledged for its emboligenic potential for causing cardiac, neurological, and vascular symptoms and increasing patient morbidity and mortality.
Case Report: This report describes the clinical case of a 27-year-old woman who entered the Emergency Department with motor aphasia and hemiparesis in the right dimidium. A brain computed tomography scan was performed at admission, which showed left frontal-parietal hypodensity. The diagnosis of ischemic stroke was made, but cerebral reperfusion therapy with intravenous recombinant tissue plasminogen activator was not instituted due to the time that had passed since ictus (15 h 40 min). On the first day of hospitalization, the patient had a fever, with no apparent infectious cause. She underwent transthoracic echocardiogram that showed a sessile, isoechoic mass adhered to the atrial surface of the anterior mitral valve leaflet, measuring 6.8×5.5 mm. Antibiotic therapy with ceftriaxone and gentamicin was initiated due to the initial diagnosis of infective endocarditis. Three blood culture samples had negative results. Given a differential diagnosis of fibroelastoma, transesophageal echocardiography and cardiac resonance imaging were performed, and the findings were compatible with a diagnosis of mitral valve fibroelastoma. After clinical discussion, the patient was referred to cardiac surgery and underwent tumor resection with anatomopathological diagnosis of papillary fibroelastoma of the heart valve.
Conclusions: Young patients with ischemic stroke must be investigated with transthoracic and transesophageal echocardiograms. Papillary fibroelastoma is potential cause of ischemic stroke in young patients, and surgical resection is curative and has excellent prognosis.

Keywords: Echocardiography, Doppler • Endocardial Fibroelastosis • StrokeFull-text PDF: <https://www.amjcaserep.com/abstract/index/idArt/930561>

Background

Primary cardiac tumors represent less than 5% of total cardiac tumors [1]. Among them, the most common tumors are myxomas and lipomas, followed by fibroelastomas [2]. However, the majority of cardiac tumors are metastatic.

Fibroelastoma is a rare benign cardiac tumor that is usually asymptomatic, but it is acknowledged for its emboligenic potential for causing cardiac, neurological, and vascular symptoms and increasing patient morbidity and mortality [2,3].

Case Report

A 27-year-old woman presenting with motor aphasia and right-sided hemiparesis was admitted to the Emergency Department. She denied any prior diseases, such as hypertension or diabetes, and had a history of previous cocaine, cannabis and light tobacco use. She was employed at a meat cold storage facility and a middle school.

A brain computed tomography scan was performed upon admission and showed hypodensity in the left frontoparietal region; the hypodensity compromised over a third of the left medium cerebral artery territory (**Figure 1A**). Ischemic stroke was diagnosed, but cerebral reperfusion therapy with recombinant tissue plasminogen activator was not started because 15 h 40 min had elapsed between the last deficit-free encounter and admission to the hospital. This time span was outside the current accepted reperfusion window.

Biochemical tests, such as viral serologies (HIV, hepatitis B and C) and syphilis, and investigation of vasculitis and thrombophilia were all within normal levels. The patient then underwent transcranial Doppler imaging that suggested intracranial stenosis of the left medium cerebral artery.

As on the first admission day, there was one fever episode without an apparent infectious cause, and the patient underwent a transthoracic echocardiogram, which showed a sessile, isoechoic mass adhered to the atrial surface of the anterior leaflet of the mitral valve, measuring 6.8×5.5 mm (**Figure 1B**). She was then started on antibiotics (ceftriaxone and gentamicin) due the initial hypothesis of infectious endocarditis. Three blood culture samples were collected, and all had negative results. The possibility of fibroelastoma as a differential diagnosis was then discussed, and transesophageal echocardiography was scheduled upon ending antibiotic treatment. The images supported the diagnosis of mitral valve fibroelastoma. After clinical discussion, evaluation for cardiac surgery was requested, and the patient underwent surgery for tumor resection (**Figure 2A**). Pathological analysis was compatible with papillary fibroelastoma of the heart valve (**Figure 2B**).

Discussion

Fibroelastoma is a rare primary cardiac tumor, corresponding to less than 5% to 10% of cases [3]. Although it usually affects cardiac valves, it is rarely related to valve dysfunction. Although infrequent, fibroelastoma can also affect other sites, such as tendinous cords, the endocardium, and papillary muscles [2,4].

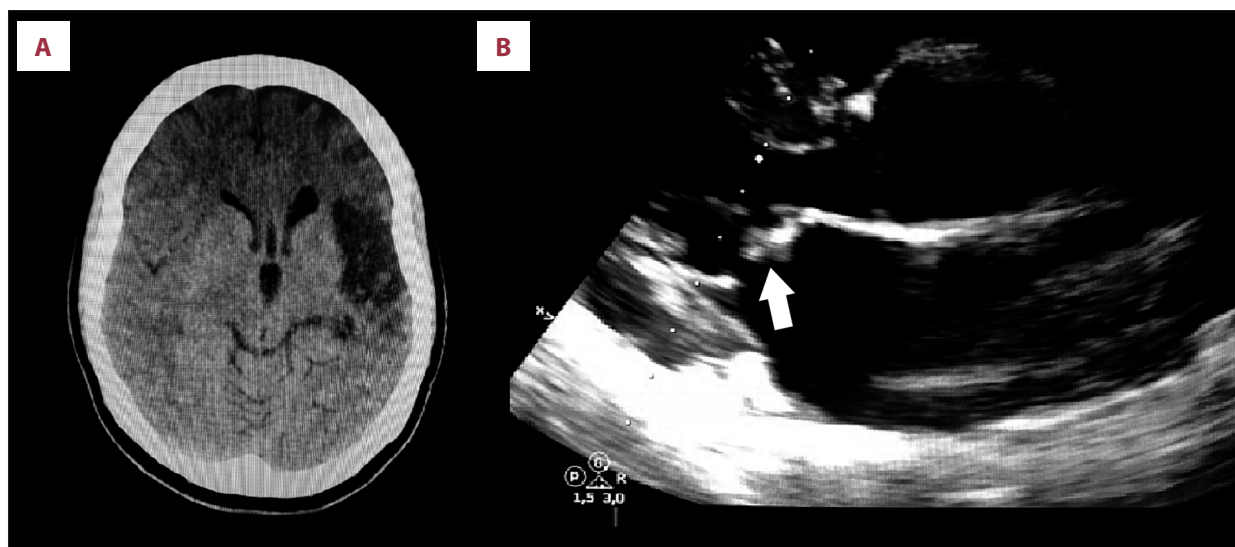


Figure 1. (A) Brain computed tomography showing a left frontoparietal hypodensity. (B) Transthoracic echocardiogram showing a sessile, isoechoic mass adhered to the atrial surface of the anterior leaflet of the mitral valve.

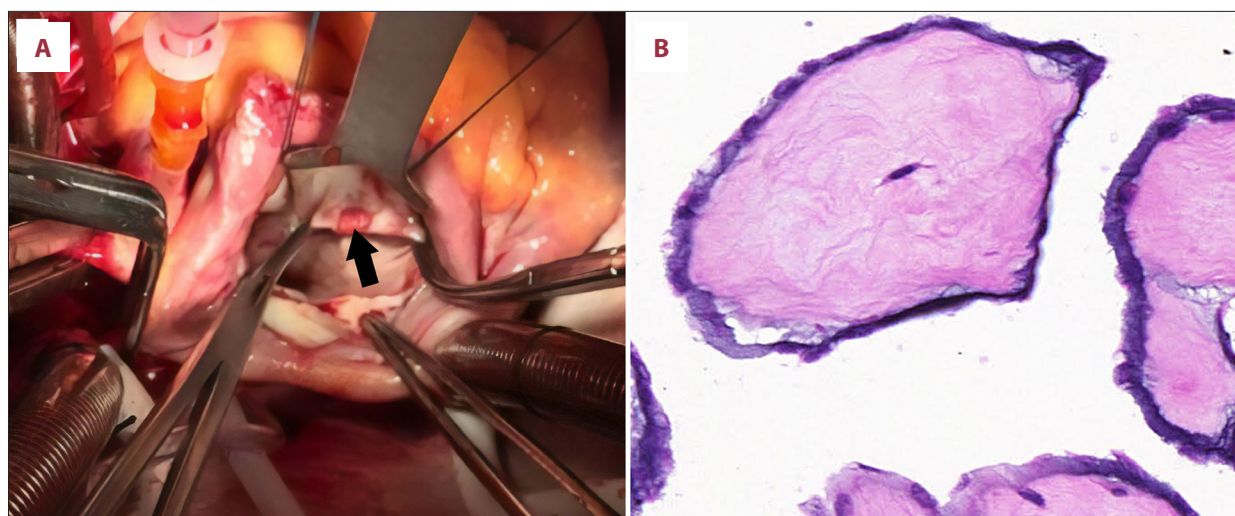


Figure 2. (A) Intraoperative view showing a mass attached to the anterior mitral valve leaflet. (B) Microscopy of the tumor showing elongated and branching papillary fronds composed of central avascular collagen and lined by hyperplastic endothelial cells.

Fibroelastoma presents as a single lesion that is small (less than 10 mm), not vascularized, and mobile, and it has a sea anemone-like appearance [4]. It more commonly affects the aortic valve, followed by the mitral, tricuspid, and pulmonary valves [5,6].

The etiopathogenesis of fibroelastoma is controversial due to its possible neoplastic origin [3], with other hypotheses suggesting origins in thrombosis, trauma, external stimuli, or even Lambl's excrescences. Histologically, a fibroelastoma is composed of a central portion of dense connective tissue that is surrounded by loose connective tissue and covered by hyperplastic epithelial cells [1,2].

Most of these tumors are asymptomatic. Nonspecific symptoms, such as chest pain, asthenia, fever, dyspnea, syncope, or rarely valve dysfunction, can occur [3]. It is important to highlight that right-side tumors can cause pulmonary embolism and arrhythmias and left-side tumors can provoke acute myocardial infarction and mesenteric, renal, or liver ischemia. Fibroelastoma can ultimately induce ischemic stroke, which is the main complication of these left-side tumors when symptomatic, as reported in this case [2,4]. This emboligenic potential is a consequence of the fragmentation of its own spicule or even of thrombi formed by platelets and fibrin adhered to the irregular tumor surface [1,4,6]. It is important to stress that, for a young patient with cardioembolic ischemic stroke, as described here, without any evidence of cerebrovascular disease and with sinus rhythm, the possibility of cardiac tumor, as well as endocarditis and mitral valve prolapse, must be considered [1].

Fibroelastoma may occur at any age but is more common among adults over 40 years old [4,5], without a sex prevalence.

Its differential diagnoses, aside from vegetation [7], thrombi, and degeneration of valvular tissue, are other cardiac tumors, such as lipoma and myxoma [3]. The diagnosis may be achieved through transthoracic echocardiogram, especially for large tumors [1,8]. For smaller tumors, transesophageal echocardiogram may help provide information about size and location [3,9].

Other examinations may help with the diagnosis, such as cardiac magnetic resonance, which provides detailed information of the tumor through the late enhancement technique [4]. It is important to highlight that the criterion standard diagnosis is made through anatomopathological study [1].

Surgical treatment is still a controversial subject in asymptomatic patients. Tumor characteristics, such as size and mobility, and the presence of symptoms [1,3] must be taken into account for decision making. If the patient presents with a patent foramen ovale and a right-side tumor, resection is recommended due to the risk of a paroxysmal embolism [3].

Our patient presented with an ischemic stroke as a complication, and after the exclusion of other possible cardioembolic sources, a fibroelastoma was considered the cause of the event. Thus, the patient was referred to cardiac surgery for tumor resection.

Surgical resection is performed at the tumor implantation base, with the aim to avoid tumor recurrence and new embolic events [1,5,10]. The procedure is curative in the majority of cases. Patients undergoing conservative treatment must remain on anticoagulants, although it is a controversial subject in the literature, with uncertain efficacy [4].

Conclusions

Young patients with ischemic stroke must be investigated with transthoracic and transesophageal echocardiograms. Cardiac masses require clinical history, blood cultures, laboratory tests, and imaging tests to establish differential diagnoses. Papillary fibroelastoma is a potential cause of ischemic stroke in young patients, and surgical resection is curative and has an excellent prognosis.

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Conflicts of Interest

None.

Anexo 4

Rodrigues LDS, Bovolato ALC, Silva BE, Chizzolini LV, Cruz BLD, **Moraes MPT**, Lourenção PLTA, Bertanha M. **Quantification of adhesion of mesenchymal stem cells spread on decellularized vein scaffold**. Acta Cir Bras. 2021 Nov 5;36(10):e361001. doi: 10.1590/ACB361001. PMID: 34755757; PMCID: PMC8598214.



Quantification of adhesion of mesenchymal stem cells spread on decellularized vein scaffold

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ABSTRACT

Purpose: To evaluate methods that improve adipose-derived stem cells (ASCs) population in decellularized biological venous scaffold for tissue engineering in blood vessels, a model in rabbits.

Methods: The ASC was expanded until the third passage. Inferior vena cava (IVC) was submitted to the decellularization process using 1% sodium dodecyl sulfate (SDS) or 2% sodium deoxycholate (SD) to compose 12 study groups (G): pure SD or SDS, exposed or not to 1% TritonX-100 (TX-100) and exposed or not to poly-L-lysine and laminin (PL). Scaffolds were covered with 1×10^5 or 1×10^6 ASCs diluted in 10 μ L Puramatrix™. The histological analysis was done by cell counting in hematoxylin and eosin (HE) and nuclei count in immunofluorescence (IF) with 4',6-Diamidino-2'-phenylindole dihydrochloride (DAPI). **Results:** The study of groups in HE and IF showed similar results. For both analyses, IVC-SD- 1×10^6 ASC and IVC-SD-PL- 1×10^6 ASC provided the best results. The IF technique showed better sensitivity than HE, with a weak agreement between them. **Conclusion:** Decellularizing agent and the number of ASC influence scaffolds cellularization response and the best protocols as those ones using SD with or without the addition of PL.

Key words: Mesenchymal Stem Cells. Endothelium. Blood Vessels. Peripheral Arterial Disease.

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Conflict of interest: Nothing to declare.

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■ Introduction

The tissue engineering of blood vessels (TEBV) represents a promising perspective of vascular substitutes in revascularization surgeries, such as coronary or peripheral arterial diseases. Many strategies to optimize the mechanisms of blood vessel production were investigated, considering specific needs to meet a demand for personalized medicine, in addition to pay attention to the need for biocompatibility¹⁻³.

On the other hand, concerning the scaffolds, additional factors must be considered, such as the maintenance of a three-dimensional (3D) structure, tissue permeability, sufficient strength to resist blood pressure, elasticity, high durability, facilitation of attachment, migration, proliferation, and cell interaction with adjacent tissues. Additionally, this material should maintain the antithrombotic activity and provide a microenvironment that imitates the natural architecture of in-vivo tissue for the seeded cells³⁻⁶. The adipose tissue (AT) is an abundant source of adipose-derived stem cells (ASCs), easy to obtain in surgery. The discovery of techniques that promote tissue differentiation, with high exhibitions of plasticity, shows that the cultivation of ASCs can contribute a great deal to TEBV⁷⁻¹².

Among the alternatives to produce scaffolds, there is the use of decellularized tissue-derived scaffolds^{9,10}. The advantages of this material are related to the essential elements contained in the organic extracellular matrix, such as collagen, elastin, proteoglycans, cell adhesion proteins, glycosaminoglycans (GAGs), which are strong promoters of cellular adhesion and differentiation. Previous experimental studies conducted by this research group have demonstrated that two protocols for decellularization of rabbit's vena cava were effective in cell removal and maintenance of the biomechanical characteristics of the 3D scaffold^{13,14}.

Although many protocols for decellularization of organs and tissues are described, including the decellularization of blood vessels, with various decellularization agents and times^{15,16} for rabbit's inferior vena cava, under a rapid decellularization method, our research team's best results were obtained using 1% sodium dodecyl sulfate (SDS) and 2% sodium deoxycholate (SD)^{13,14,17-19}.

Therefore, this study aimed to evaluate strategies that improve the number of ASC in these scaffolds as a model for future use in TEBV.

■ Methods

Animal housing conditions and tissue harvesting

The inferior vena cava (IVC) and the adipose tissue (AT) were harvested from 12 non-pregnant female adult rabbits (New Zealand). After the Committee on Animal Research

and Ethics (CEUA) approval (Process no. 1279/2018), all procedures were conducted respecting the Ethical Guidelines for Animal Experimentation from Brazilian College for Animal Experimentation and were conducted following the U.S. National Institutes of Health or European Commission guidelines.

Animals were housed under controlled conditions and fed a standard pellet diet with water *ad libitum*. The median age and weight were 3 months and approximately 2.5 kg, respectively. Before the surgical procedures to obtain the AT and IVC, animals were anesthetized with tiletamine hydrochloride/zolazepam hydrochloride (20 mg/kg, i.m.) associated with 2% xylazine chloride (4 mg/kg, i.m.). The harvest was conducted under rigorous aseptic conditions, and, at the end of the procedure, animals were euthanized with pentobarbital.

Scaffold production

The veins were fragmented in three segments of 1 cm in length each (approximately 1 cm² of luminal area), totaling 36 fragments. The decellularization was performed by two protocols, with 1% sodium dodecyl sulfate (SDS) for 2 hours and 2% sodium deoxycholate (SD) for 1 hour (both under agitation in a Shaker News Brunswick Scientific®, at 37°C). The fragments were stored in a refrigerator at 4°C in a sterile solution containing antibiotics and fungicide.

An additional step of exposition to 1% TritonX-100 (TX) for 10 minutes was performed for six scaffolds of each detergent.

Six fragments were exposed to poly-l'lysine and laminin for 30 minutes (each one), and the other six were maintained without other processing steps for each detergent.

Obtaining of adipose-derived stem cells

For the obtainment of the ASC, 2 g of AT was surgically removed from the interscapular region of the same rabbits and stored in Falcon with N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) solution containing penicillin, 100 mg/mL streptomycin, and 25 mg/mL amphotericin B (2 mmol/L l-glutamine; Invitrogen™). ASCs were acquired through enzymatic dissociation with type I collagenase (Invitrogen™). Cell culture procedures were done with an initial cell count of 2×10^4 cells/cm², obtained from five adipose tissue fragments. These cells were seeded and expanded in six-well culture plates using Dulbecco's modified Eagle's (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 25 mg/mL amphotericin B (2 mmol/L l-glutamine; Invitrogen™), 1% (v/v) minimum essential medium (MEM) essential amino acids solution (Invitrogen™), and 0.5% (v/v) of 10 mM MEM nonessential amino acids solution (Invitrogen™) until reaching the number of cells needed for the experiments. The ASC were phenotypically

analyzed through flow cytometry, using CD45, CD44, CD90, and CD11b, and through differentiation techniques in trilineage (StemPro adipogenesis, chondrogenesis, and osteogenesis kits; Invitrogen).

Cell spreading in the biological scaffold

The ASCs were washed with D-PBS, diluted with the PuraMatrix[®] peptide hydrogel (BD Biosciences), and 10 μ L was pipetted in the lumens of each scaffold. The experiment was conducted in triplicate, maintained in culture for 21 days in M199 growth media supplemented with 10% SFB and growth factors to induce differentiation in the endothelium, 10 ng/mL VEGF, 50 ng/mL β FGF, 20 ng/mL, and M199 supplemented²⁰.

The experiment was composed of 12 groups, each one in triplicate, with 1 cm² of the luminal area on scaffolds:

- group 1: IVC-SDS+1 $\times 10^5$ ASC;
- group 2: IVC-SDS+1 $\times 10^6$ ASC;
- group 3: IVC-SDS-TX+1 $\times 10^5$ ASC;
- group 4: IVC-SDS-TX+1 $\times 10^6$ ASC;
- group 5: IVC-SDS+P/L+1 $\times 10^5$ ASC;
- group 6: IVC-SDS+P/L+1 $\times 10^6$ ASC;
- group 7: IVC-SD+1 $\times 10^5$ ASC;
- group 8: IVC-SD+1 $\times 10^6$ ASC;
- group 9: IVC-SD+TX+1 $\times 10^5$ ASC;
- group 10: IVC-SD-TX+1 $\times 10^6$ ASC;
- group 11: IVC-SD+P/L+1 $\times 10^5$ ASC;
- group 12: IVC-SD+P/L+1 $\times 10^6$ ASC.

These cell-seeded scaffolds were further incubated for 30 minutes at 37°C with a 5% CO₂ incubator and a culture medium, which were placed in an appropriate Petri dish (40 $\times$ 30 mm). A minimal amount (30 μ L) of M199 medium was added to the scaffold to induce the gelling of the solution.

The scaffold spread with the cells was then transported to a 24-well cell culture plate (Corning[®] Costar[®] Ultra-Low Attachment Multiple Well Plate) and completed with another 3 mL of the M199 medium supplemented. The culture plates were kept in a humidity-controlled environment, at 37°C, and 5% CO₂, with M199, increased every two days, for 21 days total.

Analysis of scaffolding cell colonization

After the conclusion of the experimental culture, the fragments were collected and sectioned into two parts, one of them was cryopreserved in liquid nitrogen to obtain fresh histological slides to undergo immunofluorescence with 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI), and the other one was paraffinized for slide preparation and stained in hematoxylin and eosin (HE).

For cell counting in HE, the slides were analyzed under optical microscopy at x100. Each group consisted

of triplicates, three slides out of each. All viable cells were counted in five fields in a x100 optical microscope (allowing the count of all cells in fragments), totalizing 15 analysis per group. A histological slide of the freshly frozen material was cut in a cryostat and proceeded to specific immunofluorescence with DAPI to mark the nucleic acid (nuclear) of the ASCs (in blue). A statistical test of comparison and agreement for cell counts by the two forms of histological analysis was performed to ascertain whether processing methods could interfere with cell count analysis.

Statistical analysis

Statistical analysis of cell counting was done using a generalized linear model (GLM) with Poisson's distribution and a log link function, followed by Wald's chi-square test for multiple comparisons.

The Mann-Whitney test was used to perform a comparative analysis between the global cell count determined by each of the two methods of histological analysis. Continuous numerical data are expressed as median (range) according to the data distribution as determined by the Kolmogorov-Smirnov test for normality. The agreement between the cell count determined by the methods of histological analysis was evaluated using interclass coefficient correlation for two-way random effects. For statistical analysis, significant values were considered when $p < 0.05$ (Software IBM SPSS statistics version 1.0.0.1406).

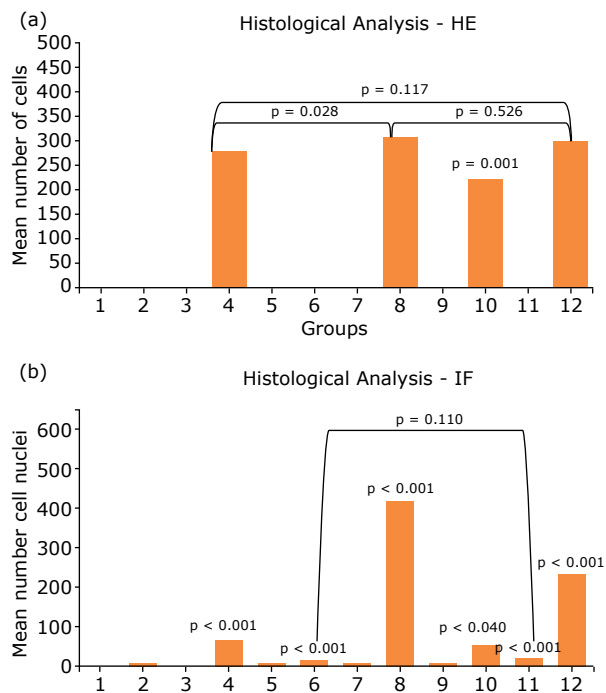
■ Results

The culture of ASC, their characterization, and the obtaining of scaffolds were performed as previously described in methods.

