UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

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ANÁLISE DAS ALTERAÇÕES MICROSCÓPICAS EM GLÂNDULAS SALIVARES MENORES E FÍGADO APÓS O TRANSPLANTE ALOGÊNICO DE CÉLULAS TRONCO HEMATOPOÉTICAS

Tese de Doutorado apresentada à Faculdade de Odontologia de Piracicaba da UNICAMP para obtenção do título de Doutor em Estomatopatologia, Área de Concentração Patologia.

Orientador: Prof. Dra. Maria Elvira Pizzigatti Corrêa

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Este exemplar corresponde à versão final da Tese defendida pelo aluno, e orientada pela Prof. Dra. Maria Elvira Pizzigatti Corrêa.

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Assinatura do Orientador

PIRACICABA, 2012

FICHA CATALOGRÁFICA ELABORADA POR
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FACULDADE DE ODONTOLOGIA DE PIRACICABA DA UNICAMP

So11a

Soares, Tânia Cristina Benetti, 1978-
Análise das alterações microscópicas em glândulas salivares menores e fígado após o transplante alogênico de células tronco hematopoéticas / Tânia Cristina Benetti Soares. -- Piracicaba, SP: [s.n.], 2012.

Orientador: Maria Elvira Pizzigatti Corrêa.
Coorientador: Cecília Amélia Fazzio Escanhoela.
Tese (doutorado) - Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Doença enxerto-hospedeiro. 2. Imunohistoquímica. I. Corrêa, Maria Elvira Pizzigatti. II. Escanhoela, Cecília Amélia Fazzio, 1958- III. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. IV. Título.

Informações para a Biblioteca Digital

Título em Inglês: Analysis of microscopic changes in minor salivary glands and liver after allogeneic hematopoietic stem cell transplantation
Palavras-chave em Inglês:
Graft vs host disease
Immunohistochemistry
Área de concentração: Patologia
Titulação: Doutor em Estomatopatologia
Banca examinadora:
Maria Elvira Pizzigatti Corrêa [Orientador]
Kunie Iabuki Rabello Coelho
Afonso Celso Vigorito
Albina Messias de Almeida Milani Altemani
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Data da defesa: 29-03-2012
Programa de Pós-Graduação: Estomatopatologia

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Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 29 de Março de 2012, considerou a candidata TÂNIA CRISTINA BENETTI SOARES aprovada.

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DEDICATÓRIA

Ao meu Senhor, Jesus Cristo que me capacitou e me sustentou para realizar este trabalho;

Aos meus pais, José Benetti e Vera Lúcia pelo carinho apoio e incentivo;

À minha irmã, Fabrícia pela paciência;

Ao meu marido, Roberto pelo amor, compreensão e apoio.

AGRADECIMENTOS

À Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas na pessoa do diretor Prof. Dr. Jacks Jorge Júnior.

Ao Prof. Dr. Ricardo Della Coletta, coordenador do programa de pós-graduação de Estomatopatologia.

À Prof. Dra. Maria Elvira Pizzigatti Correa, pelos ensinamentos, pela ajuda constante e por sua personalidade acolhedora, que incentiva seus alunos a realizarem o melhor.

À Prof. Dra. Cecília Amélia Fazzio Escanhoela, pelos ensinamentos e disposição em ajudar.

À Prof. Dra. Maria Letícia Cintra, pela disposição, ajuda incessante, paciência e pela pessoa maravilhosa que é.

À Eliana C Martins Miranda, que me atendeu em todas as solicitações de ajuda, pelo conhecimento que compartilhou e pelo trabalho minucioso e cuidadoso que sempre realizou.

Aos Profs. Dr. Márcio Lopes Ajudarte e Alan Roger por me receberem no Orocentro e me transmitirem preciosos ensinamentos da estomatologia.

Aos funcionários do Departamento de Anatomia Patológica – FCM/ UNICAMP, em especial à Ana Claudia Piazza, Adilson Piazza, Luzia Reis e Arethuza pela importante colaboração e convivência.

Aos funcionários e amigos do Orocentro – FOP/ UNICAMP, Rogério, Cida, Jeane e Elizabeth.

A amiga, colega da pós-graduação em Campinas, Ana Flávia, pela amizade e incessante ajuda.

Às colegas, companheiras no Hemocentro, Vanessa Schaira, Camila Boer, Patrícia Feio e Andréia.

Aos colegas da pós-graduação em Piracicaba, Fernanda Mariano, Lara, Camila, Sabino, Elizabeth, Felipe e Wilfredo.

À FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) e CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), pela concessão financeira que viabilizou a realização deste trabalho.

"E esta é a confiança que temos nEle, que, se pedirmos alguma coisa, segundo a sua vontade, ele nos ouve"

I João 5:14

RESUMO

A doença do enxerto contra o hospedeiro crônica (DECHc) é um processo inflamatório aloimune o qual resulta da resposta celular (células T) do doador contra o receptor em pacientes tratados pelo transplante de células progenitoras hematopoiéticas (TCTH). Os órgãos mais afetados são pele, mucosa oral, glândulas salivares e fígado. O objetivo deste trabalho foi avaliar os achados histopatológicos das glândulas salivares menores (GSM) e fígado dos pacientes afetados pela DECHc, comparando-os entre si, com as amostras destes órgãos de pacientes que não desenvolveram DECHc pós TCTH e com pacientes que não foram tratados pelo TCTH. Amostras de GSM e fígado de pacientes tratados por TCTH mieloablativo, de doadores aparentados com HLA idêntico, entre 1994 e 2006, foram analisadas. Cinquenta e sete pacientes foram selecionados, sendo 36 com DECHc oral e hepática e 21 sem DECH. O diagnóstico de DECHc foi definido por critérios clínicos (extensa/localizada), laboratoriais e de seguimento. Amostras sem alterações de pacientes não transplantados também foram avaliadas (19 de GSM e 20 de fígado). Os espécimes foram corados em hematoxilina e eosina, ácido periódico de Schiff (PAS), Tricrômio de Masson, Reticulina e Perls e tratados pela técnica de imuno-histoquímica para CD45, CD45RO, CD68, CD4, CD8, CD138 e AE1/AE3. Os critérios definidos pela classificação proposta pelo grupo de trabalho em histopatologia da reunião de consenso do *National Institutes of Health* (NIH) foram empregados para a avaliação de todas as amostras de ambos os órgãos. Nos espécimes corados pelo PAS, foi tomada a medida da área acinar das GSM, em imagens digitalizadas. No fígado de pacientes que desenvolveram DECH, foi observado aumento estatisticamente significante do número de células imunomarcadas para CD8 e CD45RO, quando comparado ao dos outros 2 grupos. Os resultados da análise dos critérios do NIH no fígado mostraram que 12 deles apresentaram correlação significante com o diagnóstico da DECH hepática e sete com a elevação sérica dos níveis de, pelo menos, uma enzima hepática e/ou bilirrubina. Foi também observado que o número de células $CD8^+$ e $CD45^+/mm^2$ nas GSM, mostrava valor preditivo para o diagnóstico de DECH no fígado. O número relativo de células $CD45^+$, $CD4^+$ e $CD8^+$ nas GSM se correlacionava com o das mesmas subpopulações celulares do fígado. Nas amostras de GSM foi observada diferença significante no número de células imuno-

marcadas para CD4, CD8, CD45 e CD45RO, quando os dois grupos de pacientes transplantados foram comparados entre si. Porém, para o CD68 e CD138, não houve diferenças. A área acinar PAS^+ das GSM mostrou diferença significante entre os três grupos. As alterações das GSM refletem aquelas do fígado, nos pacientes com a DECHc. Adicionalmente, o aumento do número de células $CD45^+$ e a diminuição de células $CD8^+$ nas GSM são preditivos do diagnóstico de DECH no fígado. Como as amostras de glândulas salivares menores podem ser obtidas com mais facilidade do que as do fígado, este achado pode ser útil na estimativa do dano hepático, em pacientes transplantados, com alterações enzimáticas sugestivas do diagnóstico de DECH. Uma vez que o número de linfócitos nas GSM de pacientes transplantados que não desenvolveram DECH parece ser semelhante ao dos indivíduos sadios, a perda inicial de ácinos é, provavelmente, resultado do condicionamento quimioterápico.

Palavras-Chave: Doença do enxerto contra o hospedeiro, imuno-histoquímica, glândulas salivares menores, fígado e transplante de células tronco hematopoiéticas.

ABSTRACT

Chronic graft-versus-host disease (cGVHD) is an alloimmune inflammatory process, which results from a donor-origin cellular response (T cells) against host tissues. The organs mostly affected are skin, oral mucosa, salivary glands and liver. The aim of this study was to evaluate the histopathological findings of minor salivary glands (MSG) and liver of patients affected by cGVHD, comparing the findings each other and with those of the patients who did not develop cGVHD after HSCT and patients who were not underwent HSCT. MSG and liver samples from patients who underwent myeloablative HLA-matched HSCT from sibling donors, between 1994 and 2006, were analyzed. Fifty-seven patients were selected, being 36 with oral and hepatic cGVHD and 21 without GVHD. The diagnosis of cGVHD was defined through clinical/ laboratory criteria and follow-up. Samples from non-transplanted patients were also evaluated (19 MSG and 20 liver specimens). The specimens were stained with haematoxylin & eosin, periodic acid-Schiff (PAS), Masson's trichrome, reticulin and Perls, and immunolabeled for CD45, CD45RO, CD68, CD4, CD8, CD138, and AE1/AE3. The criteria defined in the classification proposed by the consensus meeting of the National Institutes of Health were used for the evaluation of all samples of both organs. Digital images of the PAS stained sections were used to estimate the MSG acinar area. In the liver of patients who developed GVHD it was observed a statistically significant increase in the number of CD45RO and CD8 immunostained cells, comparatively with the other 2 groups. The results of the analysis of the NIH criteria in the liver showed that 12 of them showed significant correlation with the diagnosis of liver cGVHD and seven of them with elevated serum levels of at least one liver enzyme and / or bilirubin. The number of CD8+ and CD45+ cells/ mm^2 in the MSG showed a predictive value for the diagnosis of liver GVHD. The relative number of CD45+, CD4+ and CD8+ cells in the MSG correlated with the same cell subpopulations in the liver. Concerning MSG samples, significant differences were found in the number of CD4, CD8, CD45 and CD45RO immunolabeled cells, when the two HSCT patients groups were compared. However, for CD68 and CD138, there were no differences. Finally, the PAS+

acinar area of MSG revealed significant differences among the three groups. The changes of MSG reflect those of the liver in patients with cGVHD and immunomarked subsets of cells were predictive of GVHD diagnosis in the liver. Considering the set of clinical and laboratory findings, MSG biopsies, that are easily obtained, might help to estimate hepatic changes in cGVHD patients. Since the relative number of lymphocytes in the MSG of HSCT patients who did not develop GVHD appears to be similar to those of healthy individuals, the initial loss of acini is probably a result of conditioning chemotherapy.

Key words: Chronic graft-versus-host disease, immunohistochemistry, minor salivary glands, liver, hematopoietic stem cell transplantation.

LISTA DE ABREVIATURAS

ALL	*Acute lymphoblastic leukemia*
Allo-HSCT	*Allogeneic hematopoietic stem cell transplantation*
Alo-TCTH	Transplante de células tronco hematopoéticas alogênico
ALT	Alanina transferase
AML	*Acute myelogenous leukemia*
AP	*Alkaline phosphatase*
APC	*Antigen presenting cells*
AST	Aspartato transferase
BAAF	*B cell activating factor*
BD/PTr	*Bile ducts/Portal tract ratio*
BMT	*Bone marrow transplantation*
BT	Bilirrubina total
BU	*Busulfan*
cGVHD	*Chronic graft-versus-host disease*
CML	*Chronic myelogenous leukemia*
CsA	*Cyclosporin A*
CY	*Cyclophosphamide*
DECH	Doença do enxerto contra o hospedeiro
DECHc	Doença do enxerto contra o hospedeiro crônica
FA	Fosfatase alcalina
GGT	Gama glutamil transpeptidase
GSM	Glândula salivar menor

GVHD *Graft-versus-host disease*

HE Hematoxilina e eosina

HLA *Human Leukocyte Antigen*

HSCT *Hematopoietic stem cell transplantation*

HSCT *Hematopoietic stem cell transplantation*

Ig *Immunoglobulin*

IgA *Immunoglobulin A*

L-GVHD *Liver graft-versus-host disease*

MALT *Mucosa-associated lymphoid tissue*

MDS *Myelodysplastic disorder syndrome*

mHA *Minor histocompatibility antigens*

MHC *Major histocompatilibity complex*

MSG *Minor salivary glands*

MTX *Methotrexate*

NC *No correlation*

NIH *National Institutes of Health*

PAS *Periodic acid Schiff*

PCB *Primary biliary cirrhosis*

PNH *Paroxysmal nocturnal hemoglobinuria*

SAA *Severe aplastic anemia*

SD *Standard deviation*

SPSS *Statistical Package for the Social Sciences*

SPSS *Statistical Package for the social sciences*

SS *Síndrome de Sjögren*

TB	*Total bilirrubin*
TBI	*Total body irradiation*
TCTH	Transplante de células tronco hematopoética
Th1	Linfócitos T *herper* 1
Th2	Linfócitos T *helper* 2
Tregs	Linfócitos T regulatórios
VP	*VP-16 Etoposide*

SUMÁRIO

INTRODUÇÃO

O transplante de células tronco hematopoéticas (TCTH) é um recurso terapêutico utilizado para o tratamento de doenças hematológicas malignas além de outras doenças hematológicas não malignas e genéticas. Este tratamento apresenta a possibilidade de erradicação da doença hematológica maligna por meio do efeito enxerto contra leucemia ou enxerto contra tumor (Appelbaum, 2001). O número de transplantes alogênicos de células tronco hematopoéticas (alo-TCTH) vem crescendo, com mais de 25.000 alo-TCTH realizados anualmente em todo mundo (Ferrara *et al*., 2009). Este aumento se dá principalmente pelo desenvolvimento de novas estratégias, como o uso de sangue periférico do doador, os regimes de condicionamento não mieloablativos e o uso do cordão umbilical como fonte de células tronco, possibilitando a expansão das indicações do alo-TCTH, especialmente para aqueles pacientes mais idosos. (Welniak *et al*., 2007). A melhora na profilaxia antibiótica, nos tratamentos imunossupressores e na tipagem molecular dos doadores também tem contribuído com o aperfeiçoamento dos resultados dos transplantes (Ferrara *et al*., 2009).

Apesar de todos esses avanços, a doença do enxerto contra o hospedeiro (DECH) continua sendo a maior complicação letal (não relacionada à recaída) do alo-TCTH e está diretamente ligada à limitação de sua indicação (Welniak *et al*., 2007). Em virtude das novas tendências de transplantes que incluem os transplantes não aparentados, o número de pacientes que podem apresentar a doença do enxerto contra hospedeiro tende a aumentar significantemente (Ferrara *et al*., 2009).

Na década de 60, Billingham (1966) formulou os três requisitos básicos para o desenvolvimento da DECH: (1) o enxerto deveria conter células imunocompetentes, posteriormente reconhecidas como sendo os linfócitos T maduros presentes no enxerto; (2) o receptor deveria expressar antígenos teciduais diferentes daqueles do doador, o que permitiria às células doadas reconhecer os tecidos do receptor como estranhos; e finalmente, (3) o receptor não deveria ser apto a desencadear uma reação imune capaz de eliminar as células transplantadas (Billingham, 1966).

A DECH ocorre quando os linfócitos T do doador respondem a proteínas geneticamente definidas nas células do hospedeiro. As proteínas mais importantes são as do

Human Leukocyte Antigen (HLA), as quais são altamente polimórficas e codificadas pelo complexo principal de histocompatibilidade (*MHC – major histocompatilibity complex)*. Proteínas HLA Classe I (A, B e C) são expressas em quase todas as células nucleadas do corpo em diferentes densidades. Proteínas HLA Classe II (DR, DQ e DP) são expressas principalmente em células hematopoéticas (linfócitos B, células dendríticas e monócitos), mas também podem ter sua expressão induzida em várias outras células após um processo inflamatório ou lesão tecidual (Ferrara *et al*., 2009).

Outras diferenças geneticamente determinadas entre o doador e o receptor de células tronco hematopoéticas, como aquelas situadas fora do *loci* HLA, como os antígenos de histocompatibilidade menores (*mHA- minor histocompatibility antigens*), podem influenciar a incidência da DECH aguda (Bleakley e Riddell, 2004; Goulmy *et al*., 1996).

Os polimorfismos presente em ambos, doador e receptor, dos genes das citocinas relacionadas aos eventos inflamatórios da DECH, tais como o fator de necrose tumoral alfa, interleucina-10, interferon gama, estão implicados como fatores de risco para o desenvolvimento da DECH (Antim *et al*., 1992; Cavet *et al*., 1999; Lin *et al*., 2003; Dickinson *et al*., 2005). Estratégias futuras para encontrar o melhor doador possível provavelmente irão incorporar fatores genéticos relacionados e não relacionados ao HLA (Ferrara *et al*., 2009).

A DECH aguda acomete principalmente a pele em aproximadamente 81% dos casos, o trato gastrointestinal em 54% e o fígado em 50% dos casos (Martin *et al*., 1990). A pele é o local mais frequentemente afetado e geralmente o primeiro a ser acometido coincidindo com a "pega" do enxerto (Ferrara et al., 2009). A presença característica de *rash* cutâneo macropapular com prurido pode ser observada por toda a pele. Em casos mais graves, bolhas seguidas de ulceração podem ser vistas (Vogelsang *et al*., 2003).

O envolvimento do trato gastrointestinal é caracterizado pela presença de diarreia que pode ser acompanhada de vômitos, anorexia e dor abdominal (Ferrara *et al*., 1991). As alterações hepáticas causadas pela DECH podem ser difíceis de serem distinguidas de outras causas de disfunção hepática após o TCTH, tais como a doença

veno-oclusiva, toxicidade causada por drogas, infecções virais, sepsis ou sobrecarga de ferro (Ferrara *et al*., 2009).

A gravidade da DECH aguda é determinada pela extensão do acometimento dos três órgãos alvos principais e pode ser graduada em quatro categorias: leve, moderada, grave e muito grave. O prognóstico é desfavorável, com cerca de 25% de sobrevida, para a forma grave e de 5% para a forma muito grave (Cahn *et al*., 2005).

A doença do enxerto contra o hospedeiro crônica (DECHc) em contraste com a forma aguda, apresenta-se clinicamente com manifestações que lembram doenças autoimunes, como a síndrome de Sjögren, esclerodermia, lúpus eritematoso sistêmico e cirrose biliar primária (Shlomchik, 2007; Socie *et al*., 2010).

Em pacientes com DECHc, a pele pode exibir eritema em máculas e placas, descamações, despigmentações, lesões liquenóides, atrofia e, em alguns casos, úlceras crônicas. Pode haver o desenvolvimento de doença colestática crônica no fígado e ainda o acometimento do trato gastrointestinal, o que pode resultar em perda de peso e má nutrição (Min, 2011). A DECHc comumente produz a síndrome sicca, a qual é causada pela destruição linfocítica de glândulas exócrinas, dentre elas o fígado e as glândulas salivares (Min, 2011).

Diferentemente da DECH aguda, pouco se sabe sobre os mecanismos imunes que levam ao desenvolvimento da DECHc e da mesma forma, o grau de similaridade entre a DECHc e as diferentes doenças autoimunes. Embora a DECHc apresente manifestações de autoimunidade, ela somente ocorre em pacientes tratados com alo-TCTH (Socie *et al*., 2010).

Ambas as formas da DECH, aguda e crônica, podem ser prevenidas pela depleção dos linfócitos T do enxerto. Isso leva a crer que a resposta imunológica dos linfócitos T do doador aos antígenos alogênicos do hospedeiro é essencial no desenvolvimento da DECH crônica, porém os mecanismos imunológicos específicos da sua patogenia não são totalmente estabelecidos (Socie *et al*., 2010). A quebra da tolerância imunológica à auto antígenos e alterações na função dos linfócitos T regulatórios CD4+CD25+FOXP3+ (Tregs) seriam os fatores promotores das manifestações autoimunes da DECHc (Sprent e Kishimoto, 2001; Soiffer, 2008).

A quebra da tolerância imunológica ocorre pela agressão ao timo causada pelo regime de condicionamento pré-transplante e/ou DECH aguda, levando a uma desregulação do mecanismo de tolerância central durante a reconstituição do sistema imunológico pós-transplante (Sprent e Kishimoto, 2001).

Estudos em modelos murinos têm demonstrado que as Tregs são capazes de suprimir a DECH e que a deficiência destas células pode levar ao aumento da gravidade da doença (Socie et al., 2010). Outros estudos mostraram que pacientes com DECHc apresentaram menor quantidade de Tregs em comparação com aqueles sem DECHc (Zorn *et al*., 2005; Rieger *et al*., 2006).

Além dos linfócitos T, evidências apontam que os linfócitos B também possuem um papel importante no desenvolvimento da DECHc, semelhantemente ao que ocorre nas doenças autoimunes (Socie *et al*., 2010). A participação dos linfócitos B na patogênese da DECHc foi revelada por estudos que observaram altos níveis séricos de fator ativador de células B (*BAAF – B cell activating factor*), uma citocina que regula a autoimunidade dos linfócitos B (Srantopoulos et al., 2007). Estudos têm demonstrado que os linfócitos B respondem aos mHA ligados ao cromossomo Y, o que explicaria a alta incidência, tanto da forma aguda quanto crônica da DECH, em homens que receberam enxerto de mulheres (Randolph *et al*., 2004; Miklos *et al*., 2005). Quando paciente e doador tem o HLA e sexo compatível supõe-se que os mHA codificados por genes autossômicos sejam os antígenos alvos (Socie *et al*., 2010).

A cavidade oral representa um dos sítios de maior incidência da DECHc. Em um estudo retrospectivo realizado no Fred Hutchinson Cancer Research Center, em Seattle, EUA, foram revistos os prontuários médicos de 740 pacientes que desenvolveram DECHc após tratamento com TCTH mieloablativo. No momento do diagnóstico, a cavidade oral estava acometida em 333 (86%) dos pacientes, seguida pela pele acometida em 261 pacientes (67%) e fígado, acometidos em 136 (35%) (Vigorito *et al*., 2009).

Clinicamente, a DECHc oral apresenta-se como lesões liquenóides, eritematosas, atróficas e ulcerações. Estas alterações podem estar associadas à disfagia, alterações do paladar, restrição da abertura da boca e disfunção das glândulas salivares com

diminuição do fluxo salivar, além da presença de mucoceles (Villar *et al*., 2001; Filipovich *et al*., 2005; Pereira *et al*, 2007; Schubert e Correa, 2008; Boer *et al*, 2010).

Microscopicamente, em mucosa oral, a DECHc apresenta atrofia do epitélio com a presença de corpos apoptóticos, degeneração hidrópica da camada basal, infiltrado inflamatório (de interface e em região sub-epitelial) e exocitose (Woo *et al*., 1997; Hiroki *et al*., 1994; Shulman *et al*., 2006, Horn *et al*., 1995). Ao exame histológico das glândulas salivares, observa-se infiltrado inflamatório linfo-plasmocitário peri-ductal e intralobular, com graus variáveis de atrofia acinar e fibrose (Shulman *et al*., 2006; Alborghetti *et al*., 2005; Soares *et al*., 2005, Horn *et al*., 1995). Segundo Shulman *et al.* (2006) a DECHc ativa em glândula salivar está associada à presença de estroma periacinar e periductal com fibrose moderada ou intensa, enquanto que a presença de tecido fibroso denso com destruição acinar e ectasia ductal pode apenas representar agressão prévia. Alborghetti *et al*. (2005) mostraram as alterações do parênquima acinar das glândulas salivares menores (GSM) em pacientes com DECHc oral e que as mesmas foram persistentes mesmo após tratamento imunossupressor.

Em estudo imuno-histoquímico de GSMs acometidas pela DECHc, foi observado aumento de células CD45+, macrófagos (CD68+), e linfócitos T CD4+ e CD8+, com predomínio de células T citotóxicas (CD8+) em relação aos linfócitos T auxiliares (CD4+) (Soares *et al*., 2005). Este quadro é similar ao da Síndrome de Sjögren. Entretanto, nesta síndrome ocorre uma predominância de linfócitos T CD4+ sobre os CD8+ e o infiltrado peri-ductal é mais pronunciado que na DECHc (Harada *et al*., 1996).

Em mucosa labial de pacientes com DECHc, Soares *et al* (2005) demonstraram o aumento da presença de leucócitos CD45+, seguidos de linfócitos T CD8+, macrófagos (CD68+) e linfócitos T CD4+. A presença de linfócitos B (CD20+) foi rara nesse estudo. Outros estudos demonstraram a participação das células de Langerhans (CD1+) na forma liquenóide da DECHc em mucosa oral (Sato *et al.,* 2006; Orti-Raduan *et al*., 2009). Estudo comparativo de líquen plano oral e DECHc demostrou a importância da liberação de perforina e granzima pelas células T citotóxicas (CD8+) na agressão à mucosa oral, com maior participação da perforina na indução de apoptose dos ceratinóticos na DECHc (Pimentel *et al.,* 2010).

As alterações hepáticas podem ocorrer logo após o transplante. O diagnóstico diferencial em pacientes pós-TCTH incluem infecções virais, DECH, toxicidade por drogas, obstrução biliar, sobrecarga de ferro, hiperplasia regenerativa nodular, hiperplasia nodular focal e cirrose (McDonald, 2010; Kida e McDonald, 2012).