Histological analysis

Hematoxylin & eosin cell counting

Given the histological analysis stained with hematoxylin and eosin (HE), the cell counting showed that, by the GLM, there was a significant difference between the groups ($p < 0.001$), and, through the Wald chi-square test for multiple comparisons, the groups with the best cell adhesion were: group 8 (IVC-SD-1 $\times 10^6$ ASC) and group 12 (IVC-SD-P/L-1 $\times 10^6$ ASC), without a statistic difference between them ($p = 0.526$), followed by group 4 (IVC-SDS-TX-1 $\times 10^6$ ASC), which showed statistical lower results than group 8 (SD-1 $\times 10^6$ ASC) ($p = 0.028$) and showed no statistical difference to group 12 (IVC-SD-P/L-1 $\times 10^6$ ASC) ($p = 0.117$), followed by group 10 (IVC-SD-TX-1 $\times 10^6$ ASC), which was less than the previous three ($p < 0.001$). The other groups did not present significant cells, considered null results, and differed significantly from the other groups ($p < 0.001$). Figures 1 (a) and 2 show these results in detail.



HE: hematoxylin and eosin; IF: immunofluorescence; DAPI: 4',6-Diamidino-2'-phenylindole dihydrochloride.

Figure 1 - Analysis of histological methods. **(a)** Analysis of cell count in HE slides; **(b)** Analysis of nuclei cell count in immunofluorescence stained with DAPI.

Immunofluorescence DAPI cell nuclei counting

Given the histological analysis by immunofluorescence (IF), the cellular nuclei counting showed that, by the GLM, there was a significant difference between the groups ($p < 0.001$), and, through the Wald chi-square test for multiple comparisons, the groups with better cell adhesion were: group 8 IVC-SD-1 $\times 10^6$ ASC ($p < 0.001$), followed by group 12 IVC-SD-P/L-1 $\times 10^6$ ASC ($p < 0.001$), then by group 4 IVC-SDS-TX-1 $\times 10^6$ ASC, which showed a statistical difference for the next group ($p = 0.04$), that was group 10 IVC-SD-TX-1 $\times 10^6$ ASC. These groups were superior that group 6 IVC-SDS+P/L+1 $\times 10^6$ ASC and group 11 IVC-SD+P/L+1 $\times 10^5$ ASC ($p < 0.001$), but without differences between them ($p = 0.110$). The remaining groups showed an insignificant number of cells nuclei (mean less than eight per repetition). Figures 1 (b) and 3 show these results.

Comparative and concordance analysis of cell counts by the two histological methods

The global cell count determined by IF was higher than the number picked by HE $10.5 (0/820) \times 0 (0/640)$; $p = 0.002$. The interclass correlation coefficient was 0.315, which represents fair agreement.

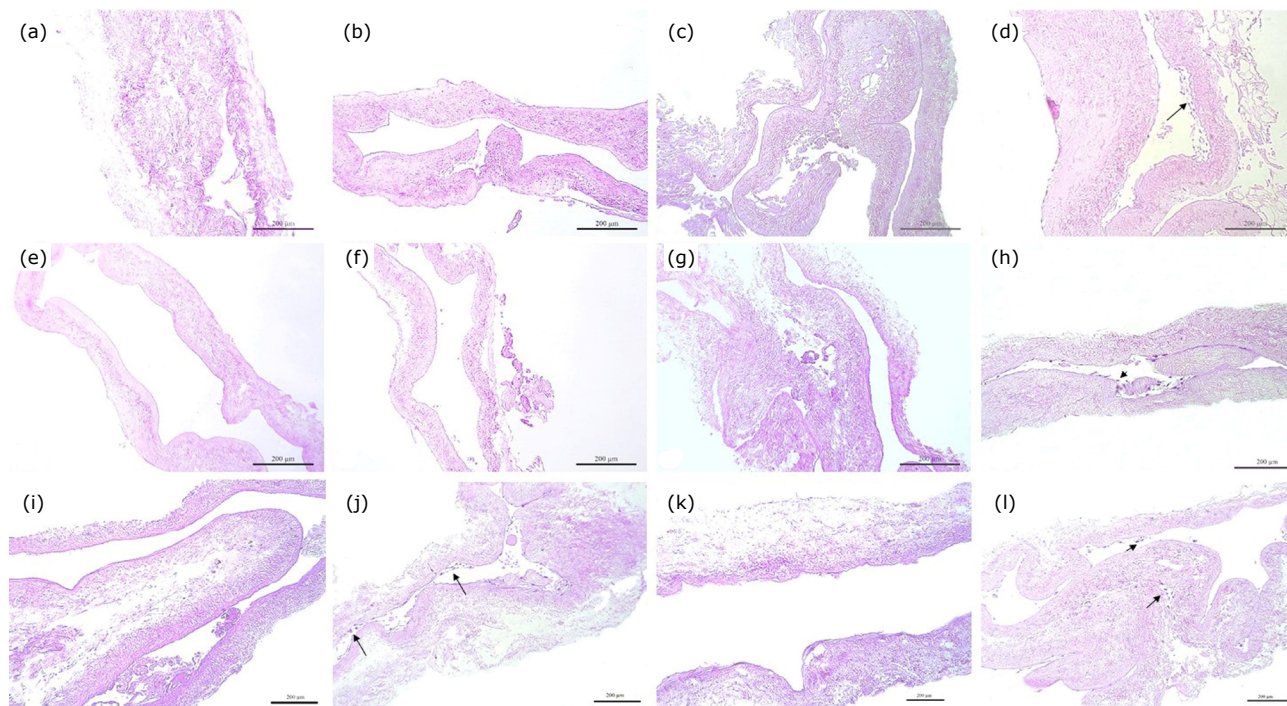
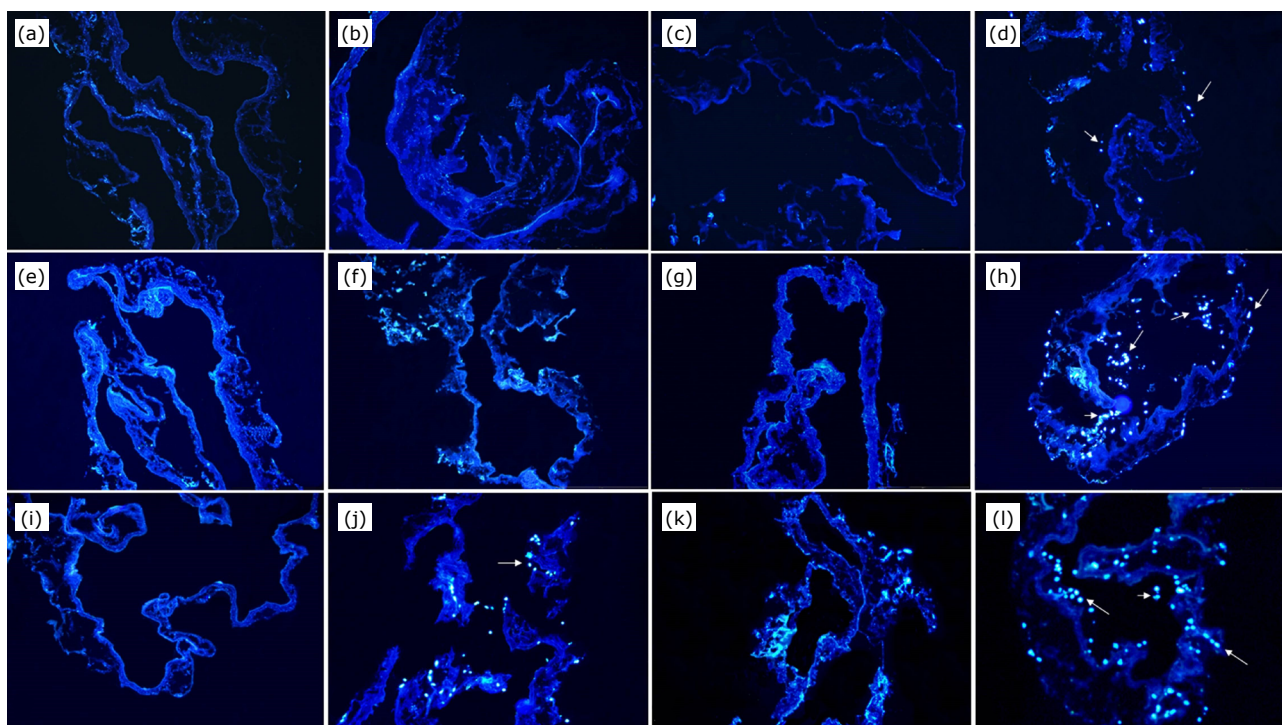


Figure 2 - Analysis of cell count (arrows) in hematoxylin and eosin (HE) slides of the groups: **(a)** Group 1 IVC-SDS+1 $\times 10^5$ ASC; **(b)** Group 2 IVC-SDS+1 $\times 10^6$ ASC; **(c)** Group 3 IVC-SDS-TX+1 $\times 10^5$ ASC; **(d)** Group 4 IVC-SDS-TX+1 $\times 10^6$ ASC; **(e)** Group 5 IVC-SDS+P/L+1 $\times 10^5$ ASC; **(f)** Group 6 IVC-SDS+P/L+1 $\times 10^6$ ASC; **(g)** Group 7 IVC-SD+1 $\times 10^5$ ASC; **(h)** Group 8 IVC-SD+1 $\times 10^6$ ASC; **(i)** Group 9 IVC-SD+TX+1 $\times 10^5$ ASC; **(j)** Group 10 IVC-SD-TX+1 $\times 10^6$ ASC; **(k)** Group 11 IVC-SD+P/L+1 $\times 10^5$ ASC; **(l)** Group 12 IVC-SD+P/L+1 $\times 10^6$ ASC.



DAPI: 4',6-Diamidine-2'-phenylindole dihydrochloride.

Figure 3 - Analysis of nuclei cell count (arrows) in immunofluorescence stained with DAPI slides of the groups: **(a)** Group 1 IVC-SDS+ 1×10^5 ASC; **(b)** Group 2 IVC-SDS+ 1×10^6 ASC; **(c)** Group 3 IVC-SDS-TX+ 1×10^5 ASC; **(d)** Group 4 IVC-SDS-TX+ 1×10^6 ASC; **(e)** Group 5 IVC-SDS+P/L+ 1×10^5 ASC; **(f)** Group 6 IVC-SDS+P/L+ 1×10^6 ASC; **(g)** Group 7 IVC-SD+ 1×10^5 ASC; **(h)** Group 8 IVC-SD+ 1×10^6 ASC; **(i)** Group 9 IVC-SD+TX+ 1×10^5 ASC; **(j)** Group 10 IVC-SD+TX+ 1×10^6 ASC; **(k)** Group 11 IVC-SD+P/L+ 1×10^5 ASC; **(l)** Group 12 IVC-SD+P/L+ 1×10^6 ASC.

Discussion

Biological scaffolds are a great source of biomaterials, with advantages such as hydrophilicity, low toxicity, and low immunogenicity, in addition to good adhesion and cell multiplication. Therefore, when using biological scaffolding, the native architecture is highly preserved, and the antigen content was removed during the decellularization process¹⁷.

The scientific literature presents several decellularizing agents and different methods. However, we seek a practical protocol, which has high efficiency and speed, but not only that, one that promotes an enhanced environment for cell adhesion and colonization, specifically for TEBV^{13,14,16,17-19,21-26}.

Our team's previous experimental studies demonstrated that rabbit's vena cava could be decellularized while maintaining an excellent structural matrix, therefore serving as a promising scaffold to receive the ASC derived from adipose tissue¹⁴. It was also determined that the process of decellularization of the vein does not

cause significant residual toxicity or loss of essential characteristics of the extracellular matrix¹⁴. However, cell adhesion and proliferation did not show regularity during the several repetitions of the method, mainly regarding the number of cells. Thus, this experiment was designed to check the possible variables that would lead to the best results: the number of cells, the type of decellularizing agent, or the addition of TritonX-100 or poly-L-lysine + laminin.

Published studies demonstrated that, for a suitable recellularization of a vascular scaffold, it is required at least 1×10^6 cells per cm^2 and at least two weeks of culture, which was also observed in our experiments with vein scaffolds seeded with 1×10^6 ASC²¹. Studies concluded that the decellularization of veins by the SDS protocol has a well-preserved extracellular matrix, membrane structure, and sufficient strength for a vascular graft²²⁻²⁴. On the other hand, Zhou *et al.*²⁵ stated that decellularization with SDS could be harmful to structural and signaling proteins, such as the collagen of some cardiac tissues that remain damaged, despite successfully

removing cells. According to our best protocols, SD was observed to be more favorable to seeded cells. Still, the TritonX-100 helped SDS protocol and increased its efficacy, assuming the third position between them^{16,24,26}. The decellularization of tissues with SD eliminates cells, preserving the extracellular matrix, and in this study it presented advantages in cellular interaction, thus making the protocol promising to produce human blood vessel scaffolds for application in TEBV.

Previous studies that used TritonX-100, an anionic detergent, to enhance the decellularizing action of other enzymatic detergents, such as SDS and SD, obtained better results on removing cells from thicker tissues, such as heart valves, in which the enzymatic or osmotic methods alone were ineffective. This association can be beneficial, because it can decrease the loss of extracellular matrix protein, balance repulsive negative ionic charges in the cells, and concomitantly decrease adverse immune response *in vivo*^{14,18,25,26}. However, ionic detergents (SDS and SD) can solubilize lipids, cytoplasmic membranes, and protein denaturation. Still, some studies point to collagen denaturation and the difficulty of removing the remaining matrix, which negatively influences the scaffolds' cytocompatibility¹⁶. This study suggested the best result of recellularization in scaffolds produced by SD protocols.

Poly-l'lysine is commonly used in cell and tissue culture as a fixation factor to enhance cell adhesion through the interaction of the polymer (positive charge) and cells/protein (negative control)^{16,27}. Laminin is a glycoprotein of fundamental importance in helping differentiation, migration, and cell adhesion, acting as a source of protein network, and organizing the extracellular matrix's formation. Our experiment with SD in association with poly-l'lysine and laminin did not improve the results, characterizing that this protocol does not need any additives to increase cell adhesion^{16,27}.

The comparison between the histological methods with fixation process for making paraffinized blocks and cuts for conventional HE histology was inferior in the detection of the sown cells compared to the method that used fresh frozen material and immunofluorescence, since the cells adhered in the lumen of the scaffolds (still immature and with little extracellular fixation matrix) are more sensitive to the physical processing required to produce paraffinized blocks. Therefore, the method of immunofluorescence with DAPI (with freshly frozen material) showed greater sensitivity in identifying cells, with a small methodological agreement after the statistical tests, following the scientific literature²⁸. Thus, for early cell adhesion assays, it is suggested that this method may be advantageous concerning conventional histology for identifying cells.

■ Conclusions

This experiment demonstrates that the decellularizing agent and the number of ASC seeded influence the recellularization response of vena cava scaffolds. In this way, it was possible to identify the best protocols that used SD with or without the addition of poly-l'lysine and laminin. In the sequence, it was observed that the addition of TritonX-100 to the SDS protocol could improve cellular adhesion. Fundamentally, the number of cells applied influenced all protocols progressively. New experiments should be carried out to evaluate the in-vivo behavior of these bioengineered products to determine their functionality.

■ Author's contribution

Conception of the study: Rodrigues LS and Bertanha M; **Acquisition and interpretation of data:** Rodrigues LS, Bovolato ALC, Silva BE, Chizzolini LV, Cruz BL, Moraes MPT, Lourenção PLTA and Bertanha M; **Statistical analysis:** Lourenção PLTA; **Manuscript preparation and writing:** Rodrigues LS, Bovolato ALC, Silva BE, Chizzolini LV, Cruz BL, Moraes MPT, Lourenção PLTA and Bertanha M; **Critical revision:** Rodrigues LS, Bovolato ALC, Silva BE, Chizzolini LV, Cruz BL, Moraes MPT, Lourenção PLTA and Bertanha M.

■ Data availability statement

Data will be available upon request.

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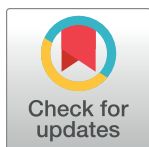
Anexo 5

Gaiolla RD, **Moraes MPT**, de Oliveira DE. **SNPs in genes encoding for IL-10, TNF- α , and NF κ B p105/p50 are associated with clinical prognostic factors for patients with Hodgkin lymphoma.** PLoS One. 2021 Mar 8;16(3):e0248259. doi: 10.1371/journal.pone.0248259. PMID: 33684151; PMCID: PMC7939322.

RESEARCH ARTICLE

SNPs in genes encoding for IL-10, TNF- α , and NF κ B p105/p50 are associated with clinical prognostic factors for patients with Hodgkin lymphoma

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Abstract

Classical Hodgkin lymphoma (cHL) is a B-cell-derived malignant neoplasia that has a unique histological distribution, in which the scarce malignant Hodgkin and Reed-Sternberg cells are surrounded by nonmalignant inflammatory cells. The interactions between the malignant and inflammatory cells are mediated by aberrantly produced cytokines, which play an important role in tumor immunopathogenesis. Single nucleotide polymorphisms (SNPs) in genes encoding cytokines and their regulatory proteins may influence the peripheral levels of these molecules and affect disease's pathobiology. In this study, we evaluate SNPs in the promoter regions of the genes encoding for two key cytokines in Hodgkin lymphoma: IL-10 (SNP/pIL10-592, rs1800872; and SNP/pIL10-1082, rs1800896) and TNF- α (SNP/pTNF-238, rs361525; and SNP/pTNF-862, rs1800630), as well as an SNP in the intronic region of the NF κ B1 gene (SNP/iNF κ B1, rs1585215), an important regulator of cytokine gene expression. We then look to their possible association with clinical and laboratory features in cHL patients. Seventy-three patients with cHL are genotyped by qPCR-high resolution melting. The SNPs' genotypes are analyzed individually for each SNP, and when more than two allelic combinations are identified, the genotypes are also divided into two groups according to proposed biological relevance. By univariate analysis, patients harboring SNP/pTNF-238 AG genotype more frequently have EBV-associated cHL compared to homozygous GG, whereas the presence of mediastinal disease (bulky and nonbulky) is more common in the pIL10-592 AC/CC group compared to the AA homozygous group. Patients with SNP/iNF κ B1 AA genotype more frequently have stage IV and extranodal disease at diagnosis. These results indicate that some SNPs' genotypes for IL-10 and TNF- α genes are associated with prognostic parameters in cHL. For the first time, the SNP/iNF κ B1 is described in association with clinical features of the disease.

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Introduction

Hodgkin lymphoma (HL) is a B-cell-derived malignant neoplasia that accounts for approximately 10% of all human lymphomas. The disease has a unique histological appearance in which the neoplastic component—the Hodgkin and Reed-Sternberg cells (H-RS)—is scarce, and tumors are mostly formed by nonmalignant inflammatory cells [1, 2]. Cure rates for the classical form of HL (cHL) reach 90% with modern treatment protocols based on chemo and radiotherapy [3–7].

Although prognostic models based on clinical and laboratory parameters for cHL are useful in clinical practice [8–10], they may not consider the disease pathobiology, which may account for their unsatisfactory performance for treatment stratification. For long-term cHL survivors, the high frequency of treatment-related late toxicity is often observed during clinical follow-up [11, 12]; furthermore, a considerable number of cHL cases relapse, including some of those initially classified as low risk [13]. Thus, data on new putative prognostic factors to accurately predict cHL behavior and outcomes are of major interest in the clinical setting.

The interactions between H-RS and inflammatory cells are mediated by cytokines, which play an important role in cHL immunopathogenesis [14, 15]. Several cytokines and chemokines have been studied in HL, but only a few are related to clinical behavior and disease features. It is worth noting that interleukin 10 (IL-10) and tumor necrosis factor alpha (TNF- α) are of great importance because they are considered major players in the regulation of inflammatory and immune responses, both known as key phenomena in the biology of HL. For instance, H-RS cells express IL-10 [16], which suppresses cytotoxic Th1 immune response [17] and contributes to the extended life span of H-RS cells [18, 19]. They also express TNF- α [20, 21], which stimulates the phagocytic and microbicidal function of macrophages and regulates the production of several other proinflammatory cytokines [22]. Elevated circulating levels of both IL-10 and TNF- α in patients with cHL were previously associated with a more aggressive disease behavior and poorer outcomes [23–28]. In 2018, His et al. reported that the IL-10 serum levels at the end of therapy distinguished patients with shorter progression-free survival and overall survival, suggesting a potential role for this parameter in identifying patients at higher risk for relapse after the initial risk-adapted therapy [29].

Single nucleotide polymorphisms (SNPs) are genetic variations that may influence the peripheral levels of cytokines, including IL-10 and TNF- α [30–33]. The cytokine production is also modulated by intracellular signaling pathways, and even their activity is affected by SNPs in their respective genes. Notably, the nuclear factor kappa B (NF κ B) pathway transcriptionally regulates numerous cytokine genes, and its abnormal activation is highly common in lymphomagenesis, including for cHL [34, 35]. The NF κ B signaling is also hijacked by the Epstein-Barr virus (EBV), which is a key event in the pathogenesis of EBV-associated cHL [36].

The NF κ B family of transcription factors includes the p65 (RelA), RelB, c-Rel, p50/p105, and p52/p100 proteins, which act as either homo- or heterodimers in mammalian cells [37]. Genetic variations in *NFKB1*, which encodes p50, the most common subunit for the NF κ B transcription factor complex, were previously associated with increased risk for HL [38]. Other associations such as higher risk for infectious conditions [39], autoimmune diseases [40], and cancers [41, 42] have also been described.

The impact of gene polymorphisms on the pathobiology and prognosis of cHL is currently a disputed topic because of the insufficient or conflicting data published. Thus, this study is aimed at evaluating possible associations of SNPs for the promoter regions of the genes encoding for IL-10 and TNF- α , as well as one SNP within the *NFKB1* gene intronic region, identified in a series of cHL patients and non-cHL controls. The results are then evaluated considering

putative associations of the SNPs' genotypes found with histologic subtypes of cHL, EBV infection status, and well-established prognostic parameters for cHL.

Materials and methods

Subjects

The study retrospectively included living patients with a diagnosis of HL between January 01, 1999 and November 30, 2010 who were treated at Botucatu Medical School's Academic Hospital. From December 1st 2010 to December 31st 2014, consecutive patients with HL diagnosis were included prospectively. Eligible patients were aged 16 years or older at the moment of HL diagnosis.

Baseline features at diagnosis (e.g., age, gender, stage, presence of bulky mediastinal mass, extranodal disease, number of involved nodal areas, and several laboratory parameters) were retrieved from electronic medical records for all cHL cases. The cHL disease stage was defined according to the Ann-Arbor classification [43], and treatment was defined according to cHL-standardized institutional chemotherapeutic protocols. By the time the enrolled patients were treated, positron emission tomography-computed tomography (PET-CT) was not available in our institution; thus, a conventional CT scan was used for imaging evaluation instead.

All cHL patients were classified according to the International Prognostic Score (IPS) [8] and the German Hodgkin Study Group (GHSG) risk group score [44]. The IPS takes into account seven risk factors associated with disease outcomes (e.g., progression-free survival and overall survival), each of them with similar and independent prognostic effect: male sex, age of 45 years or older, stage IV disease, serum albumin levels less than 4 g/dL, hemoglobin level less than 10.5 g/dL, leukocytosis with more than 15,000/mm³ white-cells count, and lymphocytopenia (lymphocytes of less than 600/mm³ of blood). Patients with up to two risk factors were classified as low-risk and those with three or more risk factors as high-risk cHL. The GHSG is largely used to guide treatment decisions for the best strategy, considering: Ann Arbor stage, number of involved nodal sites, erythrocyte sedimentation rate, presence of bulky mediastinal mass, and involvement of extranodal sites. According to the GHSG score, the cHL cases are classified as early disease stage with favorable prognosis, early disease stage with unfavorable prognostic features, or advanced disease stage. For the purpose of the statistical analysis in this study, the cHL cases were dichotomously divided into the group of patients with advanced disease and those with nonadvanced cHL (early stages of favorable and unfavorable disease).

For inferences regarding the frequencies and distribution of SNP genotypes, blood-donor volunteers matched to cHL patients according to age, gender, and skin color (as an ethnicity proxy) were also recruited to generate a non-cHL control group.

This study was approved by the Ethical Committee on Research at the Medical School of Botucatu, Sao Paulo State University (UNESP), SP, Brazil (Protocol #523/10), and informed consent was obtained for all subjects before enrollment.