As manifestações da DECH hepática em pacientes pós-TCTH tardios podem ocorrer de três formas: (1) Elevação assintomática dos níveis séricos de alanina transferase (ALT), fosfatase alcalina (FA), e gama glutamil transpeptidase como alterações laboratoriais isoladas e na ausência de icterícia, geralmente observada em pacientes com DECHc em algum outro órgão (Sullivan *et al*., 1981; Tomas *et al*., 2000). (2) Icterícia colestática de progressão lenta, que é hiperbilirrubinemia geralmente associada com FA sérica elevada, como resultado da agressão aos ductos biliares (Shulman *et al*., 1988). (3) Injúria hepatocelular aguda (DECH hepatítica), com elevação abrupta dos níveis séricos de ALT acima de 500U/L sem disfunção hepática prévia (Akpek *et al*., 2002).

Microscopicamente, a DEHCc hepática é caracterizada por agressão aos ductos biliares e infiltrado linfocítico portal (Saxena, 2011). Os achados nos ductos biliares intra-hepáticos incluem alterações degenerativas, seguidas por necrose multifocal de células epiteliais isoladas, pleomorfismo nuclear, atrofia e, finalmente, desaparecimento dos ductos (McDonald, 2006).

Histologicamente não é possível distinguir a DECH aguda da crônica, já que seus achados histológicos são semelhantes (Shulman *et al*., 2006). Porém tem sido feita distinção entre dois padrões histológicos da DECH hepática, a forma clássica e a hepatítica (Ma *et al*., 2004), sendo que esta última tende a ocorrer no momento da diminuição da terapia imunossupressora ou após infusão de linfócitos (Kida e McDonald, 2012). A forma clássica da DECH hepática se caracteriza pela agressão ao epitélio dos ductos biliares, presença de infiltrado linfocítico portal e pela estrutura hepatocelular preservada e desprovida de inflamação lobular (Akpek *et al*., 2002). A forma hepatítica da DECHc é caracterizada pela presença de hepatite lobular, com agressão relativamente leve aos ductos biliares (Akpek *et al*., 2002).

O acometimento hepático pela DECHc é comparado ao da cirrose biliar primária, e pode ser incluído na síndrome das glândulas secas (síndrome sicca),

caracterizada pela presença de olhos secos, boca seca e hiposecreção biliar e pancreática (Epstein e*t al*., 1980). Na cirrose biliar primária, assim como na DECHc, há um comprometimento multi-sistêmico, onde glândulas lacrimais e salivares estão acometidas em 70 a 100% dos pacientes (Epstein e*t al*., 1980).

As células dos ductos das glândulas salivares e dos ductos biliares são alvos preferenciais da DECH. Ambas são derivadas da endoderme e expressam alta densidade de HLA, necessários para o reconhecimento imunológico (Lauteuschlager *et al*., 1982; Nagler *et al*., 2004). A presença de linfócitos na espessura da parede dos ductos salivares e biliares danificados é evidência da linfocitotoxicidade contra antígenos de superfície destas células. A gravidade do envolvimento das glândulas salivares, tanto ao diagnóstico, como em biópsias sequenciais, poderia ter relação com o envolvimento hepático. Fatores agravantes prévios ou concomitantes também poderiam atuar.

Considerando a alta incidência da DECHc oral e hepática e seu impacto nos resultados do tratamento pelo TCTH (Pavletic *et al*., 2005; Duarte *et al*., 2005) e, considerando ainda, a relação estrutural e histológica entre o fígado e as glândulas salivares (Epstein e*t al*., 1980), a hipótese deste trabalho reside na possível correlação entre os achados histológicos da DECHc nas glândulas salivares com os do fígado e dos achados em ambos com alterações clínicas do paciente.

Desta forma, o objetivo principal deste trabalho foi analisar os achados histológicos, sob técnicas convencionais e imunoistoquímicas, nas glândulas salivares menores e no fígado dos pacientes tratados com o transplante de células tronco hematopoéticas. Tendo como objetivos específicos: (1) Correlacionar os achados histológicos em glândulas salivares menores com os do fígado; (2) Investigar alterações histológicas na GSM que sinalizam o diagnóstico de DECHc hepática; (3) Comparar as subpopulações de células inflamatórias de ambos os órgãos e a área acinar produtora de muco das GSM em pacientes com DECHc, sem DECH e pacientes não tratados pelo TCTH e (4) Correlacionar as alterações histológicas em ambos os órgãos com alterações clínicas.

CAPÍTULO 1

Salivary glands changes in hematopoietic stem cell transplantation: relationship with patients follow up

(Artigo submetido para publicação na Bone Marrow Transplantation)

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ABSTRACT

The aim of this study was to discriminate the participation of damaging events preceding hematopoietic stem cell transplantation (HSCT) in minor salivary glands (MSG) of those affected by chronic graft-versus-host disease (cGVHD); to test the potential value of histological changes in predicting cGVHD severity and overall survival of patients. MSG of 57 HSCT patients, who developed or not oral cGVHD, were studied, comparatively with 19 non-HSCT individuals. cGVHD changes were assessed according to the NIH consensus and Horn *et al* grading systems. Functional acinar area and mononuclear cells subset were set. The results showed significant increase for CD45, CD45RO, CD4, and CD8 immunomarked cells/mm^2 in cGVHD patients by comparing the 2 groups of HSCT patients. Increase in $CD68^+$ cell counts were found in non-cGVHD compared with control group. Measurements of the PAS+ area were progressively lower in the 3 groups. The "Periductal lymphocytes with exocytosis into duct" feature correlated with worse overall survival and the "Periductal lymphocytic infiltrate" feature predicted the extensive form of cGVHD. Functional acinar loss begins with the conditioning chemotoxicity. Lymphocytes migrate through the ductal epithelium, leading to progressive acinar loss, which could

explain the persistence or worsening of xerostomia in patients who develop cGVHD and could possibly exert negative influence upon overall survival.

Keywords: Graft-versus-host disease, pathology, immunohistochemistry, NIH system, morphometry.

INTRODUCTION

The oral cavity is frequently the primary source of hematopoietic stem cell transplantation (HSCT) morbidity [1]. Saliva plays a major role in maintaining oral health and function [2]. The loss of saliva leads to increased risk of dental caries, candidal superinfections, oral pain, mucosal friability and food sensitivity. Additionally, difficulties in speaking, chewing, and swallowing, as well as weight loss can also be observed [3,4]. Both, prior HSCT conditioning regimen and chronic graft-versus-host disease (GVHD) can cause salivary gland damage and inflammation, leading to xerostomia [5].

Chronic GVHD (cGVHD) is the major late complication of HSCT, and is mediated by T lymphocytes targeting various organs and tissues, including the oral mucosa and salivary glands, leading to salivary hypofunction and oral damage [6].

The oral cavity may be the principal or sole site of involvement, in 50–83% of those affected by cGVHD [7]. On a clinical basis, mucosal lesions, salivary gland dysfunction, and taste disorders are similar to those of autoimmune disorders, such as lichen planus, lupus, and Sjögren syndrome [7-9]. The typical histological findings of cGVHD in salivary glands are periductal and diffuse glandular parenchyma lymphocyte infiltrate ($CD8^+$ over $CD4^+$), atrophy or destruction of acini, and fibrosis [5, 10-15].

Despite the advent of new therapies and management of cGVHD, minimal changes in this scenario have been observed, mainly due to an incomplete understanding of the pathogenesis of this disorder and there is a lack of an adequate classification system for diagnosis and patient follow up [1, 6].

Knowing the most important histological findings for diagnosis of minor salivary glands (MSG) cGVHD and understanding the mononuclear subsets participation in salivary glands after HSCT can contribute to a better management of those patients.

The goal of this study was to assess the residual acinar area and lobular mononuclear cell subsets in order to improve the understanding of the relative participation of the conditioning regimen for transplantation and development of cGVHD after HSCT; to test the potential value of histological changes in predicting cGVHD severity and overall survival of patients.

PATIENTS AND METHODS

This was a retrospective analysis of patients who underwent ablative HLA-matched HSCT from sibling donors. After a search of the data basis of the Bone Marrow Transplantation Unity of the University of Campinas Clinical Hospital, 57 patients who fulfilled the inclusion criteria were selected. Inclusion criteria included complete medical history and lip biopsy with MSG represented. Exclusion criteria included incomplete follow-up data, and unavailable or insufficient paraffin material for new cuts. Thirty six patients with chronic oral GVHD composed the first group, and 21 patients who never developed GVHD composed the second group. Control group consisted of 19 MSG from free surgical margins of biopsies of non-inflammatory lesions in the lip. The biopsy specimens were obtained on the day in which the disease was clinically diagnosed or on the protocol day +100 post-HSCT. Samples were collected from the lower inner lip, about 10 mm beneath the vermilion border through a 4 mm-punch, under local anesthesia. Patients' clinical data are summarized in table 1.

Paraffin embedded tissue were serially sectioned at 4 µm thickness and stained with H&E and periodic acid Schiff (PAS). All specimens were assessed for histological cGVHD features according to: 1- the National Institutes of Health (NIH) consensus pathology working group (Table 2) [15] and 2- Horn *et al* [17] grading systems (Table 2).

Immunohistochemical study was performed through the standard polymeric method. Briefly, the histological sections were deparaffinized in xylene, and rehydrated. The primary monoclonal antibodies used were: CD4 (clone OPD4 at a dilution 1:100); CD8 (clone C8/144B, at a dilution 1:200); CD45 (clone 2B11+PD7/26, at a dilution 1:500); CD45RO (clone UHCL-1, at a dilution 1:700); CD68 (clone KP1, at a dilution 1:700);

CD138 (clone MI15, at a dilution 1:200), all distributed by DAKO (Dako Cytomation, Carpinteria, CA, USA). A steamer was used for epitope retrieval and the EnVision polymer (DAKO) as the reaction amplifier. Appropriate positive and negative controls were included in each essay. Staining was achieved with 3,3-diaminobenzidine tetrahydrochloride (Sigma, St Louis, Missouri, USA) and counterstaining with Mayer's haematoxylin. Unsatisfactory sections were excluded and the procedures were repeated.

Quantitative analysis was performed in 400x digital images in 10 blindly and randomly fields, either, for PAS- and immuno- stained sections of each patient. The number of ducts with lymphocytes in exocytosis into epithelium per mm^2 was also recorded, through an Olympus CH30 optical microscope (Olympus Center Valley, PA, USA), in high (x400) magnification, in the CD45 stained sections.

For the morphometric study, PAS stained digital images were analyzed using two softwares. With the LimiarK software, the digital images were segmented according to the color spectrum; using the Quant6 software, the targeted area was calculated, providing the value of PAS positive area/ total area ratio. Both LimiarK and Quant6 software were developed by Randall Luis Adam, volunteer researcher of the Institute of Computing, UNICAMP, São Paulo, Brazil. Digital images from immune-marked samples were analyzed using Imagelab 2000® (Image analysis system, 2000, Diracom, Sao Paulo, Brazil). The mean of PAS^+ relative area and the mean of immunomarked cells/mm^2 in each MSG sample were used for statistical analysis.

Statistical Package for the Social Sciences (SPSS) for Windows 14.0 was used to perform statistical analysis. The following tests were applied: Spearman's or Pearson's correlation, Anova and the Kaplan-Meier method (ref. 18). To analyze and compare actuarial curves of overall survive the log-rank test was used (ref. 19). The forward stepwise Wald test, in conjunction with the Cox regression analysis (ref. 20), was also used to assess the multivariate predictors of outcome to overall survival. P values <0.05, were considered statistically significant.

This study was approved by the Research Ethics Committee of the Faculty of Medical Sciences, University of Campinas (process 598/2002).

RESULTS

The NIH feature “acinar degeneration/interstitial fibrosis/ductal ectasia” (Figure 1) showed significant differences among patients who had or not extensive cGVHD ($p=0.03$) and, also, significant differences comparing patients who developed or not cGVHD ($p=0.03$). However, Horn’s feature “ductal dilation”, as an isolated criterium, did not correlate with the cGVHD clinical form, using Pearson’s correlation. Moreover, no significant difference in the degree of ductal dilation was found comparing patients who developed or not cGVHD.

Horn’s “periductal lymphocytic infiltrate” finding best predicted the clinical extensive form of cGVHD ($p=0.04$).

Using the Kaplan-Meyer method and Log-Rank test, the feature presenting the highest worse influence on overall survival ($p=0.007$) was demonstrated to be the NIH finding of “periductal lymphocytes with exocytosis into duct” (Figure 2). The univariate Cox analysis confirmed this result, the relative risk for dead was 5.74 ($p=0.01$; CI 95%: 1.33–24.6).