Histopathology, immunophenotype, and EBV infection status

All tumor specimens were reviewed by an experienced pathologist using tissue microarray slides (58/73 cases; 79,5%), produced as previously described [45], or archived stained tissue slides (15/73 cases; 20,5%). Unstained tumor sections were used for immunohistochemistry (IHC) with anti-CD45 (clone DD7/26, 1:250), anti-CD20 (clone L26, 1:700), anti-CD30 (clone Ber-H2, 1:100), anti-CD15 (clone C3D-1, 1:150), and anti-CD3 (clone 565; 1:800) antibodies. The EBV infection status was assessed by *in situ* hybridization against EBV-encoded RNA 1 (EBER-1), as reported elsewhere [46], and/or by IHC with anti-EBV latency membrane

protein-1 (LMP-1) (CS1-4, 1:100). All antibodies used were manufactured by Dako Cytomation (Carpinteria, CA, USA).

DNA extraction

Conventional venipuncture-drawn blood samples were obtained from all participants using 4mL BD Vacutainer™ EDTA tubes (Beckson-Dickson, Rutherford, NJ, USA). Mononuclear cells were isolated with *Histopaque*™ (Sigma-Aldrich, St. Louis, MO, USA) after centrifugation at 400xg for 30min. DNA extraction was performed using the *QIAamp DNA Blood Mini Kit*™ (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The DNA was eluted, quantified with the NanoVue™ device (GE Healthcare, Buckinghamshire, UK), and kept at -20°C until use.

SNPs genotyping

The target genes and SNPs evaluated in this study were selected based on previous data in the literature about their putative role in cHL pathogenesis. This included SNPs in the promoter regions of the *IL10* gene (NCBI's GeneID: 3586) at positions -592 (rs1800872; SNP/p*IL10*-592, C>A) and -1082 (rs1800896; SNP/p*IL10*-1082, A>G); in the promoter regions of the *TNF* gene (NCBI's GeneID: 7124) at positions -238 (rs361525; SNP/p*TNF* -238, G>A) and -862 (rs1800630; SNP/p*TNF* -862, C>A); and in the intronic region of *NFKB1* gene (NCBI's GeneID: 4790, rs1585215; SNP/i*NFKB1*, A>G). Briefly, DNA samples were normalized to 20ng/μL and amplified by real-time polymerase chain reaction (qPCR)-high resolution melting (HRM) using the Rotor-Gene™ 6000 equipment (Corbett Research, Sydney, Australia). qPCR primers were obtained from literature for SNPs/p*IL10*-592 and -1082 [47] and for SNPs/p*TNF* -238 and -862 [48, 49], while primers to evaluate the SNP/i*NFKB1* were designed by the authors (RDG and DEO) [50]. The oligonucleotide sequences and amplicon sizes are provided in [S1 Table](#). qPCR assays conditions are provided in [S2 Table](#).

The genotyping analysis was performed by comparing melting curves for the cHL case samples and controls for each SNP genotype under consideration, previously validated by conventional DNA sequencing (Sanger's method). Both HRM and conventional melting curves were used to improve the discrimination of the SNPs genotypes, and all samples were evaluated with technical duplicates.

Genotype-to-phenotype inferences

To subsidize the analysis of possible biological effects of the investigated SNPs in patients with cHL, we searched for public datasets that could present information about the expression levels of the genes encoding for IL-10, TNF-α, and NFκB in other cohorts of cHL cases. To the best of our efforts, by October 2020 no suitable datasets were found using the NCBI GEO (<https://www.ncbi.nlm.nih.gov/sites/GDS>; search query available via <https://bit.ly/3lRAafY>), ExSNP (<http://www.exsnp.org/>; last updated in 2014) [51], GWASdb v2 (<http://jjwanglab.org/gwasdb>; last updated in 2015) [52], CausalDb (<http://mulinlab.org/causaldb>) [53], and Enrichr via Harmonizome (<https://maayanlab.cloud/Harmonizome/>) [54]. Then, we performed a metasearch using the PhenoScanner v2 tool [55] to prospect the effects of the evaluated SNPs in the expression of their correspondent gene or related ones, querying their respective Reference SNP (rs) codes (rs1800872 for SNP/p*IL10*-592, C>A; rs1800896 for SNP/p*IL10*-1082, A>G; rs361525 for SNP/p*TNF* -238, G>A; rs1800630 for SNP/p*TNF* -862, C>A; and rs1585215 for SNP/i*NFKB1*, A>G) and investigating the significant associations found in the retrieved data.

Statistical analysis

The group of cHL cases and its matched control group were compared for gender and age distribution of their subjects using the chi-square and Mann-Whitney tests. The Hardy-Weinberg equilibrium was assessed using the R software with SNPAssoc package v1.9–2 [56].

The SNP genotypes were analyzed individually for each SNP. When more than two allelic combinations were identified, the genotypes were also divided into two groups according to proposed biological relevance described previously [32, 33, 57], as follows: for SNP/p*TNF* -238, GG vs AG/AA; for SNP/p*TNF* -862, CC vs AC/AA; for SNP/p*IL10*-592, AA vs AC/CC; for SNP/p*IL10*-1082, AA vs AG/GG; and for SNP/i*NFKB1*, AA vs AG/GG.

For the cHL patients, Chi-square or Fisher's exact tests were used to assess the association among SNPs/p*TNF*, SNPs/p*IL10*, and SNPs/i*NFKB1* genotypes with dichotomized clinical and laboratory variables, and the risk category, based on IPS and GHSG scores. For the univariate positive associations ($P \leq 0.05$) among genotypes and clinical or laboratory variables, a multiple logistic regression model was carried out, adjusted by known relevant prognostic factors previously described in the literature (age, clinical stage, and histological subtype).

The outcome measures were progression-free survival (PFS) and overall survival (OS). According to defined international standardization [43], PFS was defined as the time from the beginning of treatment until progression or death from any cause, while overall survival was calculated from the beginning of treatment until death from any cause. Data from patients that were alive and did not progress were censored at the time of the last visit. Survival curves were generated using the Kaplan-Meier method, and differences in PFS and OS were tested for significance by log-rank test. For survival analysis, all genotypes were grouped as indicated above.

The statistical analysis was performed using the SAS Software version 9.2 (SAS Institute Inc., Cary, NC, USA). Differences were considered significant when $p \leq 0.05$.

Results

Features of cHL patients

Eighty patients with a confirmed diagnosis cHL were originally recruited. However, seven were excluded because they were younger than 16 years old at diagnosis ($n = 5$) or due to lack of paraffin-embedded tissues for histopathological review ($n = 2$). The main features of the 73 cHL cases evaluated are presented in Table 1.

The median age of patients was 28 years (range 16–73); 42 (57.5%) patients were male, and the male/female ratio was 1.4:1. By the time of cHL diagnosis, most of the patients (42/73; 57.5%) had disease at stage I or II, 43 (58.9%) presented with B symptoms, and all except one patient had ECOG performance status ≤ 2 . After the histopathological review, 53 (72.6%) of the cHL cases were classified as nodular sclerosis and 10 (13.8%) as mixed-cellularity subtypes; the remaining cases were either lymphocyte-rich (1/73; 1.4%) or cHL unclassified (9/73; 12.3%). The EBV infection was confirmed in 21/61 (34.4%) cases. Furthermore, most cHL cases were considered as advanced disease by both IPS and GHSG scoring systems. As expected, no differences were observed regarding age and sex distribution comparing the cHL group and the control group, which comprised 73 matched non-cHL blood donor volunteers.

The majority of patients (69/73; 94.5%) was treated with doxorubicin, bleomycin, vinblastine, and dacarbazine (ABVD regimen); 2 (2.7%) patients were treated with bleomycin, etoposide, doxorubicin cyclophosphamide, vincristine, procarbazine, and prednisone (BEACOPP baseline regimen), and 2 (2.7%) with a hybrid protocol with mechlorethamine, vincristine,

Table 1. Main characteristics of cHL patients.

Features	N (%)
Gender	
Males	42 (57,5)
Females	31 (42,5)
Age	
16–44 years	60 (82,2)
≥45 years	13 (17,8)
ECOG performance ≤2	72 (98,6)
Ann-Arbor stage I/II	42 (57,5)
B symptoms	43 (58,9)
Histology subtype	
Nodular sclerosis	53 (72,6)
Mixed cellularity	10 (13,8)
Lymphocyte-rich	1 (1,3)
cHL unclassified	9 (12,3)
Bulky mediastinum mass (>10cm)	16 (21,9)
Nodal areas ≥3	33 (45,2)
Extranodal disease*	14 (19,1)
BM involvement	10 (13,7)
IPS	
0–2	26 (36)
>2	47 (64)
GHSG score	
Early favorable	4 (5)
Early unfavorable	8 (11)
Advanced	45 (62)
Missing	16 (22)
EBV infection	21/61 (34,4)

BM: bone marrow; ECOG: Eastern Cooperative Oncology Group; IPS: International prognostic score; EBV: Epstein Barr virus;

*Extranodal involvement included lung, bone, breast, palatus, adrenal gland, liver, and colon. In one patient the extranodal involvement was the only site of disease.

Fractions represent number of patients with available data.

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procarbazine, prednisone, doxorubicin, bleomycin, and vinblastine (MOPP-ABV). Involved field radiotherapy (RT) was performed as a consolidation treatment for 42 (57.5%) patients. Complete response (CR) and partial response to first-line treatment were observed in 94.5% and 3.5% of cases, respectively. Disease relapse was observed in 21.7% of patients in previous CR, and 46.1% of the relapsed patients received consolidation therapy with autologous stem-cell transplantation.

The median clinical follow-up since diagnosis was 142 months (range 16–252 months). At the time of the final analysis, 60 (82.2%) patients were alive (including 5 out of 6 transplanted). Thirteen patients (17.8%) had died: 7 (53.8%) as a result of disease progression, 3 (23%) due to infection, 1 (7.7%) due to lung cancer, and 2 (15.4%) from other reasons. The estimated PFS and OS for the studied population was 14.4 years (95% CI, 12.6–16.1) and 17.7 years (95% CI, 16.2–19.3), respectively.

Table 2. Genotype distribution of SNP/p*TNF* -238 and -862, SNP/p*IL10*-592 and -1082, and SNP/i*NFKB1* in cHL patients and controls.

Polymorphism	Genotype	Patients		Controls		P
		N	%	N	%	
<i>TNF</i> -238 (rs361525)	GG	64	87,7	68	93,2	0,25
	AG	9	12,3	5	6,8	
<i>TNF</i> -862 (rs1800630)	AA	8	11	7	9,6	0,3
	CA	29	39,7	21	28,8	
	CC	36	49,3	45	61,6	
<i>IL</i> -10-592 (rs1800872)	AA	8	11	8	11	0,98
	CA	34	46,6	33	45,2	
	CC	31	42,4	32	43,8	
<i>IL</i> 10-1082 (rs1800896)	AA	34	46,6	28	38,4	0,04
	GA	37	50,7	35	47,9	
	GG	2	2,7	10	13,7	
<i>NFKB1</i> (rs1585215)	AA	48	65,8	43	58,9	0,49
	AG	25	34,2	29	39,7	
	GG	0	0	1	1,4	

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Genotype analysis and associations with patient characteristics

There was no difference in the distribution of genotypes between the cHL group and control group for all the SNPs analyzed, except for the homozygous GG of SNP/p*IL10*-1082, which was observed less frequently in the cHL group. Genotype distribution for all the investigated polymorphisms was on the Hardy–Weinberg equilibrium. SNP/p*TNF* -238 AA and SNP/i*NFKB1* GG genotypes were not found in the cHL group, but the latter was found in one subject in the control group (Table 2).

The comparisons between SNPs' genotypes and patients' characteristics are detailed in S3 Table. The studied SNPs' genotypes were not associated with specific clinical staging (I/II versus III/IV), histological subtype, or age. Univariate analysis of SNPs/p*TNF* genotypes revealed that the AG carriers of p*TNF* -238 were more frequently associated with EBV infection when compared to homozygous GG ($p = 0,04$; Fisher's exact test). No other significant associations with dichotomized variables were observed with respect to SNPs/p*TNF*. In the multivariate analysis, the association of SNP/p*TNF* -238 GG with EBV was maintained when adjusted by age, histological subtype, and clinical stage (OR: 7.73; CI 95% 1.23–48.5) ($P = 0.029$).

Comparisons of SNPs/p*IL10* grouped genotypes of SNPs/p*IL10* with categorical variables revealed that presence of mediastinal disease (bulky and nonbulky) was more common in the p*IL10*-592 AC/CC group compared to the AA homozygous group ($p = 0,002$; Fisher's exact test). In multivariate analysis, this association remains significant when adjusted for clinical stage, histological subtype, and age (OR: 21; CI 95% 2.24–197.2) ($P = 0.008$). No differences between these groups were observed when only bulky mediastinal mass was considered. No other significant associations were observed for the remaining studied variables and SNPs/p*IL10*.

In respect to SNP/i*NFKB1*, univariate analysis showed that the AA genotype was more frequently associated with extranodal disease when compared to AG carriers. In the multivariate analysis this association was maintained when adjusted by age, and duration of symptoms (OR: 0.23; CI 95% 0.06–0.93) ($P = 0.04$). When clinical stages were analyzed ungrouped, SNP/i*NFKB1* AA patients presented more frequently with stage IV disease at diagnosis ($p = 0,02$; chi-square test), but this finding was not sustained in the multivariate analysis (OR: 0.23; CI 95% 0.04–1.26) ($P = 0.09$) when adjusted by age, gender, and duration of symptoms.

In summary, SNPs/p*TNF* AG and SNP/i*NFKB1* AA genotypes were significantly associated with important known prognostic factors in HL (EBV infection and extranodal disease, respectively), both in univariate and in multivariate analysis.

Regarding clinical outcomes, no differences in PFS and OS were observed for SNP/p*TNF* -238 GG versus AG/AA; for SNP/p*TNF* -862 CC versus AC/AA; for SNP/p*IL10*-592 AA versus AC/CC; for SNP/p*IL10*-1082 AA versus AG/GG; and for SNP/i*NFKB1* AA versus AG/GG (S1–S3 Figs).

Genotype analysis and associations with patient characteristics

To explore the possible impact of SNP/p*IL10*-592 and -1082, SNP/p*TNF* -238 and -862, and SNP/i*NFKB1* on gene expression, we searched the PhenoScanner v2 database [55] to identify datasets from previous studies in which authors identified any significant biological effect documented. The results from different datasets indicate that all SNPs evaluated regulate their own gene and/or a related one (e.g., LTA, encoding TNF- β , in the case of SNP/p*TNFs*), among others identified (S4 Table).

Discussion

Several scholars have reported that SNPs in cytokine genes may be associated with changes in the risk for developing HL [38, 58, 59], but their role in cHL biology and impact on the outcome is controversial.

Although high serum levels of TNF- α were associated with clinical features and poorer prognosis in cHL [28], data on the role of SNPs in the TNF gene in cHL are scarce. In this study, a significant association of the SNP/p*TNF* -238 AG genotype with EBV-associated cHL was found. Considering that EBV is more frequently observed in older cHL patients and MC subtype [60], the multivariate analysis was adjusted for age and histologic subtype. Previously, it was suggested that these genotypes are associated with decreased transcriptional activity because they probably affect the binding of nuclear proteins to the promoter region of the gene [32, 33]. In cHL, TNF- α is mainly produced by H-RS, and it plays a significant role in the modulation of T-cell response by recruiting Th1 cells into the tumor microenvironment and regulating the production of other proinflammatory cytokines [15]. Because Th1 response is required against EBV infection [61], it is plausible that the presence of SNPs that impair TNF- α production might adversely affect the viral clearance, allowing survival of EBV-positive H-RS malignant cells.

The prognostic value of IL-10 serum levels in HL has been previously investigated [23–26, 62]. IL-10 circulating levels seem to be influenced by SNPs/p*IL10* at positions -592 and -1082, with an impact on prognosis [31]. Here, we found that the AA genotype at SNP/p*IL10*-592 was strongly associated with less frequent mediastinal involvement (bulky and nonbulky disease). The multivariate analysis was adjusted by the histologic subtype and clinical stage, which are closely related to the pattern of nodal site involvement in cHL. Because the production of this cytokine is partially influenced by genetic background, some SNPs within the *IL10* gene may have an impact on the clinical presentation and outcomes in cHL. The presence of the A allele at either SNP/p*IL10* positions -592 or -1082 seems to be associated with lower IL-10 production compared to the G and C alleles at SNPs/p*IL10*-1082 and -592, respectively [63–65]. It has been proposed that A allele confers high ligation affinity to nuclear proteins, like PU.1 at -592 site and STAT3 at -1082 site, both of which repress IL10 gene transcription [63, 64, 66]. In this scenario, anti-inflammatory effects of IL-10 would be reduced, leading to a shift toward the production of proinflammatory cytokines, including TNF- α and IL-6, known to influence leukocyte chemoattraction (including eosinophils) and tissue redistribution, as well as

lymphocyte apoptosis [67, 68]. Altogether, these mechanisms could explain some commonly observed laboratory features in cHL patients, such as anemia, eosinophilia, leukocytosis, and lymphocytopenia, particularly in advanced-stage presentation.

Different mechanisms are involved in NF κ B activation in cHL, including mutations in the genes encoding for NF κ B inhibitors (e.g., *NFKBIA* and *NFKBIE*) [69, 70] and genomic gains of *REL* [71]. SNP/i*NFKB1* was previously shown to influence the risk of developing HL [38], but data regarding its association with biological or clinical aspects of this disease are scarce. As far as we could verify, we are the first to investigate associations between SNP/i*NFKB1* and prognostic parameters in cHL. As previously known, introns are involved in virtually every important step of RNA processing and can therefore modify the expression level of a host gene in many ways [72]. In our study, the AA genotype of SNP/i*NFKB1*, was found to be significantly associated with advanced clinical stage and presence of extranodal disease at diagnosis compared to AG genotype by univariate analysis. Extranodal presentation is uncommon in cHL occurring in 5–16% of cases and is more frequently observed in younger ages and advanced-stage disease [73]. Likewise, the longer duration of cHL related symptoms may reflect more extensive disease, including extranodal presentation [74]. Based on this, age and duration of symptoms were included as independent variables in multivariate analysis. Extranodal cHL has also been associated with lower rates of complete remission after treatment and poorer clinical outcomes [73, 75]. Our findings suggest a possible association of this polymorphism with modulation of *NFKB1* expression, which might interfere with tumor cell growth control and inhibition of apoptosis, leading to the more aggressive behavior of cHL.

The lack of expression data for the genes encoding the cytokines TNF- α and IL10, as well as *NFKB1*, may be considered a limitation of this study. Nonetheless, it is important to consider that the transcriptional levels of these genes do not necessarily reflect directly on protein levels—the most meaningful information in a pathobiological stand point. On the other hand, although the information of serum levels of cytokines would enrich our results, they may be cumbersome to evaluate because systemic cytokine levels are typically very variable, subject to a whole range of parameters (e.g., patients' age, gender, systemic inflammatory status, stage of the disease, ongoing treatments, etc.) that must be considered during data analysis. Besides, the tissue levels of TNF- α , IL10, and NF κ B proteins are not exclusively influenced by the genetic background but also by posttranscriptional regulatory mechanisms, which contribute to the result regarding cytokines' activities. As a matter of fact, the effect of gene polymorphisms in cytokine production is highly complex, with several variants possibly showing even combined effects [76]. Although this study did not provide new experimental or clinical data showing that the investigated SNPs may cause changes in the regulation of their respective genes, evidence supporting this idea is provided by the genotype-to-phenotype analysis we have performed with the Pheno Scanner v2 metasearch tool (S4 Table). Thus, this result strengthens our hypothesis that the associations between the increased frequency of some of the evaluated SNPs and clinical parameters in cHL cases have a biological background, even though the exact mechanisms and the net effect of these regulations in vivo remain to be elucidated.

In this study, the different genotypes of the studied SNPs were not associated with any specific histological subtype of cHL. Although the number of MC subtype in our study was lower than that previously described in Brazil, recently published data from the Brazilian Prospective Hodgkin's Lymphoma Registry show similar results in a representative cohort of 674 patients, reporting an incidence of 13% of MC [74].

Likewise, SNPs/p*TNF* 238 and -862, SNPs/p*IL10*-592 and -1082, and SNP/i*NFNB1* genotypes showed no association with PFS and OS, although some associations have been reported elsewhere [31, 57]. One possible explanation for these conflicting findings is the small number

of patients included in the present cohort. Notably, due to the study design, most cHL cases were retrospectively included, thus inducing a potential selection bias.

The main relevant limitation of this study is the small number of patients included. Although a larger cohort of cHL would strengthen the analysis performed, this was not achieved due to the relatively small number of patients with this disease attending the tertiary academic hospital where this study was conducted. Moreover, despite our best efforts, our retrospective data collection might be associated with potential biased analysis because we were not able to include patients who had already died or had lost follow-up upon the beginning of the study.

In conclusion, we found that some genotypes for SNPs in genes encoding for TNF- α and NF- κ B p105/p50 were associated with known prognostic parameters for cHL. Considering that genotype—phenotype assumptions must take into account possible effects of isolated versus multiple SNPs, gene–gene, and gene–environment interactions, further analysis including haplotype combinations and gene–gene interaction models may be valuable to define more precisely the pathogenetic role of polymorphisms in cytokine-encoding genes in cHL. Additionally, multicenter clinical studies would be highly desirable to assess the applicability of the obtained results in clinical practice.

Supporting information

S1 Table. Oligonucleotide sequences used to detect SNPs/pTNF at positions -238 and -862, SNPs/pIL-10 at positions -592 and -1082, and SNP/iNFKB1 using qPCR-HRM.

(DOCX)

S2 Table. qPCR conditions and cycling pattern used to amplify SNPs/pTNF at positions -238 and -862, SNPs/pIL-10 at positions -592 and -1082, and SNP/iNFKB1.

(DOCX)

S3 Table. Associations of SNPs/pTNF -238 and -862, SNP/pIL10-592 and -1082, and SNP/iNFKB1 with clinical features and histologic subtype of patients with cHL (N = 73).

(DOCX)

S4 Table. Genes regulated by the SNPs in the promoter regions of IL10 and TNF, as well as in the intronic region of NFKB1, identified in datasets retrieved from PhenoScanner v2.

(DOCX)

S1 Fig. Progression-free survival and overall survival for SNPs/pTNF- α genotypes.

(DOCX)

S2 Fig. Progression-free survival and overall survival for SNPs/pIL10 genotypes.

(DOCX)

S3 Fig. Progression-free survival and overall survival for SNP/iNFKB1 genotypes.

(DOCX)

S4 Fig. Morphology and immunophenotype of a case of classical Hodgkin lymphoma, mixed cellularity subtype (case #80).

(DOCX)

S5 Fig. Morphology and immunophenotype of a case of classical Hodgkin lymphoma, nodular sclerosis subtype (case #2).

(DOCX)

S6 Fig. Morphology and immunophenotype of a case of classical Hodgkin lymphoma, unclassified (case #78).

(DOCX)

S1 Appendix. Data on gene expression regulated by the studied SNPs obtained by Pheno Scanner metasearch.

(ODS)

S1 Data.

(XLS)

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Anexo 6

Baldi BG, Fabro AT, Franco AC, Machado MHC, Prudente RA, Franco ET, Marrone SR, Vale SAD, Cezare TJ, **Moraes MPT**, Ferreira EVM, Albuquerque ALP, Sawamura MVY, Tanni SE. **Clinical, radiological, and transbronchial biopsy findings in patients with long COVID-19: a case series.** J Bras Pneumol. 2022 Apr 29;48(3):e20210438. doi: 10.36416/1806-3756/e20210438. PMID: 35508067; PMCID: PMC9064656.



Clinical, radiological, and transbronchial biopsy findings in patients with long COVID-19: a case series

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ABSTRACT

This brief communication demonstrates the correlation of persistent respiratory symptoms with functional, tomographic, and transbronchial pulmonary biopsy findings in patients with COVID-19 who had a long follow-up period. We report a series of six COVID-19 patients with pulmonary involvement who presented with persistent dyspnea within 4-15 months of discharge. We performed transbronchial biopsies, and the histopathological pattern consistently demonstrated peribronchial remodeling with interstitial pulmonary fibrosis. Therefore, lung biopsy may be useful in the approach of patients with long COVID-19, although the type of procedure, its precise indication, and the moment to perform it are yet to be clarified.

(Brazilian Registry of Clinical Trials–ReBEC; identifier: RBR-8j9kqy [http://www.ensaiosclinicos.gov.br])

Keywords: COVID-19; COVID-19/pathology; Pulmonary fibrosis; Respiratory function tests; Biopsy.

The prevalence of pulmonary involvement in severe acute COVID-19 is high, and there is a concern regarding the occurrence of lung sequelae in the long term.^(1,2) However, the risk, prevalence, and severity of post-COVID-19 pulmonary fibrosis over time are still uncertain.^(1,3) There is still a low number of histopathological reports regarding pulmonary lesions, and they are mostly based on explants and autopsies.⁽⁴⁻¹⁰⁾ Additionally, pathological reports of interstitial lung disease secondary to COVID-19 in the long term are even scarcer.

Patients with COVID-19 may present three major lung histopathological patterns: epithelial lesions and diffuse alveolar damage (DAD); vascular injuries; and interstitial fibrosis. These patterns may coexist in the same patient during the natural history of the disease.^(9,11) The most common histopathological pattern described in acute and severe cases is DAD. Progressive phases of DAD include an early exudative pattern with edema and hyaline membrane formation, a transition to an organizing phase, followed by a fibrosing stage later.^(9,12)

A better understanding of the pulmonary sequelae of the lung lesions in the long term is warranted for determining a more targeted and optimized therapeutic proposal. The aim of this study was to report pulmonary histopathological findings obtained from transbronchial biopsy in a series of COVID-19 patients who had long follow-up periods.

We included six patients with a confirmed diagnosis of COVID-19 by a positive RT-PCR from a nasal swab specimen who presented with persistent respiratory symptoms and interstitial lung abnormalities on CT scans within 4 months of discharge at least and suspected of having post-COVID-19 pulmonary fibrosis. All patients were followed at the Hospital das Clínicas of the *Universidade Estadual Paulista* (UNESP, São Paulo State University).

This study was approved by the institutional research ethics committee (CAAE n. 31258820.5.1001.5411) and registered with the Brazilian Registry of Clinical

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Trials (identifier, RBR-8j9kqy). Bronchoscopy with transbronchial biopsy⁽¹³⁾ was performed after a multidisciplinary discussion based on the presence of symptoms or lung function impairment and persistence of interstitial lung abnormalities on CT scans. The lung samples (average size = 3.8 mm²) were evaluated by an experienced pulmonary pathologist. The final diagnosis was defined by a multidisciplinary team, mainly based on the combination of clinical, tomographic, and histopathological features. We selected patients who were neither on immunosuppressive treatment nor using systemic corticosteroids after discharge. The following variables were collected from all of the patients before the biopsy procedure: baseline dyspnea index (BDI); post-bronchodilator spirometry; DL_{CO}; TLC; Pa_{O₂} on room air; SpO₂ on room air; six-minute walk distance (6MWD); and Saint George's Respiratory Questionnaire (SGRQ) score. All patients signed the informed consent form.

Figure 1 shows CT scans of the study subjects before and after discharge, whereas Figure 2 shows the histopathological findings from the biopsy samples of the patients.

Patient 1: a 62-year-old female, former smoker with diabetes mellitus experienced COVID-19 symptoms for 15 days before hospitalization and was discharged 25 days later without oxygen supplementation. Maximal oxygen supplementation (MOS) during the use of mechanical ventilation was F_{I_{O₂}} = 90% and Pa_{O₂}/F_{I_{O₂}} < 100. Fifteen months after discharge, she presented with dyspnea (BDI = 7); SGRQ score = 32.2; and reduced DL_{CO} (57%). Spirometry, SpO₂, Pa_{O₂}, and 6MWD (440 m) were unremarkable. There was improvement from chest CT results obtained at hospital admission (Figure 1A) to those obtained at 15 months after discharge (Figure 1B), demonstrating subtle peripheral and posterior ground-glass opacities (GGO). Histopathological analysis of biopsy samples showed focal septal thickening by prominent extracellular matrix deposition associated with architectural distortion of the bronchial smooth muscle layer (Figures 2A and 2B).

Patient 2: a 69-year-old female with systemic arterial hypertension (SAH), diabetes mellitus, and dementia experienced COVID-19 symptoms for 15 days before hospitalization and was discharged 9 days later. MOS flow with nasal prong was 4 L/min. Seven months after discharge, the patient reported persistent dyspnea (BDI = 7). Post-bronchodilator spirometry, SpO₂, and Pa_{O₂} were unremarkable. However, TLC (75%), DL_{CO} (52%), 6MWD (389 m/78.8% of predicted), and SGRQ score (15.7) were reduced. CT scans at hospital admission and at 7 months after discharge are presented in Figures 1C and 1D, respectively, and there was tomographic improvement in the follow-up, although there were residual pulmonary abnormalities. Pulmonary histopathological analysis demonstrated mild hyaline peribronchial remodeling with septal extension and focal septal thickening by prominent extracellular matrix deposition (Figures 2C and 2D).

Patient 3: a 65-year-old female with hypothyroidism experienced COVID-19 symptoms for 14 days before hospitalization and was discharged 8 days later. She used a nasal prong (O₂ flow = 4 L/min). Six months after discharge, she still reported dyspnea (BDI = 9), and 6MWD was 502 m. Spirometry, SpO₂, and Pa_{O₂} were unremarkable. DL_{CO} was mildly reduced (62%), and the SGRQ score was 30.8. Comparing CT scans at hospital admission and at 6 months after discharge, there were improvements, demonstrating subtle scattered GGO (Figures 1E and 1F, respectively). Pulmonary histopathological findings highlighted prominent peribronchial remodeling with extensive extracellular matrix deposition (Figure 2E). The architectural distortion of the bronchial smooth muscle layer was highlighted (Figure 2F).

Patient 4: a 44-year-old female with asthma and SAH started having COVID-19 symptoms 7 days before hospitalization and was discharged 14 days later. She used a non-rebreathing mask with a maximum O₂ flow of 6 L/min. Four months after discharge, she presented with dyspnea (BDI = 8) and an SGRQ score of 40.3. Spirometry, TLC, DL_{CO} (76%), SpO₂, and Pa_{O₂} were unremarkable. In comparison with CT scans obtained at hospital admission (Figure 1G), only residual lesions were identified on CT obtained at 4 months after discharge (Figure 1H). Histopathologically, the architectural distortion around hyaline peribronchial remodeling promoted focal simile-desquamative reaction (Figure 2G). Note the disarray and hypertrophy of the bronchial smooth muscle layer (Figure 2H).

Patient 5: an 85-year-old male with SAH experience COVID-19 symptoms for 7 days before hospitalization and was discharged 16 days later. He used a nasal prong with MOS of 2 L/min. Ten months after discharge he still presented with reduced quality of life (SGRQ score = 62.0) and 6MWD (226 m/43.4% of predicted). He was unable to perform spirometry. SpO₂ and Pa_{O₂} were normal. The comparison of CT scans at admission and at 10 months after discharge revealed improvements, the latter showing residual pulmonary lesions only (Figures 1I and 1J, respectively). Histopathological analysis was compatible with prominent peribronchial remodeling with extensive extracellular matrix deposition and small calcification (Figures 2I and 2J).

Patient 6: a 44-year-old female with a history of hysterectomy presented with COVID-19 symptoms for 7 days before hospitalization and was discharged 21 days later. MOS with non-rebreathing mask was 10 L/min. Seven months after discharge, she reported persistent dyspnea (BDI = 7), and her SGRQ score was 41.7. Spirometry suggested a restrictive pattern. SpO₂ and Pa_{O₂} were normal, and 6MWD was reduced (375 m/69.0% of predicted). Follow-up CT scanning demonstrated an improvement in comparison with that at hospital admission (Figures 1K and 1L, respectively), showing residual pulmonary lesions only. Pulmonary histopathological analysis was compatible with peribronchial remodeling with extensive extracellular matrix deposition (Figures 2K and 2L).

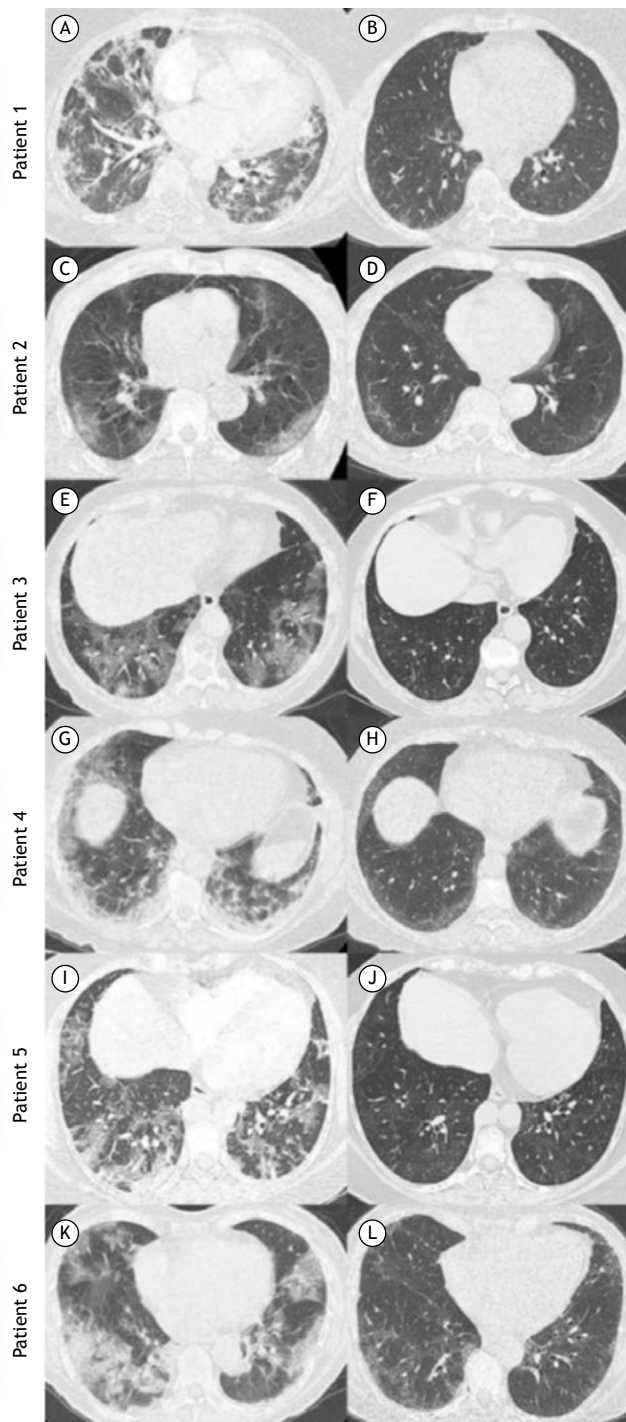


Figure 1. Chest CT scans of the patients studied. Patient 1: in A, a scan during the acute phase showing bilateral ground-glass opacities (GGO), consolidations, and parenchymal bands; in B, a scan after 15 months of follow-up showing subtle peripheral and posterior GGO. Patient 2: in C, a scan during the acute phase showing bilateral and peripheral GGO; in D, a scan after 7 months of follow-up showing subtle GGO with subpleural curvilinear lines and small dilated bronchioles in the right lower lobe. Patient 3: in E, a scan during the acute phase showing bilateral GGO and crazy-paving pattern; in F, a scan after 6 months of follow-up showing subtle scattered GGO. Patient 4: a scan during the acute phase showing bilateral and peripheral GGO and consolidations; in H, a scan after 4 months of follow-up showing subtle bilateral and peripheral GGO. Patient 5: in I, a scan during the acute phase showing bilateral GGO; in J, a scan after 10 months of follow-up showing subtle GGO and mosaic attenuation in the lung parenchyma. Patient 6: in K, a scan during the acute phase showing bilateral GGO and consolidations; in L, a scan after 7 months of follow-up showing bilateral GGO with some dilated bronchioles.

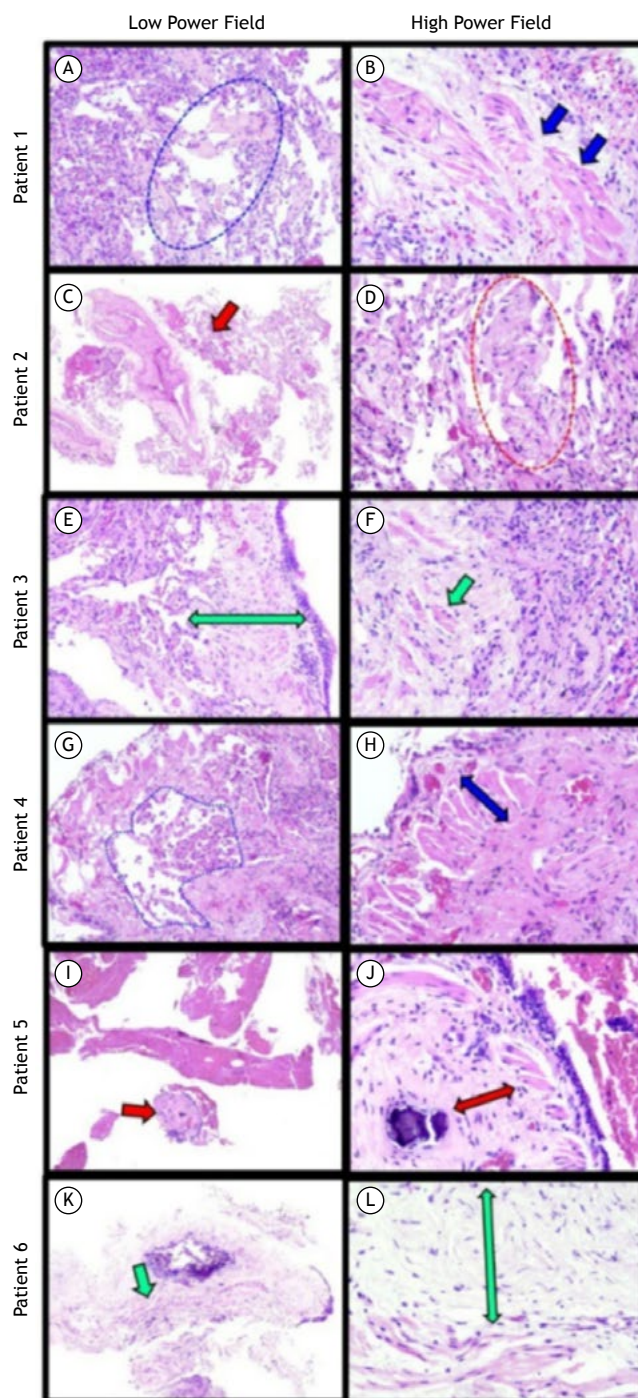


Figure 2. Histopathological panel of transbronchial biopsy samples collected from the patients studied (H&E; low power field, $\times 4$, and high power field, $\times 40$). All of the patients showed hyaline peribronchial remodeling with septal extension. Patient 1: focal septal thickening by prominent extracellular matrix deposition (blue dashed ellipse in A) associated with architectural distortion of the bronchial smooth muscle layer (blue arrows in B). Patient 2: mild hyaline peribronchial remodeling with septal extension (red arrow in C). Note the focal septal thickening by prominent extracellular matrix deposition (red dashed ellipse in D). Patient 3: prominent peribronchial remodeling with extensive extracellular matrix deposition (double green arrow in E). The architectural distortion of the bronchial smooth muscle layer is highlighted (green arrow in F). Patient 4: the architectural distortion around hyaline peribronchial remodeling promoted focal simile-desquamative reaction (area enclosed by blue dashed line in G). Note the disarray and hypertrophy of the bronchial smooth muscle layer (double blue arrow in H). Patient 5: prominent peribronchial remodeling (red arrow in I) with extensive extracellular matrix deposition (double red arrow in J) and small calcification. Patient 6: prominent peribronchial remodeling (green arrow in K) with extensive extracellular matrix deposition (green arrow in L).

Few studies have described pulmonary histopathological findings in patients with COVID-19 after a long follow-up period. Most case series have detailed features during the acute phase and based their findings on lung tissue from autopsy or transplant.⁽⁴⁻¹⁰⁾ To our knowledge, this is the first study that evaluated histopathological features obtained from transbronchial biopsies during the late stage of pulmonary involvement by COVID-19, 4-15 months after the acute infection. The main finding of our study was that the histological analysis of the six patients with long COVID-19 and persistent pulmonary abnormalities on HRCT demonstrated signs of bronchiolocentric interstitial pneumonia, most of them presenting with architectural distortion and peribronchial remodeling with extracellular matrix deposition.

Although all of the patients in our series showed tomographic improvements, dyspnea and pulmonary tomographic abnormalities still remained. Only one patient met the criteria for ARDS. However, the late functional and tomographic findings of this patient with ARDS were similar to that of the others. It is not completely clear, however, when the presence of irreversible post-COVID-19 pulmonary fibrosis is defined, and which patients may present tomographic and functional improvement over time. Additionally, it remains uncertain in which scenario after COVID-19 lung biopsy should be considered for histopathological analysis.^(1,3,14,15)

Previous descriptions of pulmonary histopathological characterization of COVID-19 were mostly obtained from autopsy and explanted lungs.^(1,5-7,9,10) The main patterns identified alone or in combination were exudative and organizing DAD, hemorrhage, thrombosis, intra-alveolar fibrin deposition, lymphoid infiltrates, and organizing pneumonia. However, in the fibrosing DAD phase, our group has already demonstrated a fibrotic phenotype with excessive extracellular matrix and collagen deposition and lung architectural distortion, corroborating other studies.⁽⁷⁻¹²⁾ Likewise, some autopsy and explanted lung cases demonstrated interstitial and bronchiolar fibrosis, with collagen deposition, bronchial metaplasia, and pulmonary vascular remodeling. Some cases have demonstrated areas of microscopic honeycombing.⁽⁵⁻⁹⁾ A recent case series reinforced the presence of diffuse interstitial fibrosis and areas of microscopic honeycombing in patients after a 4-month follow-up period.⁽⁸⁾ Our findings demonstrated some similarities, as we predominantly identified bronchiolocentric interstitial pneumonitis. However, to our knowledge, no study has described histological features and their patterns after COVID-19 with pulmonary involvement during a long follow-up period, which would add for a better understanding of this process.

Patients with interstitial lung disease secondary to COVID-19 might need to be monitored for a longer time, preferably through a multidisciplinary approach.⁽³⁾ Pulmonary lesions may persist in the long term after COVID-19, although a significant proportion of patients may present progressive functional and tomographic improvement during follow-up.⁽¹⁵⁾

Our study has limitations. First, there were a small number of patients included for robust conclusions. Second, transbronchial biopsy does not allow assessment of peripheral lesions, which may have histological patterns that are different from those identified. Third, all patients showed tomographic improvements at follow-up, and we cannot conclude that the pattern found here would be the same as those found in patients who remain stable or deteriorate during follow-up.

In conclusion, findings compatible with bronchiolocentric interstitial pneumonia were identified from transbronchial biopsies in patients with long COVID-19. Patients with pulmonary involvement secondary to COVID-19 that require biopsy during the follow-up period need to be better defined. Based on our series, transbronchial biopsy may be an initial step in the assessment of patients with post-COVID-19 pulmonary fibrosis in the presence of symptoms or lung function impairment and persistence of interstitial lung abnormalities on CT scans. Further studies are warranted to determine the histopathological patterns in a larger number of lung tissue samples obtained from transbronchial cryobiopsy or surgical biopsy, as well as in patients who remain stable or present worsening of pulmonary involvement associated with COVID-19. It will also be important to assess the indications for and responses to drug treatment in such scenarios, including the use of corticosteroids and antifibrotic drugs.

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AUTHOR CONTRIBUTIONS

BB, ATF, and SET: study conceptualization; clinical, radiological, and histological interpretation; and drafting the manuscript. RAP, ETF, ACF, MHCM, SAV, TJC, and MPTM: performance of all procedures and data collection. MVYS and SRM: radiological interpretation. EVMF, ALPA, MVYS, BB, ATF, and SET: revision of preliminary and final versions; and approval of the final version.

CONFLICT OF INTEREST

None declared.

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Anexo 7

Secondo MTS, Rodrigues LDS, Ramos LPM, Bovolato ALC, Rodriguez-Sanchez DN, Sobreira ML, **Moraes MPT**, Bertanha M. **Evaluation of Biointegration and Inflammatory Response to Blood Vessels Produced by Tissue Engineering-Experimental Model in Rabbits**. Biomolecules. 2022 Nov 29;12(12):1776. doi: 10.3390/biom12121776. PMID: 36551204; PMCID: PMC9775166.

Article

Evaluation of Biointegration and Inflammatory Response to Blood Vessels Produced by Tissue Engineering—Experimental Model in Rabbits

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Abstract: Peripheral arterial disease (PAD) is the main cause of mortality in the western population and requires surgical intervention with the use of vascular substitutes, such as autologous veins or Dacron or PTFE prostheses. When this is not possible, it progresses to limb amputation. For cases where there is no autologous vascular substitute, tissue engineering with the production of neovessels may be a promising option. Previous experimental studies have shown in vitro that rabbit vena cava can be decellularized and serve as a scaffold for receiving mesenchymal stem cells (MSC), with subsequent differentiation into endothelial cells. The current study aimed to evaluate the behavior of a 3D product structure based on decellularized rabbit inferior vena cava (IVC) scaffolds seeded with adipose-tissue-derived stem cells (ASCs) and implanted in rabbits dorsally subcutaneously. We evaluated the induction of the inflammatory response in the animal. We found that stem cells were positive in reducing the inflammatory response induced by the decellularized scaffolds.

Keywords: models; animal; peripheral arterial disease; tissue engineering; endothelium; mesenchymal stem cells; immune response; cell differentiation

1. Introduction

The treatment of peripheral arterial disease (PAD) [1] with limb-threatening ischemia is complex, and surgical revascularization is often needed [2,3]. In cases where it is not possible to use an autologous vein for a conventional bypass, synthetic arterial substitutes, such as Dacron® or polytetrafluoroethylene (PTFE) prostheses can be used [4]. The major limitations for the use of synthetic material derive mainly from infections of the prosthesis or at the incision site, as well as the high risk of contamination of the prosthesis from distant infections [3]. In addition, for below-knee prosthetic bypass there is the limitation of poor arterial outflow or incompatibility between caliber and flow [2,4]. Endovascular procedures can be conducted but sometimes have limitations too. When revascularization is indicated but not carried out, the risk of amputation greatly increases [2]. Therefore, the tissue engineering of blood vessels (TEBV) represents a promising perspective to produce personalized vascular substitutes to be used in revascularization surgeries [5]. A major challenge for TEBV to widely reach clinical practice is the production of a functional, resistant, and three-dimensional blood vessel [6,7]. For that, the basis of development requires that the scaffold (synthetic or biological) be sufficient to provide the necessary

support for the implanted stem cells to differentiate, renew the extracellular matrix (ECM) and do not induce immunological reactions [8–10].

The use of decellularized blood vessels as scaffolds presents some initial biological advantages for TEBV [6,9]. They harbor their own characteristics such as the composition and resistance of the ECM [5,9]. However, for the use of biological material as a scaffold, it is necessary to attain efficient decellularization that guarantees an adequate elimination of cell surface antigens to avoid an immune-mediated rejection of the transplanted tissue and, consequently, the risks of graft loss, including the risk of death or contamination [11–14].

This study intended to evaluate 3D structures produced by the decellularization of rabbits' inferior vena cava (IVC) with 1% sodium dodecyl sulfate (SDS) for 2 h (scaffolds), repopulated or not with ASCs (autologous and allogeneic) compared to allogeneic IVCs, implanted in the back of rabbits, maintained for 60 days, to study the inflammatory patterns and the behavior and reactions induced by these structures in the living organism.

2. Materials and Methods

2.1. Animal Handling Conditions and Tissue Acquisition

A total of 18 adult New Zealand rabbits were used, all nonpregnant females weighing between 2.5 and 3.5 kg. The IVCs were obtained from 6 donor animals, and 2 g of adipose tissue (AT) was also obtained from two of these animals to generate the allogeneic cell bank; one animal was the donor of allogeneic IVC to be used in natura at the time of implantation.