The objective counting of the relative number of ducts with lymphocytes exocytosis displayed a significant correlation with the subjective evaluation of the presence (or absence) of the NIH feature “periductal lymphocytes only with exocytosis into duct” feature ($p=0.004$). However no correlation with cGVHD diagnosis or extensive/localized form of the disease was found.

The results showed that the relative number of positive cells for CD45, CD45RO, CD4 and CD8 were incresed in cGVHD patients by comparing the 2 groups of HSCT patients (Figure 3). No differences however, were found between the control group and non- cGVHD patients for the same markers. For CD68, we found no differences between the 2 groups of HSCT patients, but there were significant differences between control group individuals and non- cGVHD patients values. Positive cells for CD68 were increased in cGVHD and non-GVHD patients by comparing with control group (Figure 3).

PAS^+ relative acinar area exhibited significant differences when the 3 groups were compared wih each other (Figure 4). We found decreased PAS+ acinar area in cGVHD group regarding HSCT patients ($p<0.001$). Also, was showed decreased PAS+

acinar area in non-cGVHD patients by comparing with control group (p=0.006). These results are summarized in table 3. After applying several tests, no significant differences were found in the number of ducts with exocytosis, comparing patients with localized or extensive cGVHD. Likewise, no differences were found for $CD138^+$ cells comparing the 3 groups of individuals (Figure 3).

DISCUSSION

The histopathological examination of MSG can aid to set cGVHD diagnosis, especially by ruling out drug toxicity and infections with atypical clinical features [21]. Correa *et al* [22] found an association between the involvement of MSG and oral mucosa in cGVHD after HSCT and decrease in patient survival. The histological features that have been reported to be associated with cGVHD on MSG are lobular and periductal lymphocytic infiltrate, acinar atrophy / destruction, and fibrosis [10-12]. Horn *et al* [17] in 1995, proposed a system for mucosal and salivary glands cGVHD diagnosis and grading. After comprehensive discussions among HSCT experts, the National Institutes of Health (NIH) consensus criteria for clinical trials in chronic graft-versus-host disease (GVHD) defined the most important diagnostic criteria for chronic GVHD. The recommendations of the NIH histopathology working group were published by Shulman *et al* [15] in 2006. With the aim of testing the value of these features in predicting cGVHD severity and overall survival of patients, we assessed all the criteria in found in the publications by Horn and Shulman (table 2), on the MSG specimens of the 3 groups of individuals. The NIH "periductal lymphocytes only with exocytosis into duct" feature was found to be important to predict cGVHD overall survival of patients. Lymphocytes exocytosis reflects high inflammatory activity and, consequently, progressive acinar loss. Xerostomia can be a distressing symptom, and decreased salivary flow may lead to reduced food intake, dental caries, and oral mucosal infection [23]. Oral dryness is associated with lower health-related quality of life [24] and can be an additional factor in the reduction of the overall survival of these patients. Although these results should be regarded with caution due to the small number of patients in our cohort, they stress the importance of improving the management of oral cGVHD inflammation in long-term survivors of allogeneic HSCT. It is necessary to

validate these results studying larger series of patients from different institutions. Significant correlation was also found between the objective counting of ducts with exocytosis and the subjective assessment (present or absent) of the "periductal lymphocytes only with exocytosis into duct". Therefore, the subjective evaluation can be considered reliable. However, the relative number of ducts with exocytosis displayed no prognostic relevance. We believe that these results can be explained by the staining used for evaluation. The subjective feature was assessed on H&E samples and only cases with significant lymphocyte exocytosis could be discerned. The counting of ducts with exocytosis was performed on CD45 immunolabeled specimens that allow easy identification of inflammatory cells permeating ductal epithelium, even when sparse. Therefore, the prognostic value of periductal lymphocytes permeating the epithelium depends on inflammatory severity.

Regarding systemic cGVHD involvement, Horn's "periductal lymphocytic infiltrate" feature was found to best predict the clinical extensive form of cGVHD. Oral cGVHD often coexists with cutaneous, hepatic or ocular cGVHD [25]. Our results are in keeping with those of Nakamura et al [10] whose results revealed the histological alterations in MSG better reflected the cGVHD status [10].

Ductal dilation has been described as one of the histological findings for cGVHD diagnosis [10-12, 17]. However, it could only be the consequence of acinar atrophy and fibrosis, due to the conditioning regimen. In fact, no significant differences were found in the degree of ductal dilation, comparing patients who developed or not cGVHD. On the other hand, when ductal dilation, fibrosis and acinar degeneration were assessed together, as recommended by Shulman *et al* [15], significant differences were found between cGVHD and non-cGVHD patient samples. This reiterates that tissue destruction caused by T cells is more important than the simple presence of ductal dilation.

Concerning immunohistochemical analysis, we found a significant increase of $CD45^+$, $CD45RO^+$, $CD4^+$ and $CD8^+$ cells in MSG of cGVHD patients, with a predominance of CD8 over CD4 phenotype, comparatively with the other 2 groups. These results reiterate the T cells role on pathogenesis of cGVHD. However, no differences were found between control group (non-HSCT patients) and non- cGVHD patients for the same markers.

Therefore, we can infer that the massive lymphocyte infiltrate was part of the GVHD process and not of the chemo-radioterapy regimen preceding HSCT. Our results also revealed the lymphocyte infiltrate related to cGVHD is not the only mechanism for salivary cells loss. PAS^+ acinar area exhibited significant differences when the 3 groups were compared to each other. A progressive loss of functional acini, probably begins for the cytotoxicity of antineoplastic and pre-transplant conditioning drugs and progress with cGVHD process. In fact, conditioning regimens have been shown to promote oral toxicity and also injure salivary glands [26], which justifies xerostomia in patients post-HSCT even when these do not develop cGVHD. The PAS relative area in non-HSCT patients corresponded to 36% of glandular parenchyma, in HSCT patients who did not develop cGVHD corresponded to 25%, and in cGVHD patients, to 3% of glandular parenchyma.

Severe involvement of MSG in cGVHD results in the total destruction of secretory units and thus permanent and profound oral dryness [27]. Imanguli *et al* (2010) [28] findings revealed the usefulness of MSG biopsy in identifying the patients most likely to benefit from cGVHD treatment. Patients in the late process of the disease, manifested by extensive destruction of MSG tissues with fibrosis and atrophy, would probably be unlikely to regain significant function. Our results, corraborate those of Imanguli *et al* (2010) [28]: a pathological examination of the MSGs is useful for diagnosis, grading of cGVHD and screening patients for treatment.

Unlike our results, Soares *et al* (2005) [14] found increased $CD68^+$ cells number in cGVHD patients, compared with non- cGVHD HSCT patients. This discrepancy might be explained by the development of previous acute GVHD. In our cGVHD cohort only 4 patients had developed previous acute GVHD. In the third phase of acute GVHD, allogeneic activated T-cells travel to target tissues and inflammation and tissue destruction occurs [29, 30]. Donor $CD4^+$ T cells can interact with donor macrophages, which present recipient antigens. Tissue macrophages can also induce donor $CD4^+$ T cells to produce inflammatory mediators and activate macrophages [31].

Significant differences were found in relative CD68+ cell numbers, comparing control group and non- cGVHD patients. The mucosa-associated lymphoid tissue (MALT) is the diffusion system of small concentrations of lymphoid tissue found in various sites of

the body, such as the gastrointestinal tract, salivary glands, eyes, and skin. MALT is populated by lymphocytes such as T cells and B cells, as well as plasma cells and macrophages. Normal lymphoid tissue is destroyed in pre-transplant conditioning, following the HSCT, immune reconstitution takes place, and successful reconstruction of the T-cell compartment is known to require one to two years [32]. However, monocytes and natural killer cells reach the normal range by day 30 [33]. All our HSCT non-GVHD biopsies were collected around day 100 post-HSCT, when the T cells had not yet fully recovered, and therefore, were not in a greater number. However, the macrophages had already been reconstituted and the cytokines released during the pre-transplant conditioning recruit these cells.

Several recent studies have pointed out the participation of B cells on cGVHD pathogenesis [34-37]. The role of B cells in cGVHD was enhanced by patient response to B cell depletion cGVHD therapy based on rituximab [38]. Autoantibody formation in patients with cGVHD has been observed, however their role in cGVHD pathogenesis has not yet been elucidated [39]. At least, the role of B cell activity in cGVHD is underscored by the observation of high plasma levels of B cell activating factor, a cytokine that appears to drive B cell auto-immunity, in patients with cGVHD [37]. Perhaps B cells might be acting as antigen presenting cells, providing constant stimulation to infiltrating T cells, leading to tissue damage [40]. Infiltration of tissue by B cells ($CD20^+$) are reported to be virtually absent in oral cGVHD [10, 14], this however, is not the case of plasma cells. In our cohort, plasma cells ($CD138^+$ cells) contributed with a significant part of the infiltrate, despite having found no differences in $CD138^+$ cells relative numbers, when the 3 groups were compared. The MSGs of the control group of our cohort were obtained from specimens of surgically removed benign conditions such as lentiginous macules, nevi and cysts. It is possible that although not in direct contact, the proximity with these benign lesions may have resulted in the increase in the number of immunocytes in these glands. With respect to the subpopulation of these cells, immunoglobulin A (IgA)-secreting plasma cells are known to be located adjacent to the duct and acini of salivary glands and predominate in the major and minor salivary glands over plasma cells producing other Immunoglobulin (Ig) isotypes

[41]. Further studies are necessary to investigate the densities and Ig class proportions of plasma cells in HSCT glands.

In summary, ours findings show that functional acinar loss begins with the conditioning chemotoxicity and may be the cause of long standing xerostomia that is observed after HSCT, even in patients not developing cGVHD. Lymphocytes migration through ductal epithelium, leading to progressive acinar loss, could explain the persistence or worsening of xerostomia in patients who develop cGVHD and might exert negative influence in overall survival.

CONFLICT OF INTERESTS

The authors declare that they have no conflicting interests.

ACKNOWLEDGEMENTS

The authors thank Mr. Adilson Abilio Piaza for his help in taking photomicrographs. This paper was supported by the Coordination for Higher Level Graduates Improvement (CAPES) and São Paulo Research Fundation (FAPESP), INCTs.

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TABLES:

Table 1. Patients' clinical data

Variable	n = 57
Age, median (range)*	28 (12-59)
Gender, n (%)	
Male	29 (51)
Female	28 (49)
GVHD	
Absent	21 (37)
Present	36 (63)
Disease, n (%)	
ALL	7 (12)
CML	31 (54)
MDS	4 (7)
AML	5 (9)
SAA	7 (12)
PNH	1 (2)
Conditioning regimen	
BU + CY	45 (78)
BU + VP-16 + CY	4 (7)
CY + TBI	5 (9)
CY	2 (4)
CY + Melphalan	1 (2)
GVHD prophylaxis	
CsA/MTX	51 (89)
CsA/Corticosteroid	6 (11)
Mean Follow-up, median (range) *	122 (8-180)
Living	36 (63)
Cause of death, n (%)	
Chronic GVHD	6 (11)
Other causes	15 (26)

ALL: acute lymphoblastic leukemia; CML: chronic myelogenous leukemia; MDS: myelodysplastic disorder syndrome; AML: acute myelogenous leukemia; SAA: severe aplastic anemia, PNH: paroxysmal nocturnal hemoglobinuria.

*Age in years.

** Follow-up in months.

Table 2. Histological cGVHD features in salivary glands according to Horn *et al* (1995) and NIH consensus systems (Shulman *et al*., 2006).