The animals were housed under controlled conditions and fed a standard pellet diet with water ad libitum. Before the surgical procedures to obtain the AT and IVC, the animals were given an intramuscular combination of ketamine 10 mg/kg (Dopalen[®], 100 mg/mL, Paulínia, SP, Brazil), xylazine hydrochloride 3 mg/mL kg (Anasedan[®] 2%, 20 mg/mL, Paulínia, SP, Brazil), and acepromazine 0.1 mg/kg (Apromazin[®] 0.2%, 200 mg/mL, Barueri, SP, Brazil). In addition, a local anesthetic injection, lidocaine 7 mg/kg (Xylocaine 1% 20 mg/mL, Cristália, Butantã, SP, Brazil) was administered at the site designed for the surgical incision. Harvesting was carried out under aseptic conditions and at the end of the procedure, the animals were euthanized with pentobarbital.

2.2. Scaffolds Production

The IVCs were sectioned into segments of approximately 2 cm in length, totaling 12 fragments. Decellularization was carried out using the protocol with 1% SDS for 2 h under shaking in a Shaker (News Brunswick Scientific[®] Edison, NJ, USA), at 37 °C. (Supplementary file S1) [9,13,15]. The fragments were stored in a refrigerator at 4 °C in a sterile solution containing antibiotics and fungicide.

2.3. Obtaining Adipose-Derived Stem Cells (ASCs)

In order to obtain autologous ASCs, the animals were anesthetized, then 2 g of AT was surgically removed from the interscapular region and stored in a conical tube containing a solution of N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) supplemented with penicillin, 100 mg/mL streptomycin, and 25 mg/mL amphotericin B (2 mmol/L 1-glutamine; Invitrogen[™], Waltham, MA, USA). ASCs were obtained by enzymatic dissociation with collagenase type I (Invitrogen[™], Waltham, MA, USA). Cell culture procedures were performed with an initial cell count of 6×10^4 cells/cm², obtained from 12 fragments of adipose tissue. These cells were seeded and expanded in 25 cm² culture flasks using Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 25 mg/mL amphotericin B (2 mmol/L of l-glutamine; Invitrogen[™], Waltham, MA, USA), 1% (v/v) of minimal essential medium (MEM), an essential amino acid solution (Invitrogen[™], Waltham, MA, USA), and 0.5% (v/v) of 10 mM MEM nonessential amino acid solution (Invitrogen[™], Waltham, MA, USA) until the number of cells required for the entire study was reached. ASCs were analyzed phenotypically by flow cytometry (FC) using CD45, CD44, CD90, and CD11b,

and by tri-lineage differentiation techniques (StemPro™ adipogenesis, chondrogenesis, and osteogenesis differentiation kits; Invitrogen, Waltham, MA, USA).

2.4. Experimental Groups

For all experiments, the vessels (with or without cells) were surgically fixed in the subcutaneous tissue over the dorsal muscular fascia. Four groups were formed (in triplicate)—group 1: implantation of a fragment of allogeneic inferior vena cava in natura (allogeneic IVC); group 2: implantation of a decellularized vein fragment with SDS without the addition of cells (decellularized IVC-SDS); group 3: implantation of a decellularized vein fragment with SDS + 1×10^5 allogeneic ASCs (decellularized IVC-SDS + allogeneic ASCs); group 4: implantation of a decellularized vein fragment with SDS + 1×10^5 autologous ASCs (decellularized IVC-SDS + autologous ASCs).

2.5. Biological Scaffold and ASCs Seeding

The ASCs (1.3×10^5) were submitted to the cytoplasmic labeling process with Qtracker 605 (CellLabeling Kits—Invitrogen™, Waltham, MA, USA). A solution containing 10 nM of Qdots (1 μ L of solution A mixed with 1 μ L of solution B, for each 1×10^5 of cells), added in 200 μ L of DMEM was used. This solution was homogenized with the cell pellet previously obtained and kept in a 5% CO₂ incubator at 37 °C for a period of 45 min, protected from light. After the incubation period, the ASCs were washed with D-PBS and diluted in PuraMatrix® peptide hydrogel (BD Biosciences, Franklin Lakes, NJ, USA). A total of 10 μ L of this solution was pipetted into the lumens of each scaffold. The experiment was carried out in triplicate, maintained for 2 h in culture for cell preadherence in a DMEM culture medium in a CO₂ incubator. With a small fragment of the scaffold with ASCs, an immunofluorescence (IF) analysis was performed with Fluoroshield™ with DAPI (Sigma, San Luis, MO, USA) to visualize the ASC nuclei to confirm the beginning of scaffold recellularization.

2.6. Surgical Experimentation

For the implantation of the grafts, the animals were anesthetized as previously described, and a surgical aseptic technique was used. A small longitudinal incision, 2 cm in length, was done in the dorsal thoracic region of the animal, dissecting up to the limit of the most superficial fascia of the latissimus dorsi, where the study fragments were fixed (only one fragment per animal) with a simple nonabsorbable stitch of NYLON 4-0 (Ethicon®, Somerville, NJ, USA) at each end of the implant, for identification at the time of explantation. The animals were kept in special housing as previously described and monitored daily, being euthanized after the 60-day observation period. The fragments were surgically collected for a histomorphological analysis.

2.7. Analysis of Interleukins and Oxidative Stress

During the monitoring period, 1.5 mL of peripheral blood was collected from the marginal ear vein of each animal before the procedure and 1, 7, 14, 30, and 60 days after the surgical procedure. Each sample was centrifuged at $2000 \times g$ for 5 min to obtain plasma, then kept frozen at -80 °C until inflammatory interleukins (IL6, TNF- α) and anti-inflammatory interleukins (IL-4, IL10) were measured by an enzyme-linked immunosorbent assay (ELISA) (all of them, Invitrogen™, Waltham, MA, USA). At the end of the study, one fragment of the implanted tissue, 0.5 cm in length, was subjected to maceration and the same interleukins were evaluated.

In addition, analyses were conducted for the detection of oxidative stress by the method of the malonaldehyde (MDA) reaction with thiobarbituric acid (TBARS) [16]. The MDA dosage of the samples was intended to assess oxidative stress variations caused by implants.

2.8. Histomorphology

The collected fragments (60 days after implantation) were sectioned into two equal parts, one being placed in 10% buffered formaldehyde to make paraffin-embedded blocks, and the other kept frozen for a fresh frozen section stained with Qtracker (red) under a fluorescence microscope, to identify the ASCs, in addition to being macerated later to measure tissue ILs.

Slides were prepared from the paraffin blocks for standard hematoxylin–eosin (HE) histology and for immunohistochemistry (IHQ) with the analysis of anti-CD31 primary marker diluted 1 in 10 (Novus Biologicals™, Englewood, CO, USA) and marked with secondary antibody Dako EnVision™ + Dual Link System-HRP for the identification of endothelial cells. The slides made with fresh material were destined for a blinded analysis of cell nuclei by immunofluorescence with the 4',6'-diamino-2-phenyl-indole (DAPI) fluoroshield sealant (Sigma™, Cotia, SP, Brazil). In addition, separate slides with anti-CD31 stained with Fluorescein-5-isothiocyanat (FITC) were made and taken for photomicrographs under a confocal microscope (LEICA™ TSC SP8, Wetzlar, Germany).

2.9. Statistical Analysis

The comparison between means at time points was conducted using a repeated measures design to assess the interaction between groups versus time points. In cases where the data presented a symmetrical distribution, an ANOVA for repeated measures was used followed by Tukey's multiple comparison test adjusted to the design. When the distribution was skewed, a gamma distribution adjustment was conducted for the same design, followed by Wald's multiple comparison tests. In all tests, the significance level was set at 5%. All analyses were conducted using SAS for Windows, v.9.4 (SAS software for Windows version 9.4, SAS Institute Inc, Cary, NC, USA).

3. Results

3.1. Animals

A total of 18 adult New Zealand rabbits were randomly used in all experiments and no one was excluded.

3.2. Vein Decellularization, ASC Characterization, and In Vivo Experimentation

HE photomicrographs show the comparative result between in natura IVC (Figure 1a) and decellularized IVC (Figure 1b). The IF-DAPI photomicrographs show the comparative result between the decellularized IVC (Figure 1c) and the decellularized IVC with ASCs seeding in the proposed model before implantation (Figure 1d).

ASCs were analyzed for phenotypic characterization by FC. The cell surface markers CD44 and CD90 showed peaks of positive events, while the hematopoietic and immunological cell markers CD45 and CD11b, respectively, were negative, confirming the cell phenotype. The trilineage differentiation challenge was positive, completing the protocol for stem cell confirmation.

The implants were performed as previously described; blood was collected for cytokine analysis and after 60 days of implantation, the animals were euthanized, and the samples were collected (Figure 1e,f).

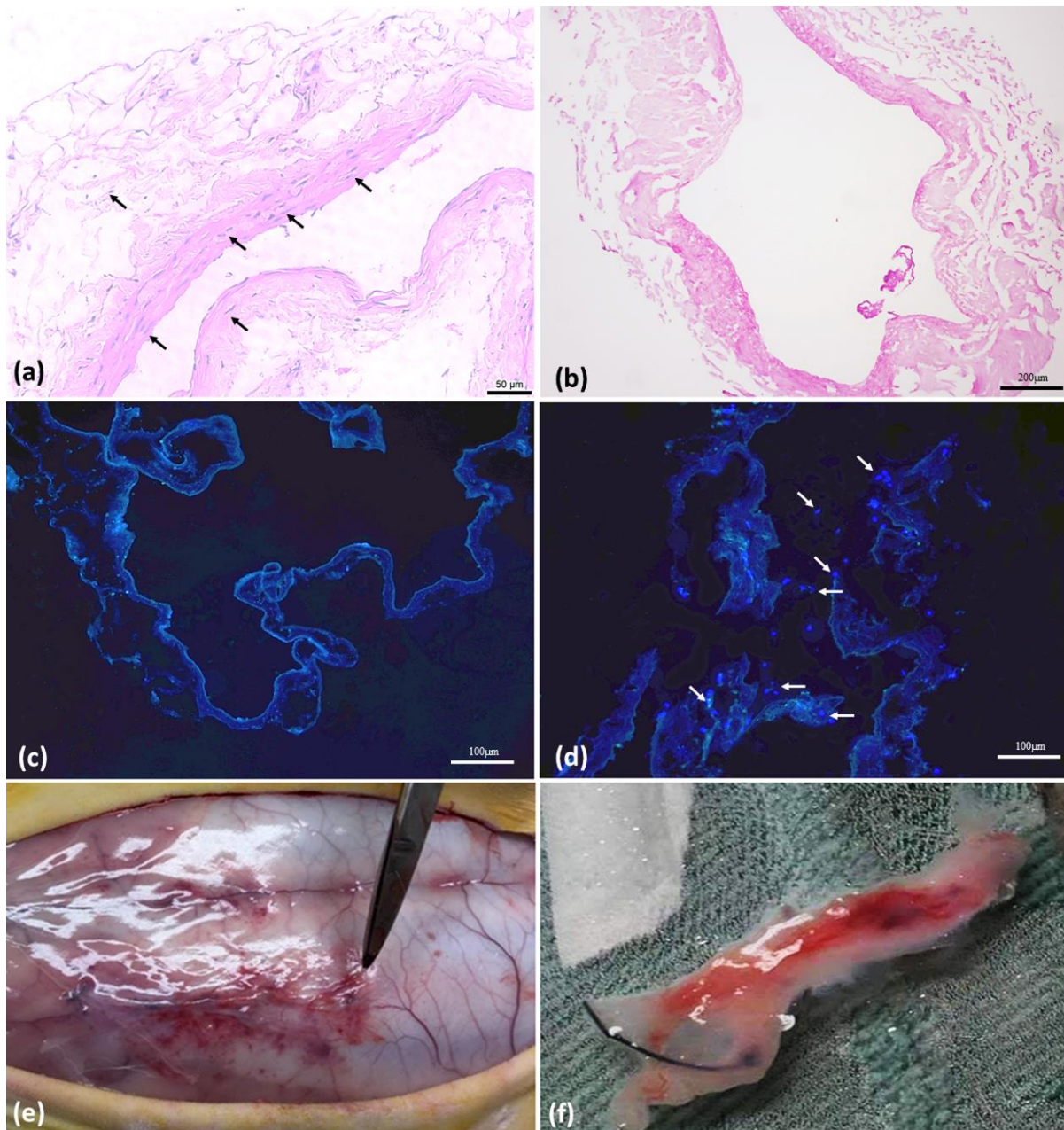


Figure 1. Pre and post implantation tissue analysis. (a). Photomicrograph in HE of inferior vena cava (IVC) in natura, magnification: 400×. (b) Photomicrograph in HE of decellularized IVC with 1% SDS for 2 h, magnification: 100×. (c) Immunofluorescence photomicrograph with DAPI of decellularized IVC with 1% SDS for 2 h, without nuclei marking, magnification: 200×. (d) Immunofluorescence photomicrograph with DAPI of decellularized IVC with 1% SDS for 2 h and seeded with 1×10^5 ASC, immediately before implantation, with nuclei marking, magnification: 200×. (e) Surgery to harvest the implanted tissue after 60 days. (f) Tissue explanted after 60 days.

3.3. Interleukin Analysis

3.3.1. Plasma

The study of serum levels of ILs showed statistically similar serum levels between the groups studied for all moments, with IL4 being the only one with a result equal to zero in all analyses, being considered not detectable under the conditions of this experiment (Figure 2).

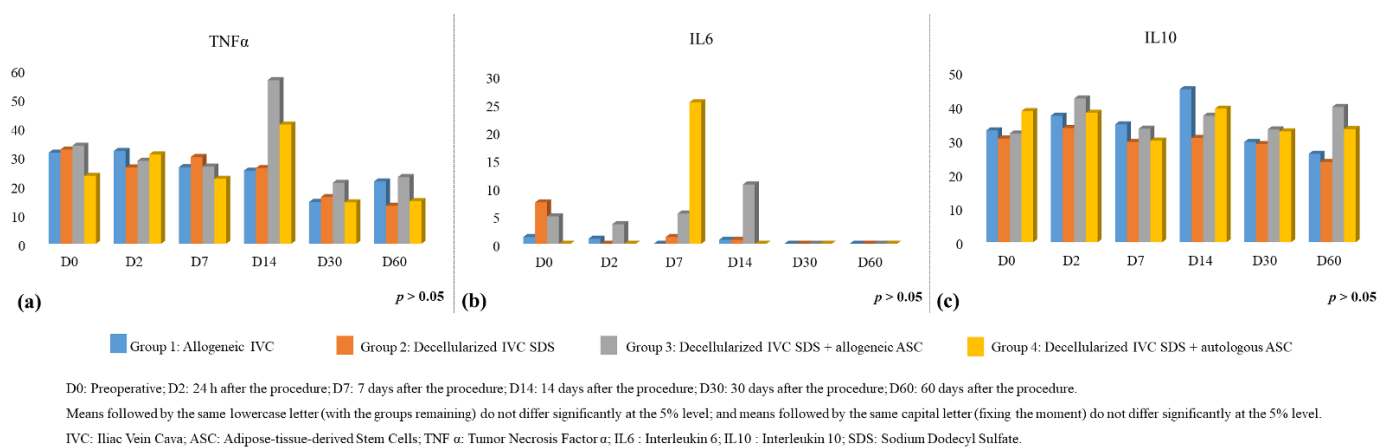
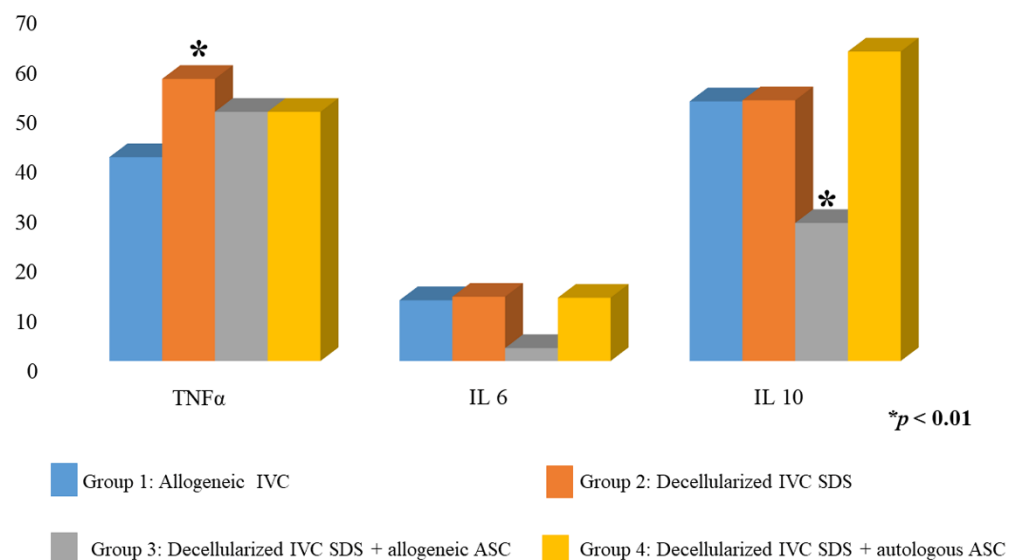


Figure 2. Analysis of plasma cytokines by Elisa. (a) Analysis of Tumor Necrosis Factor α (TNF α); (b) Analysis interleukin 6 (IL6); (c) Analysis of interleukin 10 (IL10).

3.3.2. Frozen Explant Tissue Homogenate

The analysis of ILs in frozen explant tissue homogenate at 60 days showed a result for IL4 equal to zero in all samples, considered as not detectable under the conditions of this experiment. The results for TNF α were higher for group 2 (decellularized vein), which meant a higher inflammatory response compared to the other groups which presented lower measurements ($p < 0.01$). For the values measured by the dosage of IL6, there was no statistical difference between the groups; therefore, it was not possible to use this parameter to differentiate the inflammation caused by the implants under the conditions of this experiment. For IL10, only group 3 (decellularized IVC-SDS + allogeneic ASC) was found to have a lower dose compared to the other groups, with a statistically significant difference ($p < 0.01$), suggesting a decrease in the regulation of the inflammatory IL10-mediated process in this group (Figure 3).



IVC: Iliac Vein Cava; ASC: Adipose-tissue-derived Stem Cells; TNF α : Tumor Necrosis Factor α ; IL6 : Interleukin 6; IL10 : Interleukin 10; SDS: Sodium Dodecyl Sulfate.

Figure 3. Tissue interleukin analysis (Tumor Necrosis Factor α (TNF α), interleukin 6 (IL6), and interleukin 10 (IL10)).

3.3.3. Oxidative Stress by Plasma MDA Analysis

Figure 4 shows the results of the oxidative stress analyses at all moments of the study. In general, it was not possible to find statistical differences in the serum MDA analysis in plasma; therefore, no inference could be made regarding the influence of the procedures adopted in this study and the increase or decrease in oxidative stress.

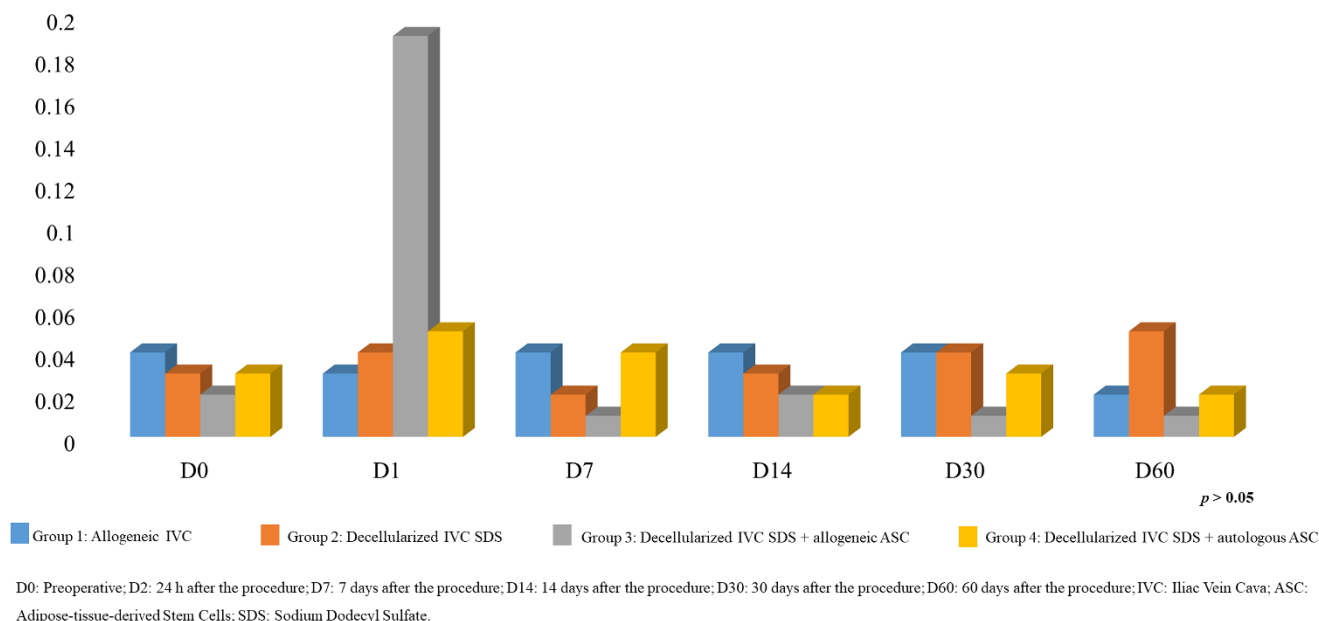


Figure 4. Malonaldehyde (MDA) analysis by moments. D0: Preoperative; D2: 24 h after the procedure; D7: 7 days after the procedure; D14: 14 days after the procedure; D30: 30 days after the procedure; D60: 60 days after the procedure.

3.4. Histomorphology

Analysis in HE of the fragments implanted for 60 days showed some endothelialization, with good cell colonization in all groups. However, some qualitative differences were observed with the same regularity in each group, such as the presence of a greater inflammatory response in group 1—allogeneic IVC, due to the presence of lymphocytes, plasma cells, and histiocytes, in addition to the persistence of native vein structures, the same endothelium, and hemorrhage; for group 2—decellularized IVC-SDS, small endothelial neoformation and immature fibroblasts (myofibroblasts), inflammatory reaction, and hemorrhage were seen; in group 3—decellularized IVC-SDS + allogeneic ASC, vascular proliferation, low inflammatory reaction, fibroblasts proliferation (myofibroblasts), and hemorrhage were observed; Unlike the others, Group 4—decellularized IVC-SDS + autologous ASCs showed greater adjacent vascular neoformation, an endothelium partially repopulating the lumen of the venous scaffold, and significantly less inflammatory reaction, except at the region of the NYLON stitch (Figure 5).

In the immunohistochemical analysis, the CD31 antibody, a vascular endothelium marker, was present in all groups; however, it occurred in slightly different patterns between the groups, corresponding to the histological findings of using HE, with a lower intensity of endothelial staining for group 1 and a less organized pattern for groups 2 and 3 as compared to group 4 (Figure 6).

Immunofluorescence to evaluate Qtracker-labeled ASCs (in red) in groups 3 and 4 (Figure 7) with overlabeling with DAPI showed that there were cells labeled with both markers in the two groups where this was possible, suggesting that they came from the ASC culture (groups 3 and 4) (Figure 8).

Another analysis by immunofluorescence was conducted with CD31 + FITC (green) and DAPI (blue), in addition to the previous staining with Qtracker (red) and it was possible to see, in confocal fluorescence microscopy, the occurrence of some endothelial cells that originated from the implanted culture since they presented the three markings simultaneously only in group 4 (Figure 9).