Histological feature	Grading
Interstitial inflammation*	Absent
	Mild
	Marked
Acinar destruction*	Absent
	Diffuse
	Complete
Ductal dilation*	Absent
	Mild
	Marked
Squamous metaplasia*	Absent
	Present
Mucous pooling*	Absent
	Present
Interstitial fibrosis*	Absent
	Mild
	Marked
Duct cell proliferation*	Absent
	Present
Periductal lymphocytic infiltrate*	Absent
	Present
Destruction of ducts*	Absent
	Mild
	Marked
Periductal lymphocytes ONLY with exocytosis into duct**	Absent
	Present
Lymphocytes (only) around & migrating into acinar units**	Absent
	Present
Periductal fibrosis**	Absent
	Present
Oncocytic metaplasia (in children)**	Absent
	Present
Periductal mixed chronic infiltrate**	Absent
	Present
Apoptotic cells in ducts/acini**	Absent
	Present
Acinar degeneration/interstitial fibrosis/ductal ectasia**	Absent
	Present
Loss of polarity of ductal epithelial cells**	Absent
	Present

Table 3. Comparative analysis of the immunostained inflammatory cells subsets relative numbers and PAS+ relative acinar area in minor salivary glands of HSCT patients and healthy individuals

	With GVHD			Without GVHD			Control group					
	N	Mean	SD	N	Mean	SD	N	Mean	SD	$P^{‡}$	$P^{£}$	$P^{€}$
CD45*	35	667.60	772.12	21	211.24	94.04	19	202.83	166.70	0.001	0.013	0.84
CD45RO*	6	527.44	353.07	9	111.45	73.62	17	85.63	60.64	0.03	<0.0001	0.38
CD4*	27	203.28	294.15	22	43.33	47.51	19	58.85	41.93	0.01	0.040	0.27
CD8*	31	488.05	436.19	21	130.58	79.21	19	193.70	203.14	<0.0001	0.008	0.21
CD68*	33	259.33	242,70	22	164.64	145.16	19	88.40	68.78	0.07	0.004	0.036
CD138*	25	307.36	271.62	21	239.98	169.30	19	213.94	249.80	0.3	0.24	0.71
PAS[#]	34	0.032	0.007	22	0.2532	0.0890	19	0.359	0.033	<0.0001	<0.0001	0.006

*cells/mm^2, [#]relative area (PAS+area/ total MSG area) [‡]with GVHD versus without GVHD, [£]with GVHD versus control group, [€]Without GVHD versus control group

FIGURES

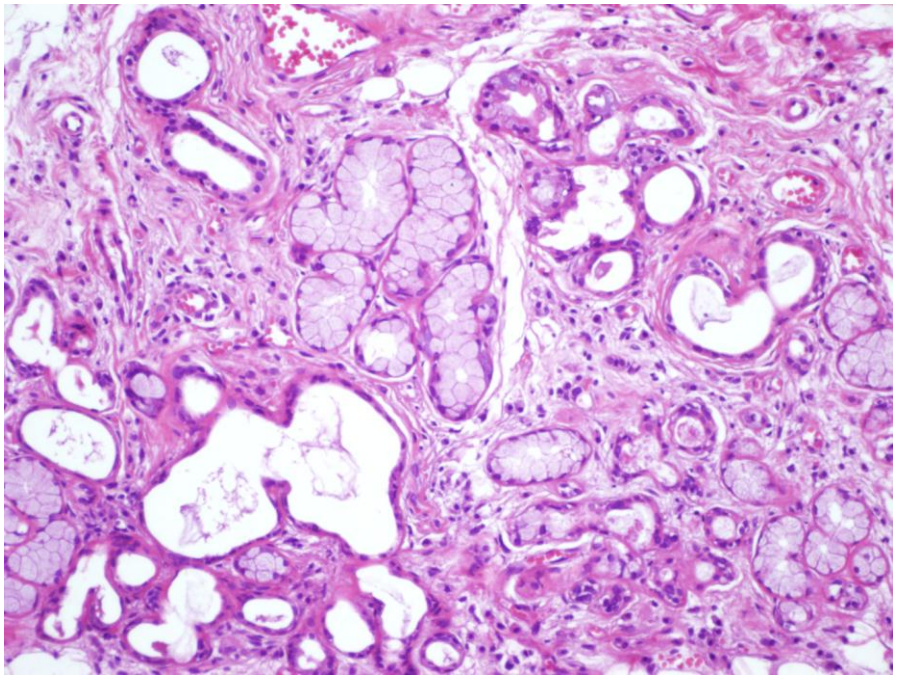

Figure 1. Minor salivary gland sample in oral chronic GVHD. The NIH feature "acinar degeneration/interstitial fibrosis/ductal ectasia" (H&E, original magnification X100).

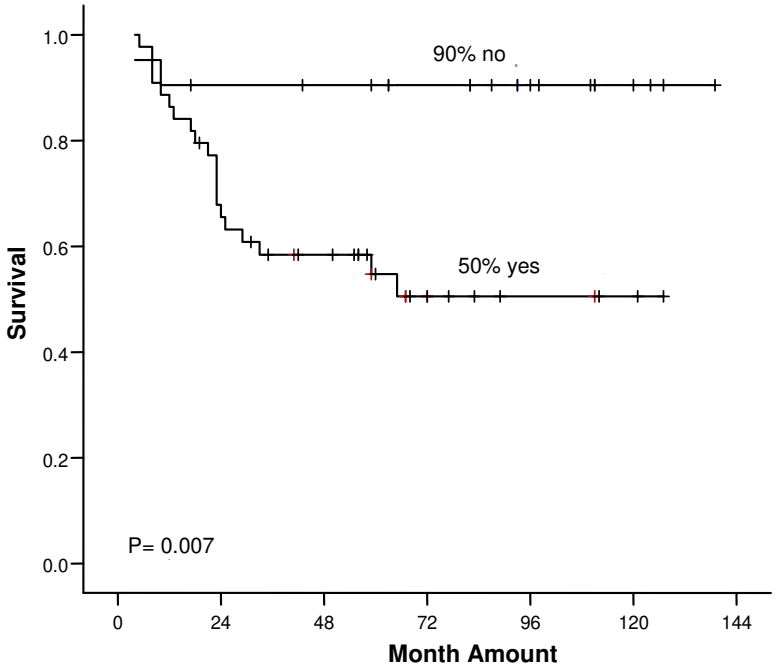

Figure 2. Global survival related to the presence (or not) of the "periductal lymphocytes only with exocytosis into duct" NIH consensus feature.

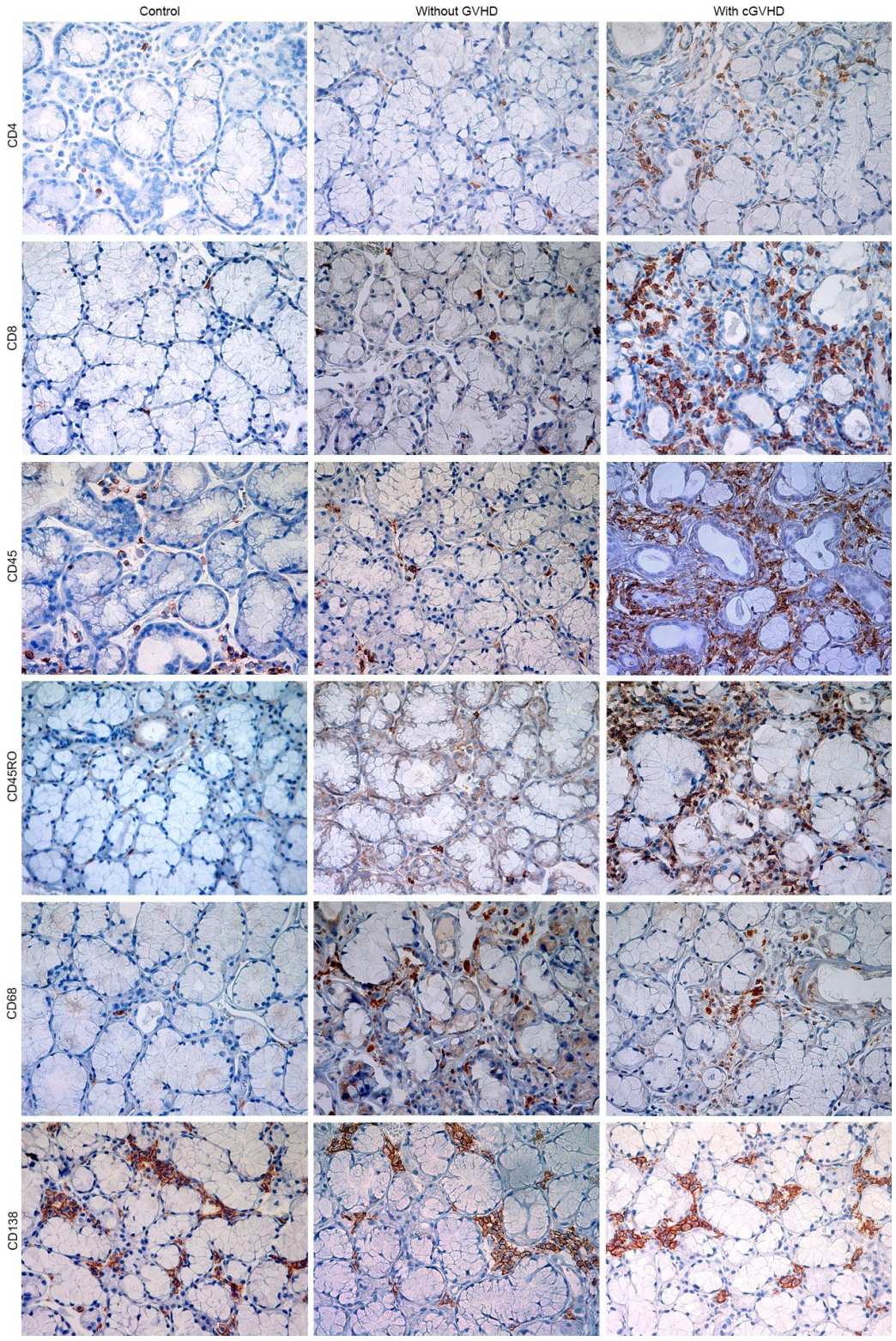

Figure 3. Immunostained minor salivary glands sections targeting for CD4, CD8, CD45, CD45RO, CD68 and CD138 in control group-, non- GVHD and cGVHD patients. Original magnification x400.

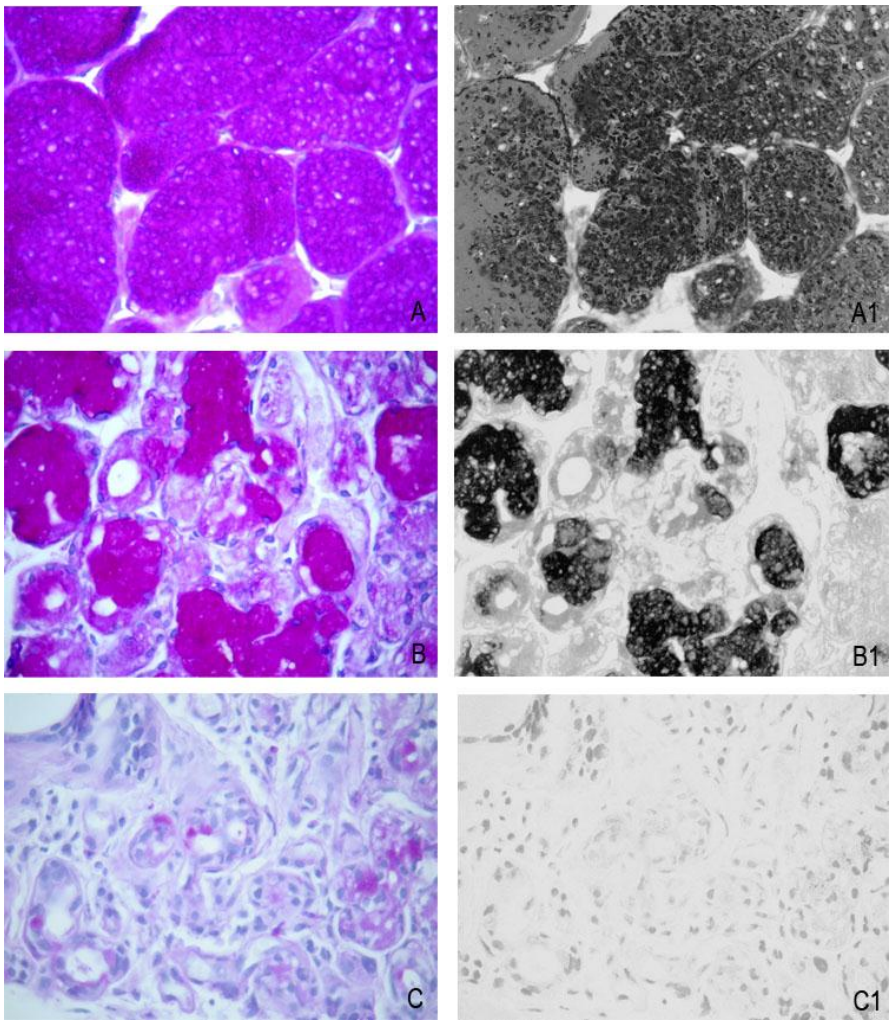

Figure 4. Digital images taken for LimiarK software analysis. A. MSG of the control group, A1. Segmented image of MSG of the control group; B. MSG of HSCT patient without oral cGVHD, B1. Segmented image of MSG of HSCT patient without oral cGVHD; C. MSG of an oral cGVHD patient, C1. Segmented image of MSG of an oral cGVHD patient. (PAS; original magnification X400).

CAPÍTULO 2

Graft-vesus-Host disease in liver: a histological and immunohistochemical study

(Artigo em preparação)

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ABSTRACT

Background and Aim: Chronic graft versus host disease (cGVHD) after hematopoietic stem cell transplantation (HSCT) is a multisystem alloimmune and autoimmune disorder. cGVHD occurs when donor T cells react to the histo-incompatible antigens of the recipient. The organs mostly affected are skin, oral mucosa, salivary glands, and liver. The purpose of this study was to analyze histological features for diagnosis of liver GVHD, the contribution of T cell subsets in liver GVHD and the correlation between the NIH Consensus Criteria for liver cGVHD and laboratory changes.