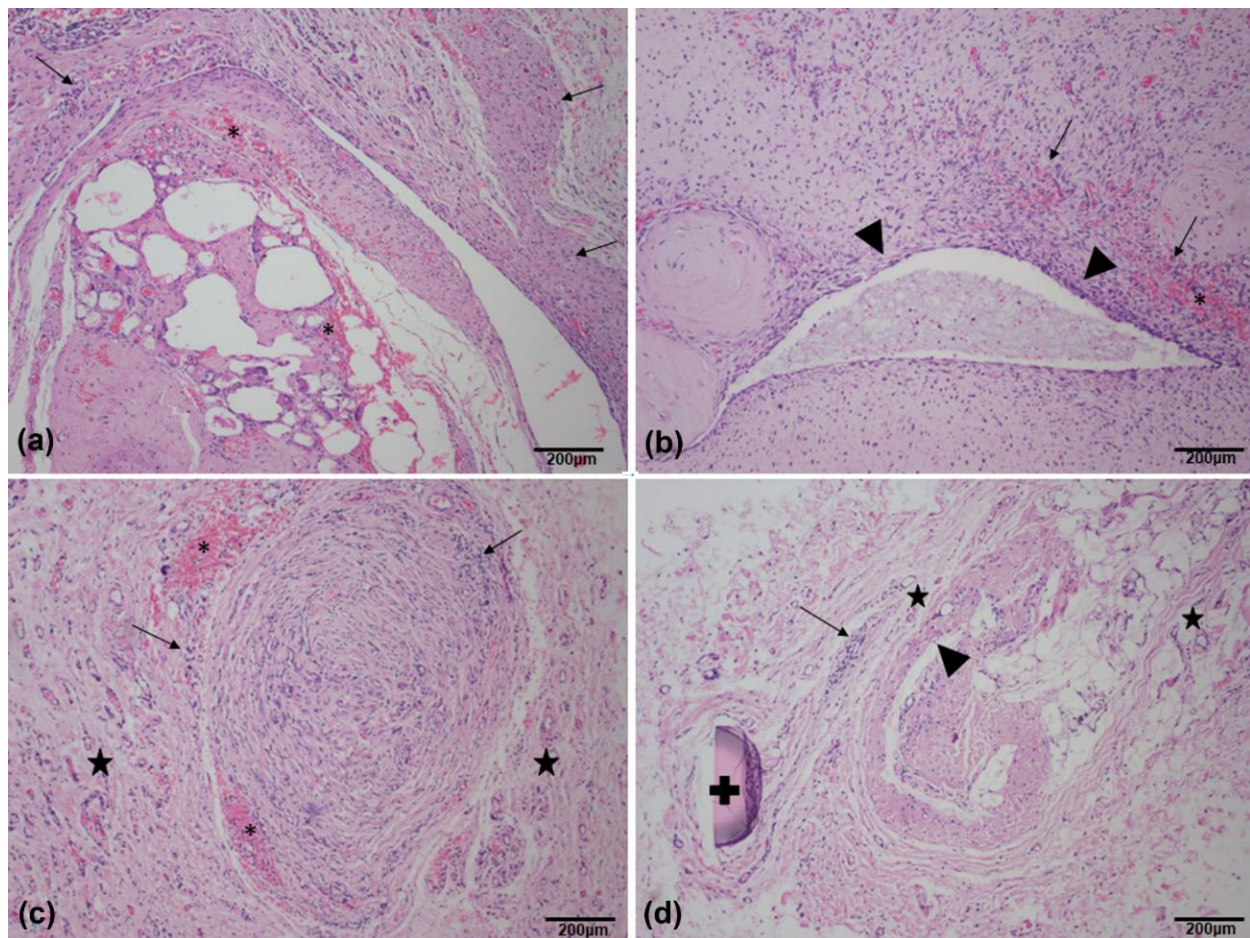


Figure 5. Photomicrography in HE, magnification: 100 $\times$. Scaffold explants at 60 days. (a) Group 1—allogeneic IVC showing inflammatory cells (lymphocytes, plasma cells, and histiocytes), persistence of native vein structures (endothelium) (arrows), and hemorrhage (asterisk); (b) group 2—decellularized IVC-SDS showing small endothelial neoformation, immature fibroblasts (myofibroblasts) (arrowhead), evidence of inflammatory reaction (arrows), and hemorrhage (asterisk); (c) group 3—decellularized IVC-SDS + allogeneic ASCs showing vascular proliferation (star), low inflammatory reaction (arrows), fibroblasts (myofibroblasts) proliferation (arrows), and hemorrhage (asterisk); (d) group 4—decellularized IVC-SDS + autologous ASCs showing greater adjacent vascular neoformation (star), endothelium partially repopulating (arrowhead), and significantly less inflammatory reaction (arrows), except at the region of the NYLON stitch (plus sign).

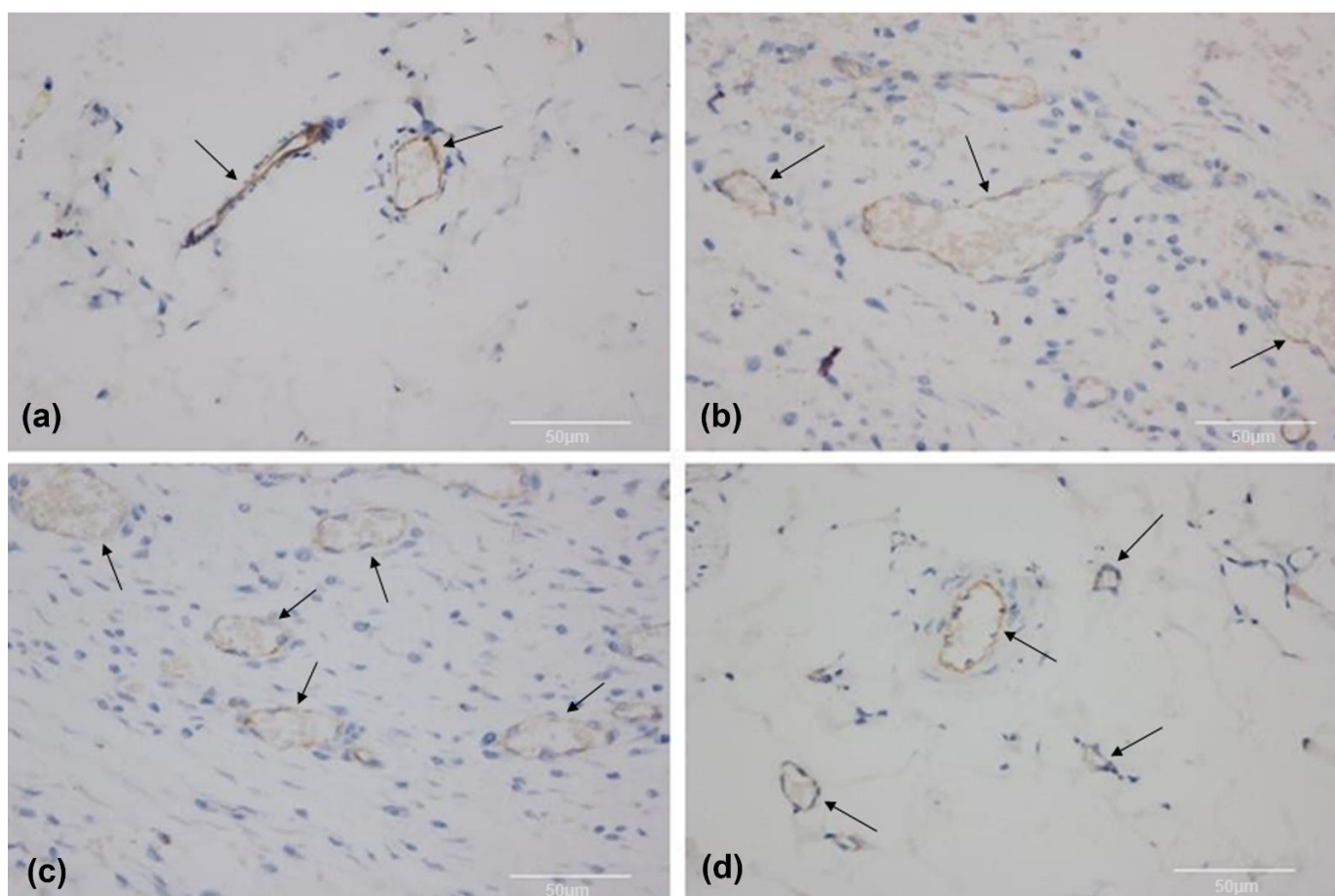


Figure 6. Immunohistochemistry, slide analysis under light microscopy with CD31 endothelium marker antibody (arrows); magnification: 400×. (a) Group 1; (b) group 2; (c) group 3; (d) group 4.

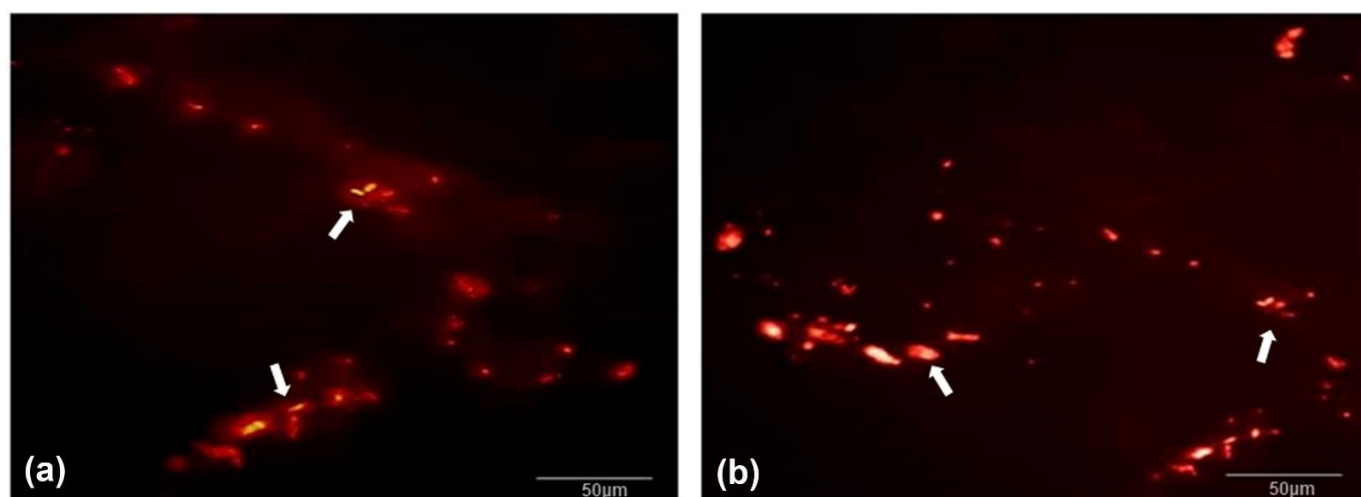


Figure 7. Analysis in fluorescence microscopy; magnification: 400×. (a) Group 3, (b) group 4, showing cell marking signals (ASC) with Qtracker (red fluorescence in nanocrystals deposited in the cytoplasm) (white arrows).

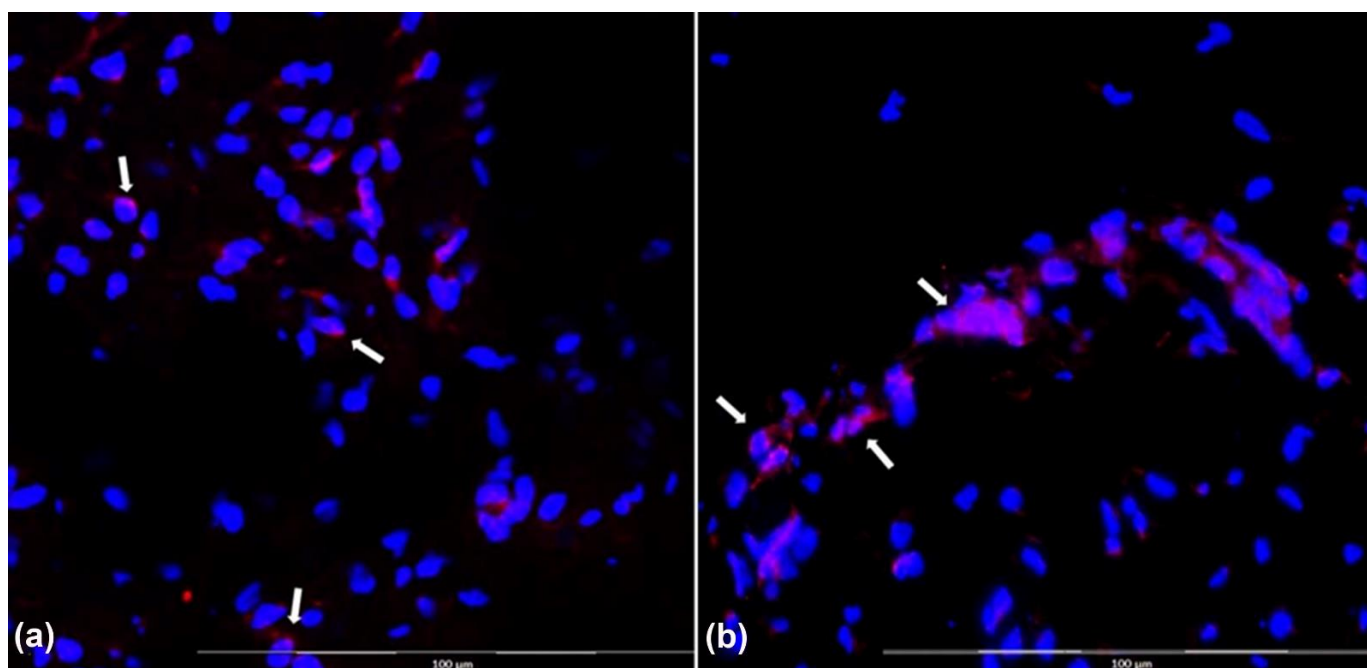


Figure 8. Immunofluorescence on frozen tissue; magnification: 200×. (a) Decellularized veins with allogeneic ASCs, cell nuclei in blue (DAPI) and red, and cytoplasm of tissue tagged ASCs (Qtracker); (b) decellularized veins with autologous ASCs, cell nuclei in blue (DAPI) and red, and cytoplasm of tagged ASCs in tissue (Qtracker) (arrows point to tagged cells) (white arrows).

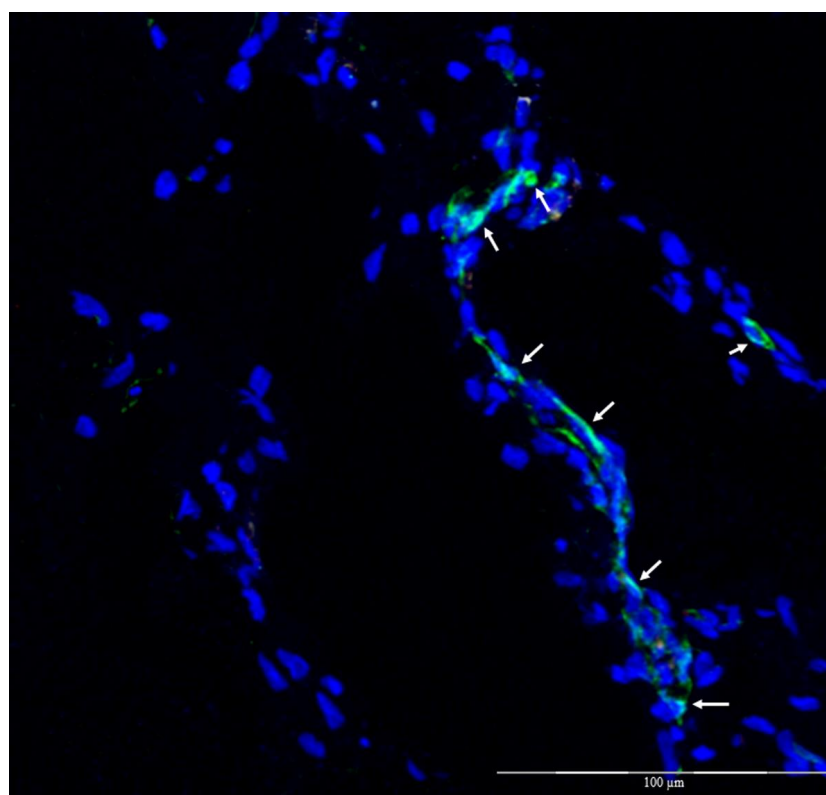


Figure 9. Immunofluorescence on frozen tissue; magnification: 200×. Decellularized veins with autologous ASCs, cell nuclei in blue (DAPI), and green, and cytoplasmic membrane labeled with anti-CD31 (FITC), only for group 4 (arrows point to labeled cells) (white arrows).

4. Discussion

The conventional treatment for PAD is clinical, aiming at the control of risk factors. In a situation of critical ischemia, however, the alternatives are endovascular treatments with angioplasty or revascularization surgeries (bridges/bypass using an autologous vein or synthetic material) [2]. However, for a portion of patients, revascularization treatments are not applied, increasing the risk for limb amputations [4]. Consequently, biotechnology becomes a partner for the development of biomimetic products, and numerous studies have pointed to the possibility of the production of organs or tissues that may be promising for the regeneration or replacement of damaged vessels, considering that there is already enough technology for the differentiation of ASCs into endothelium, smooth muscle, and other tissues [17–20].

In a review article, Afra et al. [21] compared the results of preclinical research with the use of autologous or allogeneic ASCs for the development of blood vessels and concluded that autologous cells are preferred. Our work goes in the same direction, pointing out the advantages of the use of autologous cells. In that same review, the authors studied the potential of ASCs in engineering functional blood vessels compared to other cell types, such as bone marrow mononuclear cells, endothelium precursor cells, adult autologous smooth muscle cells, autologous endothelial cells, embryonic stem cells, and pluripotent stems [21]. It was concluded that ASCs were still the preferred cells for tissue engineering of blood vessels, mainly because of their multipotent potential and ease of obtaining them in quantity. These reasons also guided our work. ASCs can also secrete a broad spectrum of bioactive macromolecules that help the regeneration of injured tissues and have immunomodulatory properties [21].

The most suitable scaffolds for tissue engineering of blood vessels should be able to withstand vascular stress without degeneration, do not promote thrombotic events, present good interaction with adjacent tissues and implanted cells, and enable nutrient transport through the porosity of their wall until they are fully developed [20]. More than one of our previous studies [15] have determined that the method used in the production of a biological scaffold produced by the decellularization of the vena cava in the rabbit model meets these requirements, and the current study demonstrates that the interaction with autologous ASCs promotes a favorable environment, generating the ideal adaptive conditions when it is implanted on the animal's back.

The immune response triggered by the implantation of scaffolds in the *in vivo* animal model varied between groups and according to the form of analysis. For the evaluation of serum results, no significant differences were found between the groups at the times analyzed, which could be explained by the volatility of the production of these cytokines after the first hours of surgery, the small aggression caused by the surgery, and by the small amount of tissue implanted. For the analysis of the homogenate of part of the tissue collected 60 days after implantation, unlike the serum analysis, there was some difference between the groups even in the latest period assessed. A greater proinflammatory reaction, mediated by TNF α , was found in group 2, which contained only decellularized tissue compared to the other groups, but this was not seen in the assessment of IL6. Such finding is expected since the scaffold basically behaves like a foreign body [22]. However, a decrease in the IL10-mediated anti-inflammatory action was seen in the animals of group 3, which received allogeneic ASCs, which may be a sign of a delayed rejection reaction [23,24].

Interestingly, our experiments did not detect serum or tissue IL4 in any group. IL4 was previously selected to assess whether ASCs would have any anti-inflammatory activity in the experiment with biological scaffolds. However, we could not find significant levels of IL4 in any of our experiments; perhaps this can be explained by the volatility of the cytokine in the samples, the sensitivity level of the test, or the animal model used, or technical problems in the experiment; however, this question remains open [25]. Regardless, the immunomodulatory role of ASCs has already been established in experiments of this nature, and it is likely that they perform this function while not differentiating in other cell types [26–29].

Regarding the cellular differentiation of ASCs and the structural organization of these neovessels, the implants of groups 3 and 4, composed of decellularized vena cava + ASCs, were more promising. The presence of these red-marked cells, derived from viable cultures, was detected in the tissue even after 60 days of implantation.

5. Conclusions

The group with a decellularized vena cava scaffold (SDS) recellularized with autologous ASCs showed a lower host immunogenic response considering serum and tissue cytokines. This group also showed the best histological analyses, with a greater cellular organization and endothelial differentiation compared to using allogeneic tissue, unpopulated scaffolds, or allogeneic-ASCs-repopulated scaffolds.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biom12121776/s1>, Supplementary S1: Decellularization protocol.

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

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Data Availability Statement: All survey data can be requested from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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Anexo 8

Ribeiro MCO, Lemos JVB, **de Toledo Moraes MP**, Oliver FA, Alvarez M, Silva GF, Qi X, Romeiro FG. **Hepatic epithelioid hemangioendothelioma: how fast does it grow and which findings could have prevented diagnostic delay? - a case report.** Transl Gastroenterol Hepatol. 2023 Jan 25;8:12. doi: 10.21037/tgh-22-48. PMID: 36704649; PMCID: PMC9813661.



Hepatic epithelioid hemangioendothelioma: how fast does it grow and which findings could have prevented diagnostic delay? – a case report

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Background: Hepatic epithelioid hemangioendothelioma (HEHE) is a rare neoplastic disease of varied presentation and unspecific radiological signs in the early stages. The diagnostic delay can lead to metastatic disease, thus increasing the tumor burden and reducing the treatment options. HEHE is usually deemed a slow-growing tumor, but its speed of growth is poorly reported and still unknown.

Case Description: In this case report, we documented a HEHE diagnosed in a young woman who had complaints of abdominal pain, weight loss and bloating for a long time. The typical findings observed in histological studies were not promptly recognized in the histological analyzes, even after two laparoscopic-guided liver biopsies, delaying the diagnosis until extrahepatic tumor spreading. Findings observed in computed tomography, magnetic resonance imaging and histological studies are presented. The coalescence of nodules and the rising of giant masses, occupying large parts of the liver in a specific time span, were registered and quantified. As opposed to prior reports, the results show that hepatic HEHE can grow rapidly, reinforcing the need of early diagnosis, thus avoiding the complications presented herein.

Conclusions: The findings observed via radiological and histological imaging that could have avoided the diagnosis delay are depicted and discussed, showing that HEHE can rise faster than previously documented.

Keywords: Liver cancer; extrahepatic metastasis; imaging; liver neoplasms; case report

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Introduction

Hepatic epithelioid hemangioendothelioma (HEHE) is a rare sarcoma with a varied presentation, from indolent nodules to aggressive tumors. The primary sources of the tumor tissue are pre-endothelial or vascular endothelial cells (1). Despite a similar tumor having been found in the lung and reported in 1975, the first epithelioid hemangioendothelioma description was in 1982 when the authors report an atypical vascular tumor that had been frequently misdiagnosed as carcinoma (2,3). Nearly 50% of epithelioid hemangioendothelioma are found in association with a preexisting vessel, so their microscopic appearance is of infiltrative cords of epithelioid endothelial cells implanted in a myxohyaline stroma growing within the vessel or around it (3). The tumor cells are usually positive for CD31, CD34, and cytokeratin, with a translocation that allows confirmation of the diagnosis, denoted t(1;3) WWTR1-CAMTA1 fusion, which can be detected by fluorescence in situ hybridization (FISH) (3). Less commonly, the fusion found is YAP1-TFE3, detected by TFE3 immunoreactivity (4). While HEHE has been known as a slow-growing tumor of indolent nature (5-7), this is the first case report in which the tumor growth was measured, showing nodules that enlarged and spread to other organs in a given time span. We present the following case in accordance with the CARE reporting checklist (available at <https://tgh.amegroups.com/article/view/10.21037/tgh-22-48/rc>).

Case presentation

All procedures performed in this study were in accordance with the ethical standards of the institutional research committee and with the Helsinki Declaration (as revised in 2013). Written informed consent was obtained from the patient's legal guardian for publication of this case report and accompanying images. A copy of the written consent is available for review by the editorial office of this journal. The local ethics committee (named "Comitê de Ética em Pesquisa") approved the development of this study (Protocol No. 5.235.006).

The patient is a 17-year-old female from the interior of São Paulo state, Brazil. Her first complaints were related to the mass effect caused by the liver tumor, such as abdominal pain and bloating during the last 3 years. She also reported weight loss in recent months. Painful hepatomegaly was described in her medical appointments since 2018. The first abdominal ultrasonography was performed in September

2018 and showed hypoechoic hepatic nodules of 12×31 mm. A magnetic resonance imaging (MRI) on February 8, 2019, revealed 11 hyperintense nodules in T2, filled by liquid material and peripheral enhancement by the contrast medium, thus suggesting necrotic content, which would be the first sign that the nodules deserve further investigation (*Figure 1*). The largest nodule measured 6.3×6.1×4.0 cm, and was located in the liver segment VI. At that time, she already had an enlarged spleen, reaching 13.5 cm. Her blood tests were unremarkable.