Methods: This restrospective study included liver biopsy samples of 36 cGVHD patients who underwent allogeneic HSCT after myeloablative conditioning and subsequently required systemic treatment for cGVHD and concomitant infection or medication toxicity had been ruled out. Biopsy sections were stained for H&E and for immunohistochemical technique targeting CD45, CD4, CD8, CD68, CD45RO and CD138. The samples were analyzed based on NIH criteria for liver cGVHD. Digital images were analyzed through

Imagelab® software for subsets T cells quantification. Liver biopsies from 20 healthy non-HSCT subjects were analyzed and considered as the control group.

Results: An increased number of CD8 and CD45RO immunomarked cells were observed in cGVHD liver tissue, compared with the liver biopsies from the control group. A positive correlation was found between immunomarked cells/mm^2 in portal areas and the NIH cGVHD criteria for "final" diagnosis of cGVHD. Nevertheless, twelve histological features found in liver and increase of the serum levels of aspartate transferase, alanine transferase and alkaline phosphatase showed positive correlation with liver cGVHD NIH diagnosis.

Conclusion: The results suggested that T cells (CD8+ and CD45RO+) play an important role in the pathogenesis of liver GVHD. Five NIH cGVHD Consensus Criteria were found to correlate with liver cGVHD diagnosis. Significant correlation was found between changes in the liver enzyme levels and GVHD diagnosis. Our findings support the liver NIH cGVHD Consensus Criteria for histological liver cGVHD for the improvement of GVHD diagnosis.

INTRODUCTION

Chronic graft versus host disease (cGVHD) remains a major cause of late morbidity and mortality after allogeneic hematopoietic stem cell transplantation (allo-HSCT) (Martin et al., 1990; Lee et al., 2002). The incidence of cGVHD following allo-HSCT ranges from 25% to 80%, the occurrence of cGVHD is associated with immune dysfunction and thus, a risk of infection and reduced quality of life (Baird and Pavletic, 2006), despite the fact that cGVHD is also associated with lower relapse rate by the graft versus-leukemia effects (Lee et al., 2002). The syndrome has features resembling autoimmune disorders and affects a wide spectrum of organs, resulting in systemic manifestations that can involve skin, oral cavity, liver along with other sites (Lee et al., 2002; Vigorito et al., 2009).

Liver dysfunction is a frequent complication of hematopoietic stem cell transplantation (HSCT), occurring in 50–72% of patients with mortality rates ranging between 4 and 15% (Ho et al., 2004; Strasser, 1999; El Sayed et al., 2004; Tomas et al., 2000). Among earlier post-HSCT hepatobiliary complications are sinusoidal obstruction

syndrome, cholestatic disorders (cholangitis lenta, acute graft-versus-host disease, and drug-induced liver injury, fungal and bacterial infections, and acute hepatocellular injury. Latterly, hepatobiliary complications are cGVHD, chronic hepatitis C or B, cirrhosis and iron overload (McDonald, 2010).

Liver cGVHD (LcGVHD) is characterized by the destruction of bile duct epithelium followed by progressive cholestasis, with elevations in serum alkaline phosphatase (AP), γ-glutamyl transpeptidase and serum bilirubin levels. It resembles primary biliary cirrhosis (PBC) clinically and histologically (Strasser et al., 2000). The diagnosis of liver cGVHD is based on the presence of clinical jaundice and changes in liver enzymes, but sometimes, liver biopsy can be useful as a complimentary diagnosis when other complications related with the HSCT should be ruled out (Strasser et al., 2000).

The goal of this study was to analyze the most relevant histopathological features for LcGVHD diagnosis, to correlate the NIH final diagnosis for LcGVHD with laboratorial changes and, to identify the leucocytes subsets in the liver cGVHD.

PATIENTS AND METHODS

This was a retrospective analysis which enrolled the 36 patients who survived 100 days or more after allo-HCT between 1994 and 2006. These patients underwent an allogeneic HLA- matched HSCT after myeloablative conditioning, who had submitted to a liver biopsy due to the clinical suspicion of LcGVHD. Clinical and epidemiological data were obtained from the medical chart.

The hepatic involvement of cGVHD was diagnosed by simultaneous clinical and laboratory parameters and by liver biopsy according to Shulman et al., 2006. Viral hepatitis was excluded by standard serological tests for hepatitis A, B, C. Herpes simplex infection as well as varicella zoster were excluded by absence of cytopathologic features of viral inclusions. Each patient's recent medication history was reviewed to assess potential hepatotoxic drug reactions. The patients' clinical data are summarized in table 1.

Histological study

Liver biopsy was performed by the percutaneous needle biopsy. The tissue specimens submitted to subsequent histologic analysis were fixed in 10% buffered formalin

and paraffin-embedding. Tissue blocks were serially sectioned with 4 µm of the thickness. The histologic sections were stained with hematoxylin-eosin, Masson's trichrome, reticulin and Perl's stain (special histochemical were performed with standard methodologies). All histological specimens were subsequently reviewed by one expert hepatic-pathologist by conventional light microscopy. The histologic features were assessed semiquantitatively according to two previously described coding systems (Quaglia et al., 2007; Shulman et al., 2006), with some modifications (Table 2). The histopathologic diagnosis for LCGVHD was carried out as proposed by National Institutes of Health (NIH) consensus pathology working group criteria on cGVHD (Shulman et al., 2006). The Final diagnosis of NIH consensus integrates the histopathologic results and the clinical context, and is summarized in 1 to 4 categories: no GVHD, possible GVHD, consistent with GVHD and definitive (unequivocal) GVHD.

Immunohistochemical study

The bile ducts/ductular reaction were evaluated with AE1/AE3 immunostains. Mononuclear subsets were evaluated with CD4, CD8, CD45, CD45RO, CD68 and CD138. Histological sections (4 µm thick) were obtained from the paraffin-embedded tissue specimens, then deparaffinized in xylene, and rehydrated. The immunohistochemistry was performed using the standard polymeric method. The primary antibodies used were monoclonal mouse anti-human from DakoCytomation/DAKO, directed to: 1- cytokeratin, clone AE1/AE3, dilution 1:100; 2- T-helper cells (CD4+), clone OPD4, dilution 1:100; 3- cytotoxic T cells (CD8+), clone C8/144B, dilution 1:200; 4- Leucocyte Common Antigen (CD45), clone 2B11+PD7/26, dilution 1:150; 5- CD45RO, clone UHCL-1, dilution 1:100; 6- Macrophages (CD68), clone KP1, dilution 1:700; 7- Plasma cell (CD138), clone MI15, dilution 1:200. All antibodies were from DAKO (Dako Cytomation, Carpinteria, CA, USA). A steamer was used for epitope retrieval and the EnVision polymer (DAKO) as the reaction amplifier. Reactions were performed using appropriate positive and negative controls. Staining was achieved with 3,3-diaminobenzidine tetrahydrochloride (Sigma, St Louis, Missouri, USA) and counterstaining with Mayer's haematoxylin. Unsatisfactory sections were excluded and the procedures were repeated.

For quantitative analysis, digital images were obtained through a Power Shot A630 Canon camera connected to a Nikon Eclipse E200 microscope, under a 40x objective. The number of captured microscopic fields ranged with the size and amount of portal tracts by liver samples. Thus, enough fields were photographed in order to sufficiently analyze all or ideally, ten complete portal tracts. Digital images were analyzed through Imagelab® software (Image analysis system, Diracom, São Paulo, Brazil) for cell quantification and portal tracts measurements. The mean number of immunomarked cells/mm^2 by portal tract was used for statistical analysis. Additionally, a control group for evaluation of mononuclear subsets, liver biopsies from 20 healthy non-HSCT subjects, whose were potentials donors for liver transplantation, was analyzed.

Statistical Analysis

To perform statistical analysis, Statistical Package for the Social Sciences (SPSS) for Windows 14.0, was used and P values <0.05, were considered statistically significant. The tests applied were, the Spearman or Pearson correlation, Student t-test and Kaplan-Meier method (Kaplan and Maier, 1958), which analyzed and compared actuarial curves of overall survive using the log-rank test (Mantel and Haenze, 1958).

RESULTS

The number of complete portal tracts ranged from 1 to 15 (median 4). Twenty-three biopsies had from 1 to 5 portal tracts, 8 biopsies had 6-10 portal tracts and 5 samples had more than 10 portal tracts. The biopsy day post-HSCT ranged from 77 to 2380 (median 257) with 2 biopsies taken before 100 days post-HSCT.

Seven histological features showed correlation with at least one liver serum test (aminotransferases or bilirubin). Decreased bile duct/portal tract ratio (BD/PTr) was correlated with Total bilirubin (TB) (r=33%, p=0.04), alanine transaminase (ALT) (r=37%, p=0.02) and aspartate transaminase (AST) (r=41%, p=0.01); lobular inflammation and lobular inflammation type (lymphomononuclear or polymorphonuclear) were correlated with AST (r=35%, p=0.03; r=45%, p=0.006 respectively) and AP (r=52%, p=0.001; r=42%, p=0.01 respectively); bile ducts infiltrated by lymphocytes was correlated with ALT (r=53%, p=0.001) and AST (r=46%, p=0.004); focal necrosis in parenchyma, bile

duct epithelium degenerative changes and periportal interface hepatitis were correlated only with AP (r=44%, p=0.007; r=36%, p=0.03 and r=35%, p=0.03 respectively) (Table 3).

In addition to these histological findings, other 5 histological features showed correlation with NIH consensus final diagnosis for LcGVHD. Bile duct epithelium degenerative changes, portal inflammation, lobular inflammation and focal necrosis in parenchyma showed a stronger correlation with NIH consensus final diagnosis (r=66%, p<0.0001; r=54%, p=0.001, r=51%, p=0.001 and r=51%, p=0.001 respectively). Furthermore, NIH final diagnosis were correlated with lobular inflammation type (r=46%, p=0.004), bile ducts infiltrated by lymphocytes (r=43%, p=0.01), confluent necrosis (r=42%, p=0.01), decreased bile duct/portal tract ratio (r=40%; p=0.01), periportal interface hepatitis (r=39%, p=0.01), central perivenulitis (r=36%, p=0.03), eosinophilic portal inflammation (r=36%, p=0.03) and endothelitis (r=33%, p=0.04) (Table 3).

The histological findings of bile duct epithelium degenerative changes were positive in 83% of all cases, most of them showing focal involvement. Results were correlated with bile ducts infiltrated by lymphocytes (r= 74%, p<0,001), decrease bile ducts/portal tracts ratio (r=43%, p = 0.009) and lobular inflammation (r = 34%, p = 0.03). Portal inflammation, the most frequent histologic feature (94%), showed mild or moderate involvement in most of cases. In addition to the features previously described, the following histologic features were observed in more than 60% of the samples: Lobular inflammation (83%), Reticulo-endothelial iron (83%), focal necrosis in parenchyma (75%), liver cell ballooning (69%), fibrosis (67%), bile ducts infiltrated by lymphocytes (64%) and hepatocellular iron (64%). The interface hepatitis was present in exactly 50% of the cases. HOwever, chronic cholestasis and arteritis were observed in only one sample (3%), whereas periductal lymphoid aggregates were not observed.

The following histologic features were the less frequent in the assessed samples: apoptosis (39%), confluent necrosis (33%), endothelitis (31%), central perivenulitis (28%), periductal fibrosis (14%), decrease BD/PTr (14%), intrahepatocytic cholestasis (14%), eosinophilic portal inflammation (14%), ductular reaction (11%), canalicular cholestasis (11%), microvesicular steatosis (11%), macrovesicular steatosis (6%). Although infrequent (11%), canalicular cholestasis, together with the lobular

inflammatory infiltrate, containing lymphocytes and polimorphonuclear cells, showed to influence overall survival (p=0.03 and 0.008, respectively). The frequency and degree of each histological criterion assessed are shown in Table 2.

Correlation between eight histologic features and at least one of three inflammatory subsets (CD8, CD45 and CD138) was found. Lobular inflammation showed correlation with 3 leucocytes subsets, CD8+ T cells (r=50%, p=0.008), CD138+ cells (r=49%, p=0.01) and CD45+ cells (r=37%, p=0.05). Bile duct epithelium degenerative changes and bile ducts infiltrated by lymphocytes were correlated with CD45+ cells (r=57%, p=0.002; r=56%, p=0.002 respectively) and CD8+ T cells (r= 45%, p=0.01; r=41%; p=0.01 respectively). Ductular proliferation and central perivenulitis were correlated with CD45+ cells (r=43%, p=0.02; r=46%, p=0.01 respectively), focal necrosis in parenchyma showed correlation with CD8+ T cells (r=47%, p=0.005) and CD138+ cells (r=45%, p=0.02). Periportal interface hepatitis and portal inflammation was correlated with CD8+ T cells (r=52%, p=0.005; r=40%, p=0.04 respectively) (Table 4). CD4, CD45RO and CD68 showed no correlation with any histological feature. An increased number of immunomarked for CD8 ($p<0.0001$) and CD45RO (p=0.01) were observed in cGVHD patients when compared with control group (Table 5 and Figure 1).