In March 2019, she was submitted to a laparoscopic liver biopsy at a local hospital. The first analysis by the local pathologists was "No signs of malignancy. The histologic specimen is formed by granulation tissue with marked fibrosis and hyalinization, embracing and compressing biliary ducts and hepatocytes. Some of these cells contain vacuolated cytoplasm and are disposed in cords". However, the sample already had findings compatible with HEHE such as cords of epithelioid tumor cells, and intracytoplasmic vacuoles containing red blood cells (shown in *Figure 2A,2B*). At that moment, no malignancy had been suspected and no immunohistochemical assay was carried out. Only when the patient was forwarded to our hospital in July 2021 the immunoreaction for CD31 was observed on the same sample from the first liver biopsy (*Figure 2C*).

In August 2019, the patient reported fever and worsening abdominal pain. Intermittent ultrasonography assessment was maintained until reaching new signs of tumor growth. Then, a computerized tomography (CT) was done in May 2020 showing that the tumor located in the liver segment VI had dimensions of 12.9×7.3 cm (shown in *Figure 3*). In February 2021, the patient was submitted to a new MRI that showed delayed enhancement of the tumors by the gadolinium.

In April 2021, a second laparoscopic liver biopsy was done. Unfortunately, the histologic assessment was still deemed inconclusive by the local pathologists, who reported no malignant tissue in spite of the HEHE findings. Ascitic fluid was also assessed, but no malignant cells were found. At that time, she reported a dull pain in her knees. Due to the lack of a diagnosis at that time, the patient was forwarded to our hospital in July 2021, with painful hepatomegaly, asymptomatic splenomegaly, and pain in both knees, but no respiratory complaints. The histologic tumor samples were reassessed and the HEHE findings were finally recognized. Additional staining for CD31 was performed, thus confirming the diagnosis (shown in *Figure 2C*).

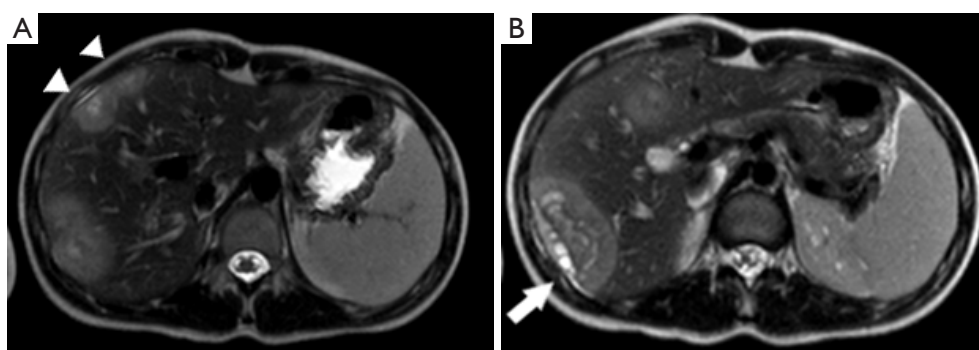


Figure 1 Abdominal resonance magnetic imaging in February, 2019. (A) White target sign in which the bright central core is surrounded by a peripheral slightly hyperintense halo on T2-weighted images (white arrowheads). (B) Subcapsular lesions on T2-weighted images with capsular retraction (white arrow).

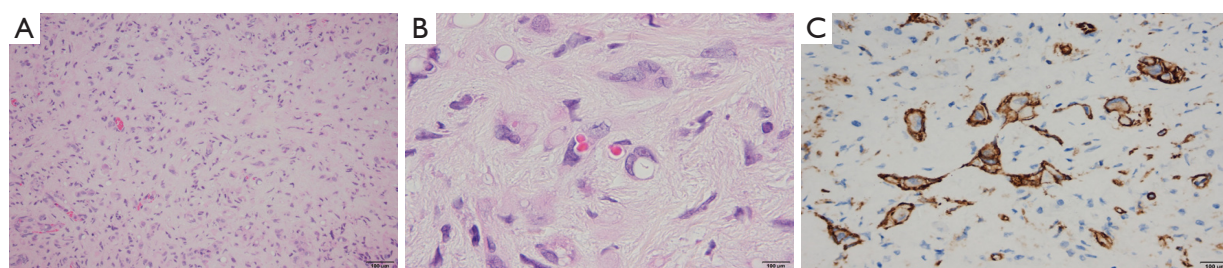


Figure 2 Histologic findings from the sample obtained through laparoscopic biopsies. (A) Hematoxylin-eosin staining showing cords of epithelioid tumor cells within a characteristic myxoid to hyaline matrix. (B) Hematoxylin-eosin staining displaying intracytoplasmic vacuoles. These spaces represent immature vascular lumina and may contain erythrocytes. (C) Immunohistochemistry showing the expression of CD31, an endothelial marker.

A chest CT showed 7-mm nodules diffusely distributed in both lungs, associated with longitudinal fissure thickening (shown in *Figure 4*). A new MRI showed an enlarged liver, containing infiltrative tumors occupying most parts of the right lobe and part of the liver segment III, causing retraction of the liver capsule on segment VI, similar to the findings observed in the last CT (shown in *Figure 5*). The tumor was heterogeneous and led to hepatic vessels distortion. It was filled by nodules with a target aspect, reaching up to 17 mm, with progressive enhancement during the contrast medium injection.

In the same month, bone scintigraphy was carried out, revealing bone metastasis. In spite of good outcomes reported in a large cohort of patients with metastatic HEHE submitted to liver transplantation and liver resection (8), multi-organ disease prevents liver transplantation in Brazil, and liver resection could not be performed due to the hepatic spreading of the tumors. Therefore, no surgical treatment was done. Ten sessions of

radiotherapy was done and sorafenib was started due to the benefits reported in another case (9). Despite prior reports considering thalidomide as another option (9,10), the drug was not used because the effect on vascular endothelial growth factor (VEGF) is not as powerful as that observed with sorafenib (9). The treatment was well tolerated by the patient, who adhered properly to the medical recommendations.

Aiming to estimate the tumor growth between the two MRIs carried out in 2019 and 2021, a 2.5D volumetry of the liver was made using 3D Slicer® 4.11 on the MRI T2 weighted arterial images (11). The segmentation employs a supervised semiautomatic nodule segmentation algorithm based on open-source software. First, seed points were placed within the liver region to initialize the segmentation using a region-growing algorithm. When the image contained multiple nodules, a seed point was placed inside each nodule in axial, sagittal, and coronal directions. The contour propagation was governed by a smoothing process,



Figure 3 Abdominal tomographic findings in May 2020. The tumors were typically seen as multiple hypoattenuating nodules in both hepatic lobes, coalescing to form larger confluent lesions with peripheral or subcapsular distribution (A). In (B), a halo (or target pattern) of enhancement can be seen around a larger lesion (white arrow). In (C,D), capsular retraction can be seen near two subcapsular tumors (white arrowheads). In (E), a portal vein branch tapers and terminates just within the edge of a tumor denoted as “lollipop sign” (black arrow).

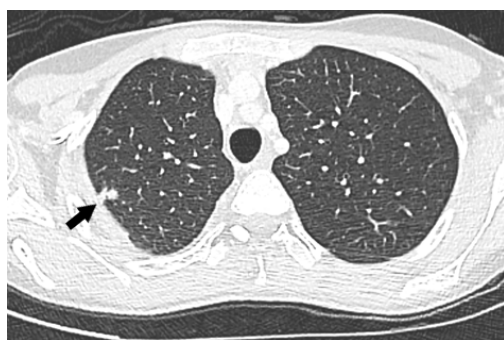


Figure 4 Chest tomographic findings in April 2021. Hyperdense nodules of 7 mm were observed in both lungs (black arrow).

which minimizes the contour curvature by a Gaussian filter of size 5 pixels. A final analysis was carried out and, if a segmented region mismatches the liver (or tumor) area, it was manually cropped or added to the segmentation. The results are shown in *Table 1* and in *Figures 6,7*.

As explicit in *Table 1*, the liver volume measured in February 2019 increased by 27.12% in July 2021. In the same period, the tumor volume grew from 9.26% to 70.27% of the liver. *Figures 6,7* show that many nodules

coalesced, thus changing the nodules to giant tumors and causing hepatomegaly in this time span. The tumor cells presented TFE3 immunoreactivity (shown in *Figure 8*), suggesting the presence of the YAP1-TFE3 fusion, a distinct histological HEHE pattern in which mature lumina and epithelioid eosinophilic cells are observed (4).

Discussion

Three HEHE clinical findings could be used to suggest it as a possible diagnosis. First, the liver is the most commonly affected organ. Second, the tumor is more common among women. Third, abdominal pain is the main symptom. Lau *et al.* and Mehrabi *et al.* reported the largest reviews, containing 206 and 434 cases, respectively (12,13). In the study by Lau *et al.*, the liver was involved in 99 cases (32%), the lung in 89, and the bones in 48. The patients' age at the diagnosis ranged from 7 to 81, and the female proportion was 61%. Single-organ involvement occurred in 131 patients, of which 21% had only liver tumors. Pain was the most common symptom, occurring in 74% of the symptomatic patients (12). Right upper quadrant

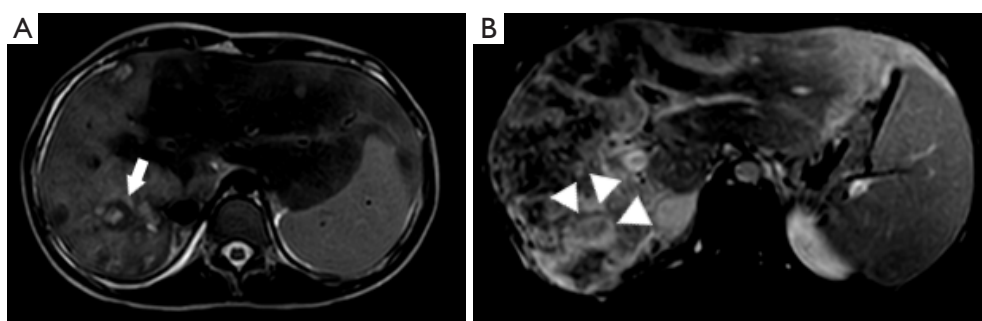


Figure 5 Abdominal resonance magnetic imaging in July 2021. In (A), a T2 axial image shows heterogeneously increased signal intensity, with a white target sign in which the bright central core is surrounded by a peripheral slightly hyperintense halo (white arrow). In (B), the gadolinium-based contrast agent shows lesions with either a peripheral halo or a target-type enhancement pattern, with a thin peripheral hypointense rim (white arrowheads).

Table 1 Liver and tumor volumes estimated from the magnetic resonance imaging in 2019 and 2021

Analysis	2019	2021	Ratio 2021/2019
LV (cm ³)	949.581	1,207.15	127.12%
TV (cm ³)	87.96	848.225	964.33%
Ratio TV/LV	9.26%	70.27%	

LV, liver volume; TV, tumor volume.

pain, hepatomegaly, and weight loss are the most common symptoms, and the male-to-female ratio is 2:3 (13). Despite the estimated prevalence of less than one in 1 million (8), dealing with hepatic nodules requires knowledge on HEHE as a possible diagnosis, especially when the three most common symptoms are present.

In such cases, a multidisciplinary team is needed to evaluate the imaging exams. In 2019 the patient already had tumors that could be easily distinguished from hemangiomas or other non-threatening lesions, as shown in *Figure 1*. Peripheral nodules with central hypodensity, calcifications, and capsular retraction are common signs of HEHE in CT and MRI images (14). Capsular retraction was already observed, suggesting malignant compromise. Of note, it occurs in less than 25% of patients, and signs of portal hypertension may also be observable due to the tumors' growth (14). Since the patient had painful hepatomegaly and the radiological images were not recognized as HEHE, the decision to perform a biopsy at that time was correct.

As shown in *Figure 2*, the liver biopsies had typical findings to make the diagnosis, with cords of epithelioid tumor cells, intracytoplasmic vacuoles containing red blood cells and

endothelial markers in immunohistochemistry (3). However, since the images in hematoxylin-eosin staining (HE) were not properly identified in the first evaluations, immunohistochemistry was not carried out, and the pathology conclusions were mistaken. Only when skilled pathologists assessed the samples was the disease diagnosed and further confirmed by immunohistochemistry. Regrettably, diagnostic mistakes are not rare in EHE, and this case shows how the lack of an experienced pathologist can be deleterious, especially assessing liver nodules. The two initial histological analyzes overlooked the tumor cell cords, mistakenly assuming that they were hepatocytes. As a result, no further staining or immunohistochemistry assay was carried out, and the presence of a malignant disease was wrongly denied. After two laparoscopic-guided biopsies suggesting no malignancy, the pediatricians withheld the diagnostic investigation. Certainly, a consultation with a more experienced pathologist could have avoided this terrible mistake. CD34 is expressed in more than 90% of the vascular tumors, showing a good sensitivity for initial evaluation, but is not specific to diagnose HEHE (1). CD31 is more specific and could be enough to confirm the diagnosis if the other signs of HEHE had been identified (1). In some cells, intracytoplasmic vacuoles can displace the nucleus, the so-called blister cells, leading to a signet ring-like structure (14,15). A common finding in liver HEHE is the fibrotic stromal background (15). Unfortunately, none of these signs were recognized or described after two laparoscopic-guided biopsies.

The same problem occurred with the radiological findings. HEHE can appear as multiple nodules in the initial stages, but they tend to coalesce to form large lesions that

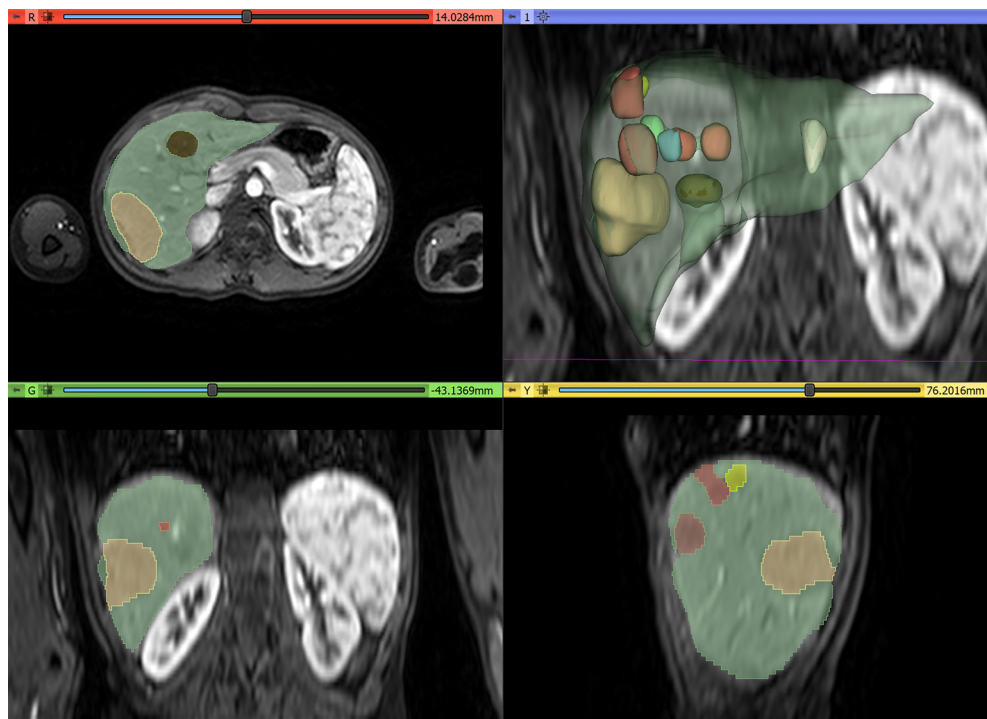


Figure 6 Volumetric images obtained from the magnetic resonance imaging in February 2019. The normal liver parenchyma is shown in green whereas the tumors are presented in other colors. Left upper quadrant: axial view. Right upper quadrant: three-dimensional view. Left lower quadrant: coronal view. Right lower quadrant: sagittal view.

grow diffusely into the liver (14,16). Despite the signs of malignancy observed in 2019, the disease was not diagnosed, allowing spreading to other organs. Bone involvement can cause pain, neurological symptoms, and even fractures, whereas pulmonary disease is usually manifested as coughing (16). Most patients with the single-organ disease receive surgical treatment and have no recurrence in 5 years (15). On the other hand, multi-organ disease and tumors with poor or moderate cell differentiation are findings of worse prognosis (15,17). Since the first symptom of our patient was painful hepatomegaly, maybe she could have been submitted to a curative treatment. However, the metastasis led us to offer only palliative (radiotherapy) and off-label (sorafenib) options, which were well-tolerated and provided relief from the bone pain, but did not change the liver and lung disease.

While the nodular type can be non-specific, the diffuse HEHE type is considered a slow-growing tumor, occupying the liver periphery, and not leading to capsular bulging (13). However, the time elapsed between the first symptoms and the diagnosis is usually long, leading to massive tumor growth, usually occupying both liver

lobes when the disease is finally recognized (13). For the first time, the tumor growth was precisely estimated, showing that in a 29-month period the liver commitment was markedly changed, increasing 964.33% and leading to significant liver enlargement (127.12%). Fan *et al.* reported a fast-growing HEHE in which one of the tumors increased 9.45% within 2 months (18). However, only the growing of the largest lesion was documented in a short time span, thus reducing the measurement accuracy. As a result, the term “slow-growing tumor” seems inadequate for HEHE in patients as the one reported herein. This case is a clear example that a rapid growth can be observed in multinodular disease when its first signs are not recognized.

In spite of the limitations of a case report, this is the first time that HEHE growth was accurately measured in a given period. The results show that each sign of the disease must be recognized as soon as possible, thus avoiding diagnostic delay in similar cases.

It was also demonstrated that HEHE nodules are able to occupy the liver and spread to other organs in a short time span, a finding that is not compatible with a slow-growing tumor.

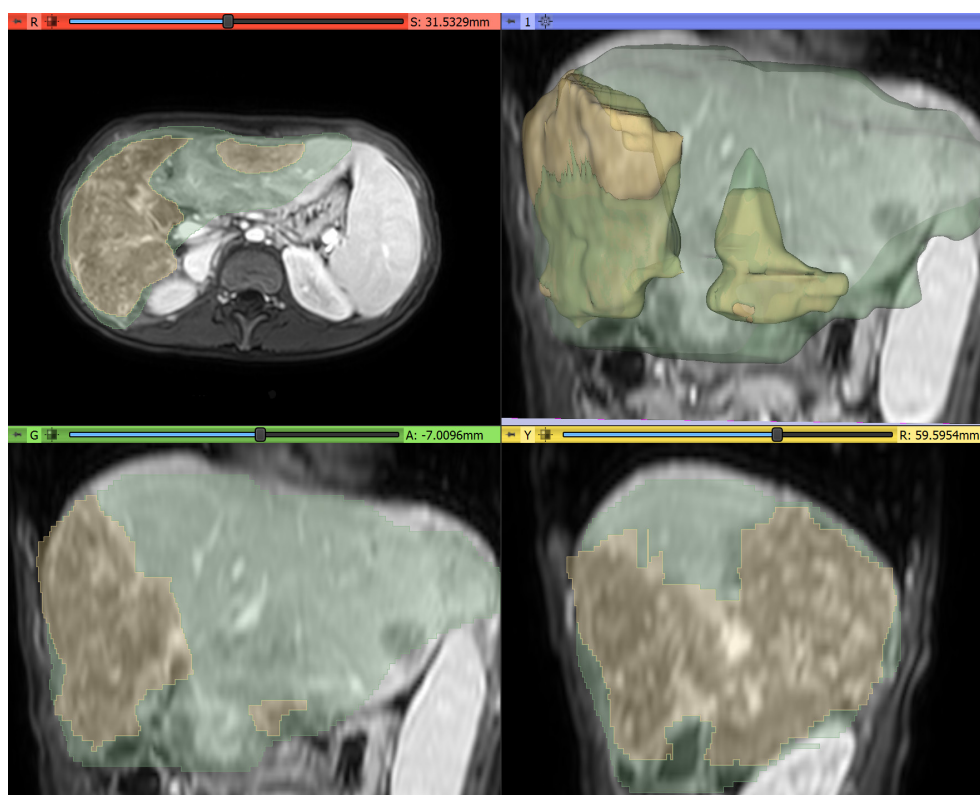


Figure 7 Volumetric images obtained from magnetic resonance imaging in July 2021. The normal liver parenchyma is shown in green whereas the tumors are presented in other colors. Left upper quadrant: axial view. Right upper quadrant: three-dimensional view. Left lower quadrant: coronal view. Right lower quadrant: sagittal view.

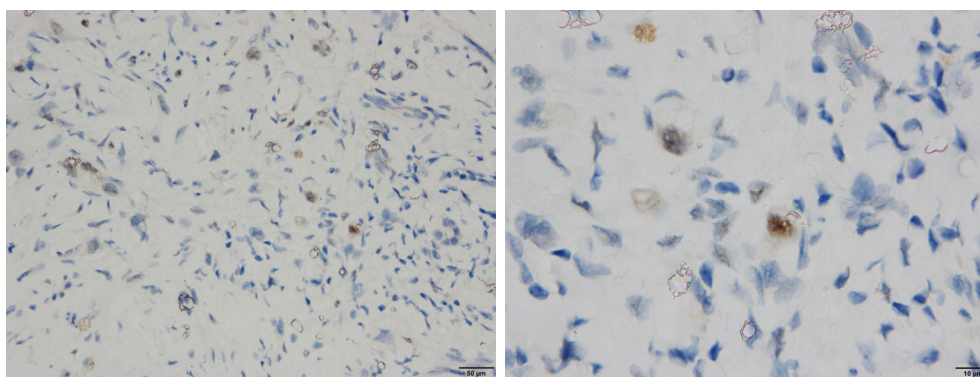


Figure 8 Weak nuclear immunoreactivity for TFE3 suggesting the presence of the YAP1-TFE3 fusion.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study were in accordance with the ethical standards of the institutional research committee and with the Helsinki Declaration (as revised in 2013). Written informed consent was obtained from the patient's legal guardian for publication of this case report and accompanying images. A copy of the written consent is available for review by the editorial office of this journal. The local ethics committee (named "Comitê de Ética em Pesquisa") approved the development of this study (Protocol No. 5.235.006).

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Anexo 9

Campos MAG, Vasques LF, de Medeiros RG, Cutrim EMM, Favarin AJ, Silva SEM, Silva GEB, **Moraes MPT**, Zanatta ML, Queiróz DAR. **Malignant lung PEComa (clear cell tumor): rare case report and literature review.** Front. Oncol., 20 September 2023. doi.org/10.3389/fonc.2023.1260844.



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Malignant lung PEComa (clear cell tumor): rare case report and literature review

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Clear cell tumors of the lung (CCTL), or “sugar tumors” of lung, are very uncommon lesions and are mostly benign perivascular epithelioid cell (PEC) tumors with no specific morphologic features. Fewer than 100 cases have been reported; the aggressive nature demonstrated in sporadic reports has rarely been described in the literature. Although the course is generally described as benign, eight reported cases showed malignant behavior. We report a case of a PEC with a malignant presentation in a young man, correlating the main characteristics of the tumor with other cases reported in the literature to better elucidate this rare presentation. We also performed a literature review of reports on benign and malignant CCTL cases, with a focus on clinical, imaging, and immunohistochemical differentiation. CCTLs are rare tumors that require histopathological and immunohistochemical confirmation; to date, criteria that can predict malignant evolution are lacking.

KEYWORDS

PEComa, sugar tumor, pulmonary cancer, cathepsin K, HMB-45

Introduction

Clear cell tumors, or “sugar tumors”, are very uncommon lesions (1) and are mostly benign perivascular epithelioid cell (PEC) tumors (2, 3). PEC tumors are a rare group of mesenchymal neoplasms histologically and immunohistochemically distinct from other neoplasms, with an abundance of periodic acid-Schiff (PAS)-positive glycogen (3). They are expressed in the bladder (4), uterus (5), breast (6), ovary (7), vagina (8), kidney (9), prostate (10), pancreas (11), jejunum (12), soft tissues (13) and lung (14). Due to this wide diversity, these tumors lack specific morphologic features (2).