NIH final diagnoses for LCGVHD showed a positive correlation with CD45+ (r=46, p=0.01) e CD138+/mm^2 (r=47%, p=0.02), and also a positive correlation with the increase of the following liver enzyme levels: ALT (r=47; p=0.004), AP (r=38; p=0.02) and AST (r=33; p=0.05).

DISCUSSION

cGVHD is the most common non-relapse complication after allo-HCT. Although GVHD is associated with a potent antileukemia effect, cGVHD is also associated with significant morbidity and treatment-related mortality. cGVHD affects many organs and tissues, such as the skin, oral mucosa and liver, among others, resembling the autoimmune diseases (Hidaka et al., 2010).

Classically, LcGVHD presents with cholestatic disorder, and liver function test derangement with an elevation of bilirubin and AP levels (Ma et al., 2004; Yasmineh et al.,

1989). However, hepatitic LcGVHD differed significantly from classic liver GVHD regarding the pattern of hepatic serum enzymes, with higher AST and ALT levels at disease onset and higher AST, ALT, and ALP levels at the peak of enzymatic changes, however the level of bilirubin was comparable in these two situations (Ma et al., 2004).

Our results showed correlations between some histological findings and increased levels of bilirubin, AP, AST and ALT. Among them, a greater number of histologic findings that make up the framework of hepatitic LcGVHD were correlated with altered liver enzymes; these are lobular lymphocytic infiltrate, lobular lymphocytic infiltrate with polymorphonuclear cells, bile ducts infiltrate by lymphocytes and focal necrosis in parenchyma. Only one characteristic findings of classic LcGVHD, decreased BD/ PTr, was correlated with TB, AST and ALT. Bile Ducts degenerative changes, present in both forms of the LcGVHD were associated with altered levels of AP. All laboratory liver tests studied reflected at least one of the histological hepatic changes correlated with diagnosis of LcGVHD. These results are in agreement with previous studies which demonstrated the correlation between the histopathologic and clinical diagnosis of LcGVHD, showing that a comprehensive assessment of the laboratorial liver tests changes is needed for diagnosis of liver cGVHD. (Ma et al., 2004; Strasser et al., 2000; Shulman et al., 1988).

A study conducted by Duarte et al, was the first to report the prognostic value of histological findings for LcGVHD (Duarte et al., 2005). They showed that a high level of lobular inflammation and a low level of liver cell ballooning biopsy specimens were independent favorable prognostic factors for non-relapse mortality rate and overall survival (Duarte et al., 2005). In our study we found a prognostic value in two histological findings: canalicular cholestasis and type of lobular infiltrate. We observed a worse prognostic for overall survival when canalicular cholestasis was present when he lobular infiltrate was composed by lymphomononuclear cells. This result should be viewed with caution as the number of patients studied was small and the groups are unbalanced, amongst the samples, only 4 (11%) showed canalicular cholestasis; among biopsies with lobular inflammation (83%), only 9 of them (25%) were comprised for lymphomononuclear infiltrate.

Bile duct epithelial degenerative changes are an important histological diagnostic criterion of LCGVHD and its presence in classical and hepatitic LCGVHD was one of the most frequent histologic feature found in the studied sample (83%) and showed a higher correlation with the LCGVHD NIH final diagnosis ($r=0.66$, $p<0.0001$). According to Quaglia et al, bile duct epithelial degenerative changes culminate in bile ductal loss, which reinforces the histological diagnosis of LCGVHD (Quaglia et al., 2007). In agreement with these results, we observed a correlation of the bile ducts infiltrated by lymphocytes and decrease bile ducts/portal tract ratio with NIH final diagnosis of LcGVHD. Other histological features had a greater correlateion with NIH diagnosis for LcGVHD, such as: lobular inflammation, focal necrosis in parenchyma, portal inflammation, periportal interface hepatitis, confluent necrosis, central perivenulitis, eosinophilic portal infiltrate and endothelitis. These results suggest the need of observing those histological features in the liver biopsy assessment for diagnosis of LCGVHD, especially central perivenulitis, an important criterion in liver allograft rejection. Central perivenulitis, characterized by inflammation and necrosis around the central venules, associated with severe acute cellular rejection and late cellular rejection of liver allograft. Although infrequent (present in 28% of cases) the correlation of central perivenulitis with LcGVHD diagnosis showed that this histologic feature may have been underestimated in the histological context of LcGVHD.

Fibrosis, one of the histological features that traditionally have been associated with chronicity of GVHD (Shulman et al., 1988; Shulman et al., 1980), showed no correlation with LcGVHD NIH diagnosis even though present in 67% of cases. LcGVHD is not a fibrogenic process (Quaglia et al., 2007). Reticulo-endothelial iron and hepatocellular iron showed a significant frequency among our cases, however none correlated with NIH LcGVHD diagnosis. Sucak et al suggest that the number of transfusions in these patients is a contributing factor to iron overload, although the amount of iron transfused by blood products per se does not explain the iron overload of the patients (Sucak et al., 2008). Decreased utilization of iron due to ineffective erythropoiesis, release of iron from the injured tissues, increased intestinal absorption of iron secondary to mucositis and GVHD, are other possible causes of iron overload in post-HSCT patients (Kamble et al., 2006).

In many target organs, such as minor salivary glands and skin, CD45+ lymphocytes with initially a CD4 and later a CD8 phenotype are easily identified by immunohistology (Sale, 2005). Our results showed the significant increase of memory T cells (CD45RO+) and cytotoxic T cells (CD8+) on portal tracts when compare to portal tracts from normal patients. No significant difference was observed between the groups for auxiliary T cells (CD4+). As occurs in minor salivary glands, the CD8+ T cells have predominance over CD4+T cells.

The CD8 subset showed correlation with histologic features of LCGVHD such as, bile ducts degenerative changes, bile ducts infiltrate by lymphocytes, portal inflammation, lobular inflammation and focal necrosis in parenchyma, reflecting topographic and qualitative participation in the inflammatory infiltrate on LcGVHD. Leucocytes CD45+ and CD138+, despite not being increased in LcGVHD, showed correlation with some histological findings and NIH final diagnosis. CD45 showed correlation with bile ducts degenerative changes, bile ducts infiltrate by lymphocytes, ductular proliferation, lobular inflammation and central perivenulite. Plasma cells (CD138+) were correlated with lobular inflammation and focal necrosis in parenchyma. The role of B cell activity in cGVHD is underscored by the observation that high plasma levels of BAFF (B cell activating factor), a cytokine that appears to drive B cell autoimmunity, were noted in patients with cGVHD (Sarantopoulos et al., 2007). Clinical data on the therapeutic benefit of rituximab indicate that B cells might be pathogenic in chronic GVHD (Cutler et al., 2006; Zaja et al., 2007). These observations, however, do not clarify whether B cells play a role in priming donor T cells (as antigens presenting cells) or function as effectors due to dysregulation of donor T-helper cells (Toubai et al., 2008).

Although, thirty-six biopsy samples were analyzed, and the number of complete portal tracts ranged from 1 to 15, a mean of 5,5 portal tracts by sample. The ideal number of portal tracts for liver assessment should be 10, according to Shulman et al (2006). The inadequate amount of portal tracts in the sample was a limitation found in this study; only 5 samples (14%) had 10 or more portal tracts. This limitation has been found by other authors (Sloane et al., 1980; Shulman et al., 1988; Quaglia et al., 2007) since samples are usually collected by needle biopsy, obtaining a small sample.

CONCLUSION

Our results suggested that T cells CD8+ and CD45RO+ play an important role in the pathogenesis of liver GVHD, and that the participation of plasma cells (CD138+) should be further investigated. The most important histopathological features on LcGVHD diagnosis was the bile duct epithelium degenerative changes and lymphocytes permeating the ductal epithelium, decreased bile duct/portal tract ratio, presence of lobular inflammation regarding the lymphomononuclear infiltrate, focal necrosis in parenchyma, portal inflammation and the presence of eosinophils in inflammatory infiltrate, periportal interface hepatitis, confluent necrosis and central perivenulitis. Significant correlation was found between changes in laboratorial liver tests and cGVHD diagnosis, as well as in the histological features that compose the criteria for NIH diagnosis of LcGVHD. Our findings support the view that the histological findings of the NIH Consensus are helpful in improving the diagnosis of GVHD.

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ACKNOWLEDGMENTS

This paper was supported by the Coordination for Higher Level Graduates Improvement (CAPES) and Research and Extension Support Foundation (FAPESP) n° 2006/59917-7 .

TABLES

Table 1. Patients' clinical characteristics.

Variable	n = 36
Age, median (range)	29.5 (12-59)
Male: female, n (%)	21 (58) : 15 (42)
Disease, n (%)	
CML	24 (66.7)
ALL	4 (11)
SAA	3 (8.3)
AML	2 (5.6)
MDS	2 (5.6)
PNH	1 (2.8)
Conditioning regimen, n (%)	
BU + CY	31 (86.1)
BU + VP + CY	3 (8.3)
CY + TBI	1 (2.8)
CY	1 (2.8)
Graft, n (%)	
Bone marrow	21 (58.3)
Peripheral blood	15 (41.7)
GVHD prophylaxis, n (%)	
CsA/MTX	32 (88.9)
CsA/Corticosteroid	4 (11.1)
Serum liver tests, n (%)*	
Total Bilirubin	16 (44.4)
Aspartate transaminase	27 (75)
Alanine transaminase	26 (72.2)
Alkaline phosphatase	34 (94.4)
Mean Follow-up, median (range) **	122 (8-180)

ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic disorder syndrome; AML, acute myelogenous leukemia; SAA, severe aplastic anemia; PNH, paroxysmal nocturnal hemoglobinuria; VP, VP-16 Etoposide; BU, Busulfan; CY, cyclophosphamide; TBI, total body irradiation; CsA, Cyclosporin A; MTX, Methotrexate.

* Serum level > 2 times above the upper normal limit.

** Follow-up in months.

Table 2. Histological features assessed in liver.

Histological feature	Grading	Frequency, n (%)
Bile duct epithelium degenerative changes**	1 = Absent	6 (16.7)
	2 = Focal Involvement (few ducts)	20 (55.6)
	3 = Diffuse Involvement (most ducts)	10 (27.8)
Lymphocyte exocytosis through bile ducts*	1 = Absent	13 (36.1)
	2 = One or more lymphocytes in one bile duct	18 (50.0)
	3 = lymphocytes in more than 1 bile duct	3 (8.3)
	4 = lymphocytes in all bile ducts	2 (5.6)
Periductal lymphoid aggregates*	1 = Absent	36 (100)
	2 = Present	0
Periductal fibrosis*	0 = Absent	32 (88.9)
	1 = Focal Involvement (few ducts)	4 (11.1)
	2 = Diffuse Involvement (most ducts)	0
Ductular reaction*	1 = Absent	32 (88.9)
	2 = Mild and focal (up to six ductules < 50% of portal tracts)	4 (11.1)
	3 = More than 6 ductules in < 50% of portal tracts or up to 6 ductules in > 50% of portal tracts	0
	4 = More than 6 ductules in > 50% of portal tracts	0
Bile duct/portal tract ratio***	1 = 1,8 - 0,9	31 (86.1)
	2 = 0,8 - 0,5	5 (13.9)
	3 = 0,4 - 0,1	0
	4 = <0,1	0
Canalicular cholestasis*	1 = Absent	32 (88.9)
	2 = Occasional identified plugs	2 (5.6)
	3 = Easily identifiable bile plugs in most centrilobular areas	2 (5.6)
	4 = Widespread canalicular cholestasis	0
Intrahepatocytic cholestasis***	1 = Absent	31 (86.1)
	2 = Present	5 (13.9)
Chronic cholestasis**	1 = Absent	35 (97.2)
	2 = Present	1 (2.8)
Portal inflammation***	1 = Absent (scarce lymphocytes)	2 (5.6)
	2 = Mild, some or all portal tracts	16 (44.4)
	3 = Moderate, some or all portal tracts	14 (38.9)
	4 = Moderate/marked, all portal tracts	3 (8.3)
	5 = Marked, all portal tracts	1 (2.8)
Type of portal inflammation	1 = lymphomononuclear	9 (25)
	2 = lymphocytes and polymorphonuclear cells	25 (69.4)
Periportal interface hepatitis***	1 = Absent	18 (50)
	2 = Mild (focal, few portal tracts)	13 (36.1)
	3 = Mild/Moderate (focal, most portal tracts)	1 (2.8)