The lungs are an uncommon site for PEC tumors, known as clear cell tumors of lung (CCTL) (15), and such PEC tumors in the lung are usually benign, with malignancy exceedingly rare (9). Although fewer than 100 cases have been reported, sporadic reports have described CCTLs with very rare aggressive nature (3). A silent, indolent, and asymptomatic presentation may pose a diagnostic challenge for pathologists and oncologists (16).

CCTLs exhibit nested histological features similar to other PEC tumors. They consist of uniform round to polygonal epithelioid cells with clear or eosinophilic cytoplasm and well-defined borders. CCTLs are surrounded by prominent thin-walled vascular channels. Although the course of CCTL is typically described as benign, eight cases have shown malignant behavior (17). We present a case of CCTL with a malignant presentation, correlating the main characteristics of the tumor with other cases reported in the literature to better elucidate this rare presentation.

Case description

A 49-year-old man, hypertensive and a smoker, presented to the primary healthcare department with complaints of dyspnea on exertion and daily chest pain for the past year, with gradual worsening in the last three months, in addition to a weight loss of 10 kg. The patient denied coughing, hemoptysis, fever, or a family history of cancer.

Computed tomography (CT) of the chest showed a solid, calcified, upper left paratracheal lesion (191 cm³) with central necrosis in close contact with the ipsilateral subclavian artery without a cleavage plane, in addition to multiple randomly distributed nodules in the lung parenchyma and enlarged lymph nodes in the para-aortic chain and thin left pleural effusion

(Figure 1). A lithic image of the T1 vertebral body indicated possible secondary neoplastic involvement; however, bone scintigraphy and positron emission tomography-CT (PET-CT) revealed a low probability of bone metastasis. No secondary lesions were observed in any of the abdominal organs.

The anatomopathological examination of the transbronchial biopsy showed the presence of neoplasia caused by epithelioid cells with abundant clear and granular cytoplasm, well-defined cell borders, and round and hyperchromatic nuclei, without the presence of prominent nucleoli (Figure 2). There were mild cellular atypia and foci of hemorrhage, necrosis, and absence of significant mitotic activity. Immunohistochemical (IHC) analysis revealed negativity for pan-cytokeratin (AE1, AE3, TTF1, RCC, MiTF, S-100, CK7, CK8, CK20, and desmin) and positivity for muscle markers and melanocyte antibodies (HMB-45, smooth actin muscle, CD10, and Melan-A) and Cathepsin K. These findings were indicative of a CCTL.

The challenges of verifying diagnosis and scheduling medical appointments, combined with suboptimal patient adherence, led to delayed treatment initiation. Only, five months after the biopsy, the patient underwent 2000 cGy doses of radiotherapy targeting the left lung in five fractions over one week and three cycles of doxorubicin 75 mg/m² within three months. However, owing to his compromised performance status and the presence of multiple nodules, surgical resection could not be performed. He presented with deterioration of the clinical picture and progression of pulmonary nodules in number and size two months after starting treatment, in addition to an increase in mediastinal lymph nodes and bilateral pleural effusion.

One month later, the patient was admitted to the emergency room with dyspnea, desaturation, and severe chest pain. Additionally, he had mild respiratory alkalosis associated with microcytic and hypochromic anemia without leukocytosis or normal platelet levels. He had normal electrolyte and renal function, increase in transaminase levels by about three times the upper normal value, and slight hypoalbuminemia, without changes in bilirubin or coagulogram. C-reactive protein was 8.9 mg/dl (reference value, < 1 mg/dl), with no reports of infectious symptoms. Serological tests for hepatitis, HIV and syphilis were negative. Chest radiography and CT revealed a significant progression of the lung masses (Figure 3). There was no evidence of other complications such as massive pleural effusion, pulmonary embolism, or pneumothorax, which would justify respiratory worsening in addition to the progression of tumor masses. Owing to the rapid progression of his condition, the patient underwent palliative sedation and died approximately 48 h after admission. A timeline specifying the entire clinical evolution of the patient is shown in Figure 4.

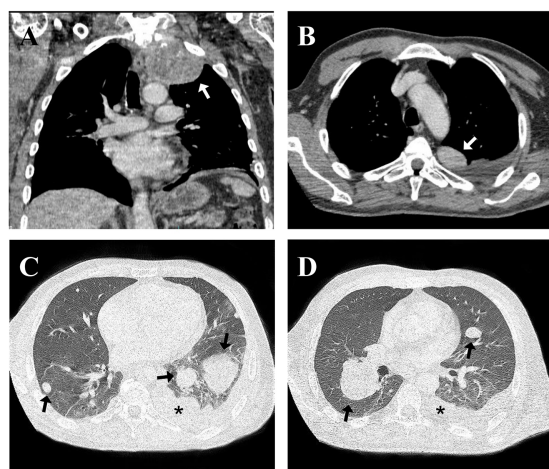


FIGURE 1

Initial coronal (A) and axial (B) computed tomography pulmonary angiogram reconstruction of the chest performed showing a mass in upper left paratracheal side (white arrow in A, B) associated with multiple randomly distributed nodules in the lung parenchyma (black arrow in C, D), the largest of them in the middle third of the lung right, enlarged lymph nodes in the para-aortic chain and thin left pleural effusion (black asterisk in C, D).

Discussion

Herein, we present a rare case of malignant CCTL. It is an uncommon and usually benign neoplasm composed of uniform round to polygonal epithelioid cells with clear or eosinophilic cytoplasm, well-defined borders, and prominent thin-walled

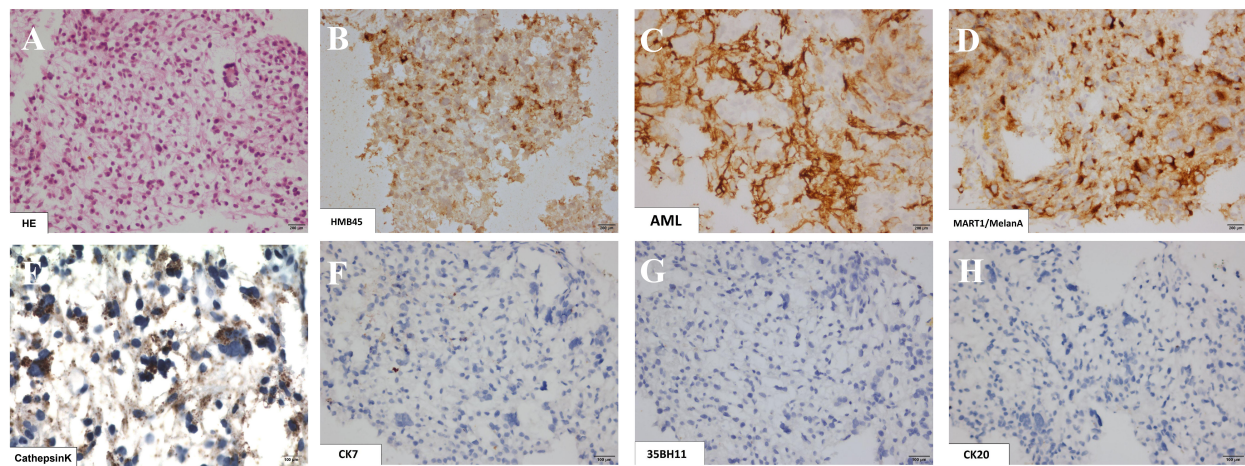


FIGURE 2

Histological sections of lung biopsy showing in hematoxylin-eosin staining the presence of clear cell tumors represented by epithelioid cells with abundant clear and granular cytoplasm, with well-delimited cell borders and round and hyperchromatic nuclei, discreet cell atypia and absence of significant mitotic activity (A). The immunohistochemical study revealed co-expression of markers: HMB45 (B), smooth muscle actin (C), Melan-A (D) and Cathepsin K (E), and negativity for cytokeratins CK7, CK8 and CK20 (F, G, H, respectively).

vascular channels (1), as observed in our case. Throughout the clinical and imaging evaluation, the malignant presentation of the patient was clear.

CCTL has no established epidemiology, clinical characteristics, diagnoses, or treatments. It is commonly confused with lung carcinoma or metastases from clear-cell carcinoma of the kidney (18). Patients are usually > 50 years old, but cases from 9 to 75 years of age have been reported (15, 19), with a similar prevalence between the sexes (20).

Among the benign manifestations of CCTL, an asymptomatic condition with an accidental diagnosis is common. However, as the size of this neoplasm can vary from several millimeters to approximately 16 cm (15), symptoms such as chest pain, cough,

shortness of breath, hemoptysis, or fever may be observed in larger masses, especially if the size exceeds 2.2 cm (21–24).

We performed a literature review of the cases of benign and malignant CCTL (Table 1). Cases of CCTL with malignant behavior during the course of the disease and its outcomes have been reported sporadically. Certain characteristics, such as diameter greater than 2.5–3.0 cm, presence of symptoms, metastases, extensive necrosis, and abundant mitoses, are associated with a malignant presentation (24). Of the 76 benign occurrences found, necrosis was present in only 6.5% of the cases and mitotic activity was present in 4.0%. The largest mean mass diameter was 2.8 cm. In malignant cases, necrosis and mitotic activity were significantly more frequent, occurring in 50% and 12.5% respectively, and the mean diameter of the mass was 6.7 cm, which is 2.3 times greater than that of benign masses.

The site that was most affected by the primary nodule was the lower lobe of the lung. In this case, an extensive lesion was observed in the left upper lobe, characterized by necrosis and abundant vascularization, in addition to other scattered nodules in the parenchyma.

CCTL has already been associated with other diseases, such as tuberous sclerosis complex (79), Birt-Hogg-Dube Syndrome, and essential thrombocythemia (26, 27). However, in the malignant cases, it was not possible to determine the presence of risk factors strongly related to CCTL (Table 2).

PEC tumors can manifest in the bladder, uterus, breast, ovary, vulva, vagina, kidney, prostate, pancreas, jejunum and soft tissues, often with benign behavior (2, 14). PEC tumors originating in soft tissues and gynecological have some findings suggestive of malignancy (8), being indicative in the presence of 2 or more criteria: > 5 cm, infiltration, high-grade atypia, necrosis, vascular invasion, mitoses > 1/50 high power fields. Some authors (80) suggest greater specificity for PEC tumors of gynecological origin with at least 4 or more of these characteristics. But WHO followed the guidance of at least 3 criteria for malignancy (81).

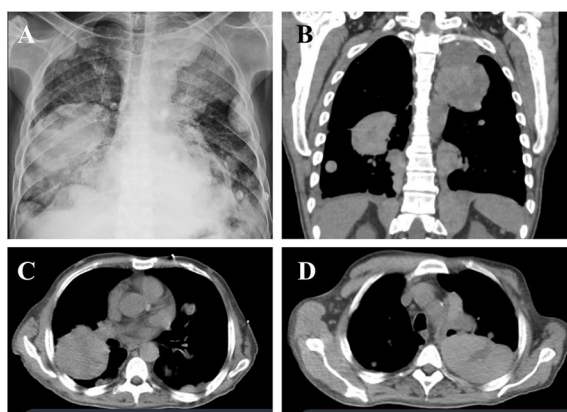
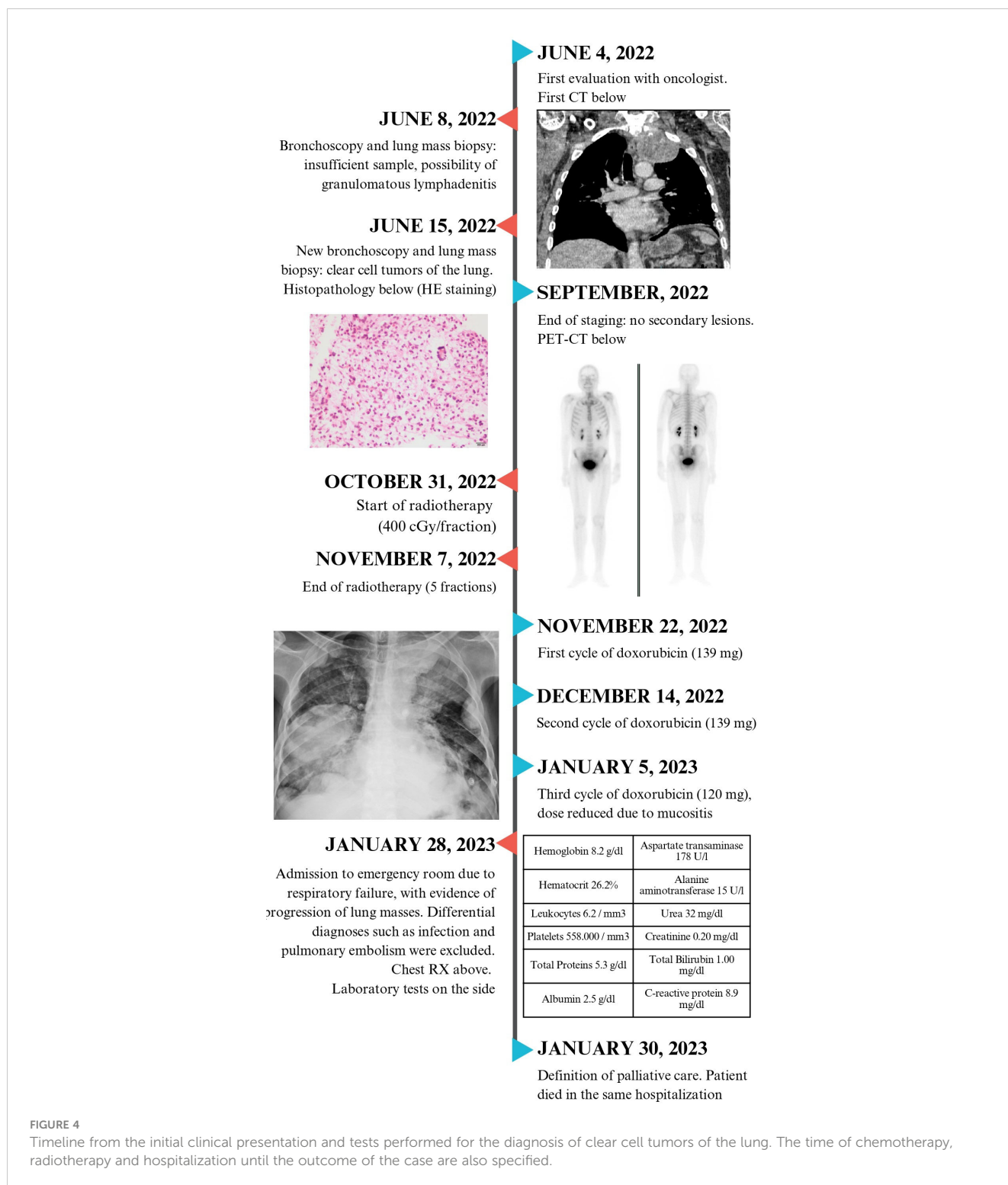


FIGURE 3

Radiographic images showing extensive pulmonary masses on chest X-ray (A) and computed tomography of the chest in coronal (B) and axial planes (C, D), suggesting disease progression. A solid tumor, calcified upper left parasternal lesion with central necrosis, close contact with ipsilateral subclavian artery, without a cleavage plane and a tumor with similar characteristics on the lower right.



There are currently no well-defined diagnostic methods for malignant CCTL. Zhao et al. proposed major and minor criteria for differentiating between benign and malignant manifestations (17). Imaging characteristics are nonspecific, and the tumor can appear in any lobe (although more commonly in the bilateral lower lobes) with a well-demarcated nodule without cavitation or calcification (24). The presence of a single and intraparenchymal pulmonary nodule is common to several diseases in addition to CCTL, such as

bronchogenic carcinoma and oat cell carcinoma, others tumors such as angiomyolipoma, and with metastases from clear cell carcinoma of the kidney, breast, liver, ovary, uterus and cervix, adrenal and melanoma (28, 82). Due to the nonspecificity of the image, histopathological and immunohistochemical is highly recommended for the CCTL characterization (19)

At optical microscopy the CCTL cells are round or oval, have clear cytoplasm strongly stained by PAS due to the presence of

TABLE 1 Comparison of features between benign and malignant cases of perivascular epithelioid cells (PEC) tumors.

Characteristic	Benign (81) ^a n (%)	Malignant (8) ^b n (%)
Sex		
Male	45 (55.5)	3 (37.5)
Female	36 (44.4)	5 (62.2)
Range age	49 (10 – 75) years	60 (49 – 75) years
Symptoms		
Not reported	31 (38.2)	2 (25.0)
Cough	7 (8.6)	3 (37.5)
Chest pain	13 (16.0)	2 (25.0)
Dyspnoea	8 (9.8)	0 (0.0)
Absente	26 (32.0)	0 (0.0)
Pulmonary tumor site		
Lower right	16 (19.7)	2 (25.0)
Lower left	22 (27.1)	4 (50.0)
Upper left	14 (17.2)	0 (0.0)
Superior right	13 (16.0)	1 (12.5)
Middle right	6 (7.4)	1 (12.5)
Middle left	4 (4.9)	0 (0.0)
Range of the largest diameter of the lesion	2.78 (0.4 - 12) cm	6.7 (3 - 16.2) cm
Concurrent nodules		
Single node	75 (92.5)	5 (62.2)
Two nodules	2 (2.4)	1 (12.5)
More than two nodules	4 (4.9)	2 (25.0)
Borders		
Regular	65 (80.2)	5 (62.2)
Irregular	4 (4.9)	3 (37.5)
Comorbidities		
Not reported	9 (11.1)	3 (37.5)
COPD	4 (4.9)	0 (0.0)
SAH	8 (9.8)	0 (0.0)
Dyslipidemia	3 (3.7)	0 (0.0)
Essential Thrombocythemia	2 (2.4)	0 (0.0)
Absente	46 (56.7)	5 (62.2)
Smoking		
Not reported	33 (40.7)	2 (25.0)
Present	19 (23.4)	0 (0.0)
Absent	29 (35.8)	6 (75.0)

(Continued)

TABLE 1 Continued

Characteristic	Benign (81) ^a n (%)	Malignant (8) ^b n (%)
Biomarkers		
HMB45	48 (59.2)	5 (62.2)
Vimentin	21 (25.9)	4 (50.0)
S-100	22 (27.1)	3 (37.5)
Melan-A	15 (18.5)	3 (37.5)
CD34	11 (13.5)	1 (12.5)
NSE	11 (13.5)	0 (0.0)
SMA	4 (4.9)	1 (12.5)
CD56	2 (2.4)	1 (12.5)
Necrosis		
Not reported	25 (30.8)	1 (12.5)
Present	5 (6.1)	4 (50.0)
Absent	51 (62.9)	3 (37.5)
Mitotic activity		
Not reported	16 (19.7)	1 (12.5)
Present	3 (3.7)	1 (12.5)
Absent	62 (76.5)	6 (75.0)
Vascularization		
Not reported	18 (22.3)	–
Present	55 (67.9)	6 (75.0)
Absent	8 (9.8)	2 (25.0)

COPD, chronic obstructive pulmonary disease; SAH, systemic arterial hypertension.

^aReferences (1, 3, 18, 19, 21, 24–78).

^bReferences (14, 17, 23, 36, 74–77).

glycogen, mild nuclear pleomorphism and a characteristic plexus of prominent sinusoidal vessels, but mitotic activity and necrosis are absent (19, 24). This characteristic pattern helps to differentiate CCTL from other conditions, such as angiomyolipoma and adrenal carcinoma (28, 82).

Tumor cells show reactivity for HMB-45 in about 90% of cases, similar to most mesenchymal tumors, and do not express cytokeratins (AE1/AE3, CK7, CK8 and CK20) and epithelial membrane antigen (EMA) in 90% of cases, a fact that facilitates differentiation from other types of tumors (18, 19, 28), as observed in our case. The immunoreactivity of renal cell carcinoma to EMA, cytokeratins, CD10 and RCC are useful for the differentiation of CCTL, despite having vimentin as a positive marker in common. However, in the case of atypical melanomas, positivity to HMB-45 and S-100 present in both, does not allow elucidation of the diagnosis (28). Cathepsin K, the main marker for differentiating PEC tumors from other neoplasms, was diffusely positive in 5 cases of CCTL described in the literature (82), demonstrating the superiority of this marker over HMB-45 in differentiating CCTL

TABLE 2 Malignant cases of perivascular epithelioid cells (PEC) tumors reported in literature.

Reference	Sex/Age	Diagnostic exam	Treatment	Survival
Gaffey M.J. 1990 (36)	Male/66	unreported imaging exam Excisional biopsy	Surgical resection	Metastatic lesions remaining after 13 years. Death after 4 years of recurrence
Jeremy R.P. 2006 (77)	Female/53	CT Excisional biopsy Immunohistochemistry	Surgical resection Resection of brain metastases	Not reported
Ting Ye 2010 (76)	Female/50	CT Excisional biopsy Immunohistochemistry	Surgical resection	Not reported
Benedict Yan 2011 (23)	Female/75	CT Excisional biopsy Immunohistochemistry	Surgical resection	No signs of recurrence after 10 years. Death without clear cause
Hyun-ju Lim 2013 (14)	Male/60	PET/CT Excisional biopsy Immunohistochemistry	Surgical resection	Metastatic lesions remaining after 1 year
Yinghua Song 2017 (75)	Female/49	CT Excisional biopsy Immunohistochemistry	Surgical resection	Clinically stable during follow-up
Jiyong Wu 2019 (74)	Male/67	CT Excisional biopsy Immunohistochemistry	Not reported	Not reported
Jikai Zhao 2019 (17)	Male/54	CT Excisional biopsy Immunohistochemistry	Surgical resection Chemotherapy (paclitaxel and carboplatin)	Clinically stable during follow-up

CT, computed tomography; PET-CT, positron emission tomography-CT.

from other lung tumors. Thus, the co-expression of HMB-45 and cathepsin K markers ensures a more accurate diagnosis.

Furthermore, other markers such as S-100, vimentin, CD34, Melan-A, microphthalmia factor, neuron-specific enolase, cathepsin B, and CD1a can be expressed (18, 20, 28). CD10 may be expressed in other types of non-pulmonary PEComas (83, 84). Our patient tested positive for HMB-45, Melan-A, smooth muscle actin, and CD10, and tested negative for cytokeratins.

Complete surgical resection is probably the most effective therapeutic method at present (85). Radiotherapy may be considered for patients with surgical contraindications (20). Chemotherapy should be administered for malignant manifestations, as in the reported case. CCTLs upregulate the mammalian target of rapamycin (mTOR) signaling pathway; therefore, mTOR inhibitors can be used in such cases (86, 87).

Despite being described in the literature, mitotic activity and some biomarkers may not differ between malignant and benign CCTLs. Except for lesion size, clinical characteristics and complementary examinations cannot predict a worse development. Close follow-up can identify when CCTL cases may exhibit unexpected and malignant behaviors.

Conclusion

Herein, we report the clinical evolution of a patient with malignant CCTL. Although this form of presentation is extremely

rare in the literature, it is not yet possible to establish clinical, immunohistochemical, or histopathological criteria to predict the prognosis and possible malignant evolution. Although described in some studies, mitotic activity does not differentiate between benign and malignant tumors. The size of the initial lesion, presence of multiple lesions, and patient’s clinical evolution seem to be the only comparative parameters.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

Written informed consent was obtained from the proxy for the patient described in the manuscript.

Author contributions

MC: Conceptualization, Methodology, Writing – review & editing. LV: Data curation, Formal Analysis, Investigation, Writing – original draft. RM: Data curation, Formal Analysis, Investigation, Writing –

original draft. ÉM: Conceptualization, Methodology, Writing – original draft. AF: Conceptualization, Methodology, Writing – original draft. SS: Conceptualization, Methodology, Writing – original draft. GS: Supervision, Visualization, Writing – review & editing. MM: Data curation, Visualization, Writing – review & editing. MZ: Data curation, Investigation, Visualization, Writing – review & editing. DQ: Conceptualization, Investigation, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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