	4 = Moderate (continuous around <50% of tracts or septa)	2 (5.6)
	5 = Severe (continuous around >50% of tracts or septa)	2 (5.6)
Apoptosis*	1 = Absent	22 (61.1)
	2 = One apoptotic body per 10X objective	13 (36.1)
	3 = Two to four apoptotic bodies per 10X objective	1 (2.8)
	4 = More than four apoptotic bodies per 10X objective	0
Focal lobular necrosis***	1 = Absent	9 (25)
	2 = Few foci	12 (33,3)
	3 = Several Foci	15 (41.7)
Confluent necrosis***	1 = Absent	24 (66.7)
	2 = Focal confluent necrosis	7 (19.4)
	3 = Bridges of confluent necrosis	5 (13.9)
Liver cell ballooning*	1 = Absent	11 (30.6)
	2 = Occasional ballooned hepatocytes usually with centrilobular location	13 (36.1)
	3 = Easily identifiable ballooned hepatocytes in most centrilobular areas	11 (30.6)
	4 = Widespread liver cell ballooning	1 (2.8)
Fibrosis***	1 = Absent	12 (33.3)
	2 = Portal fibrosis	20 (55.6)
	3 = Portal–portal fibrous septa	4 (11.1)
	4 = Predominance of areas with incomplete nodules or septa	0
	5 = Predominance of nodules / cirrhosis	0
Macrovesicular steatosis***	0 = Until 5% parenchyma	34 (94.4)
	1 = 5-33% parenchyma	2 (5.6)
	2 = 34-66% parenchyma	0
	3 = > 67% parenchyma	0
Microvesicular steatosis***	0 = Absent	32 (88.9)
	1 = Focal	4 (11.1)
	2 = Form contiguous sheets	0
Lobular inflammation***	0 = Absent	6 (16,7)
	1 = Mild	15 (41.7)
	2 = Moderate	14 (38.9)
	3 = Marked	1 (2.8)
Type of lobular inflammation	1 = Lymphomononuclear	9 (25)
	2 = Lymphocytes and polymorphonuclear cells	21 (58.3)
Hepatocellular iron Accumulation***	0 = Absent or hardly discernible at 400X	13 (36.1)
	1 = hardly discernible at 250X and easily identifiable at 400X	7 (16.7)
	2 = Discrete granules easily identifiable at 100X	5 (13,9)
	3 = Discrete granules easily identifiable at 25X	8 (22.2)
	4 = Visible masses at 10X	3 (8.3)
Reticuloendothelial iron	1 = Absent	6 (16.7)
	2 = Present	30 (83.3)

Endotheliitis*	1 = Absent	25 (69.4)
	2 = Focal	10 (27.8)
	3 = Moderate	1 (2.8)
	4 = Diffuse	0
Central perivenulitis	1 = Absent	25 (71.4)
	2 = Present	10 (28.6)
Eosinophilic portal inflammation	1 = Absent	28 (82.4)
	2 = Present	6 (17.6)
Arteritis*	1 = Absent	35 (97.2)
	2 = One artery	1 (2.8)
	3 = More than one artery	0
	4 = All arteries	0

*Histologic feature assessment according Quaglia et al., 2007.
** Histologic feature assessment according Histopathology forms of Shulman et al., 2006.
***Histologic feature assessment according Burt et al., 2007.

Table 3. Correlation of the histological features analyzed with hepatic enzyme levels and histologic L-GVHD diagnosis.

Histologic features	Correlation % (*p*)				
	TB	ALT	AST	AP	LcGVHD
Bile duct/portal tract ratio	33 (0.04)	37 (0.02)	41 (0.01)	NC	40 (0.01)
Lobular inflammation	NC	NC	35 (0.03)	52 (0.001)	51 (0.001)
Lobular inflammation Type	NC	NC	45 (0.006)	42 (0.01)	46 (0.004)
Bile ducts infiltrated by lymphocytes	NC	53 (0.001)	46 (0.004)	NC	43 (0.01)
Focal necrosis in parenchyma	NC	NC	NC	44 (0.007)	51 (0.001)
Bile duct epithelium degenerative changes	NC	NC	NC	36 (0.03)	66 (<0.0001)
Periportal interface hepatitis	NC	NC	NC	35 (0.03)	39 (0.01)
Portal inflammation	NC	NC	NC	NC	54 (0.001)
Confluent necrosis	NC	NC	NC	NC	42 (0.01)
Central perivenulitis	NC	NC	NC	NC	36 (0.03)
Eosinophilic Portal inflammation	NC	NC	NC	NC	36 (0.03)
Endothelitis	NC	NC	NC	NC	33 (0.04)

TB, total bilirubin; ALT, alanine transaminase; AST, Aspartate transaminase; AP, alkaline phosphatase; NC, No correlation.

Table 4. Correlation between histological features of liver cGVHD and leucocytes subsets.

Histologic features	Correlation % (*p*)		
	CD8	CD45	CD138
Bile duct epithelium degenerative changes	45 (0.01)	57 (0.002)	NC
Bile ducts infiltrated by lymphocytes	41 (0.01)	56 (0.002)	NC
Ductular reaction	NC	43 (0.02)	NC
Portal inflammation	40 (0.04)	NC	NC
Periportal interface hepatitis	52 (0.005)	NC	NC
Lobular inflammation	50 (0.008)	37 (0.05)	49 (0.01)
Focal necrosis in parenchyma	47 (0.005)	NC	45 (0.02)
Central perivenulitis	NC	46 (0.01)	NC

NC, No correlation.

Table 5. Leucocytes subsets in the liver portal tract of patients with cGVHD and control group.

	With GVHD			Control group			
	Mean*	St Deviation*	N	Mean*	St Deviation*	N	p
CD8	2397.61	1358.19	27	839.29	440.53	17	<0.0001
CD45RO	2208.11	1143.50	16	1247.65	902.18	18	0.01
CD45	2915.45	2017.86	28	2077.14	1089.96	19	0.1
CD4	1516.92	935.91	27	1219.15	711.58	20	0.2
CD68	963.08	629.07	21	780.80	540.05	19	0.3
CD138	362.12	485.54	25	261.23	248.30	15	0.4

*Cells/mm^2

FIGURES

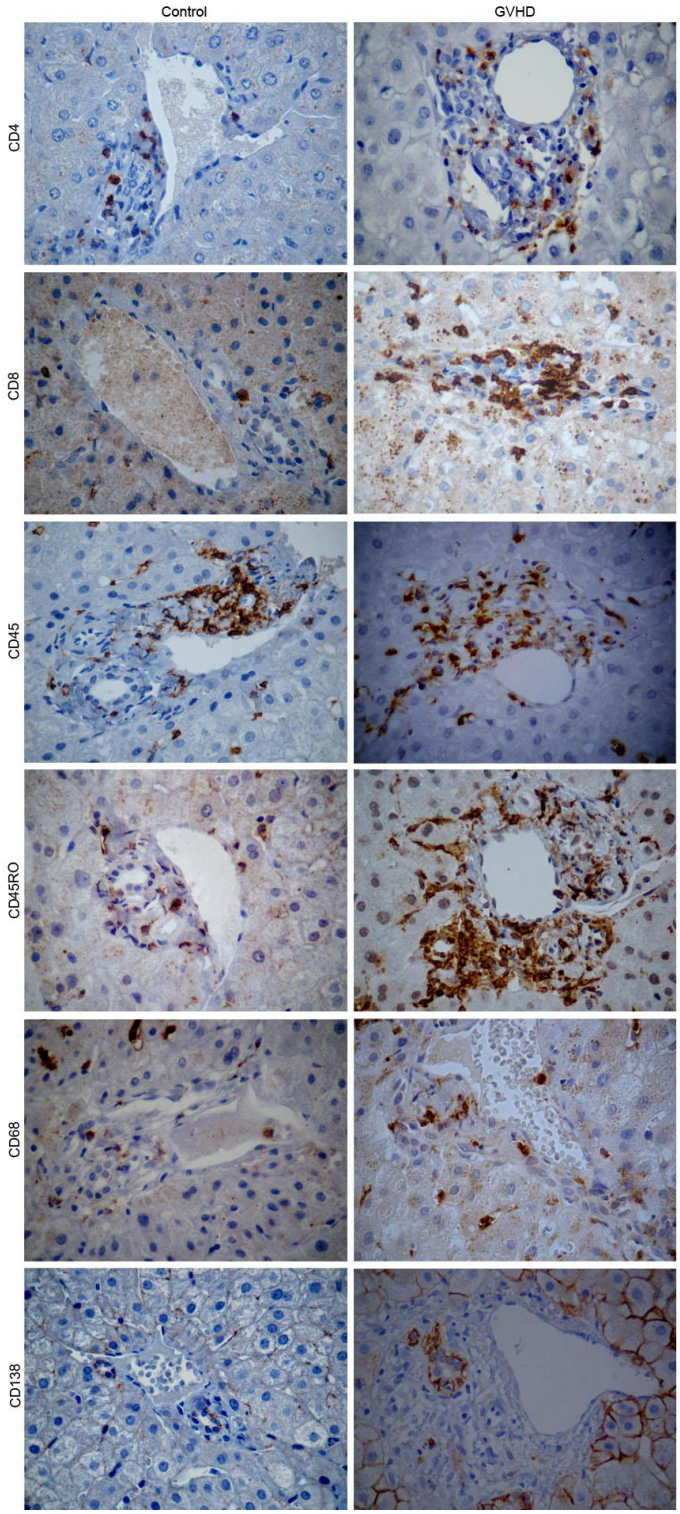

Figure 1. Immunostained liver sections targeting for CD4, CD8, CD45, CD45RO, CD68 and CD138 in control group- and cGVHD patients. Original magnification x400.

CAPÍTULO 3

Chronic GVHD after hematopoietic stem cell transplantation: Histological and imunohistochemical correlation between liver and minor salivary glands changes
(Artigo em preparação)

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ABSTRACT

Background: Chronic GVHD (cGVHD) after hematopoietic stem cell transplantation (HSCT) is a multisystem alloimmune and autoimmune disorder characterized by immune dysregulation, immunodeficiency, and impaired organ function. The aim of this study was to compare mononuclear cell subsets in minor salivary gland (MSG) and liver samples of patients with cGVHD after HSCT and to identify MSG histological changes that might be predictive of severity in the liver involvement by GVHD.

Methods: Liver and MSG biopsies of 36 cGVHD patients were studied. The patients had undergone ablative HLA- matched- sibling HSCT. Histological sections were stained for H&E and immunohistochemical technique targeting CD45, CD4, CD8, CD138, and CD68. Digital images were analyzed through *Imagelab 2000*® software.

Results: Clinical changes resulting from cGVHD in the oral cavity were prior or, at least, simultaneous, to clinical and laboratorial liver changes. Diagnosis of liver cGVHD categories 3 or 4 from NIH consensus were correlated with serum levels > 2 times above

the upper normal limit of ALT, AST and AP. A positive correlation was found between the relative proportion of CD4, CD8, and CD45 immuno-labeled cells in MSG and liver biopsy samples. Higher numbers of CD45+ and CD138+ cells/mm^2 in portal tract correlated with NIH diagnoses 3 or 4 for liver cGVHD. Higher relative numbers of CD45+ and lesser of CD8+ cells in MSG, were found to be predictive of severity in liver GVHD histological changes.

Conclusion: MSG mononuclear infiltrates mirror those of liver in cGVHD. Considering the set of clinical and laboratory findings, MSG biopsies, whicht are easily obtained, could help estimate hepatic changes in cGVHD patients.

INTRODUCTION

Chronic graft-versus-host disease (cGVHD) is a multiorgan disorder with features of immunodeficiency and autoimmunity [1]. This disorder can affect any major organ system, but most commonly affects skin, oral-, vaginal-, and conjunctival mucosa, salivary and lacrimal glands, and the liver [2]. Clinically, cGVHD symptoms resemble those of autoimmune- and other immunologic disorders, such as scleroderma, Sjögren syndrome, primary biliary cirrhosis, wasting syndrome, bronchiolitis obliterans, immune cytopenias, and chronic immunodeficiency [3, 1].

Eighty per cent of patients with liver involvement by GVHD have increased serum levels of alkaline phosphatase, bilirubin and aminotransferases [5]. Histologically, typical liver GVHD is characterized by bile duct damage, portal lymphocytic infiltrate, and centrilobular necrosis. Other features include marked lobular hepatitis, hepatocyte unrest, and sinusoidal inflammation with perivenular necroinflammatory foci. Intrahepatic bile ducts injury begins with degenerative changes, followed by multifocal necrosis of isolated epithelial cells, nuclear pleomorphism, atrophy, and finally, the disappearance of ducts [5-7]. Late changes such as cholestasis, biliary cirrhosis, and chronic hepatitis may develop [8,9].

Common clinical oral findings in cGVHD include erythema, mucosal inflammation and atrophy, lichenoid changes, mucoceles, hyperkeratotic plaques, perioral fibrosis, and infections related to hiposalivation [10-12]. The histological presentation of

salivary glands cGVHD includes lymphohistiocytic infiltrate on the glandular parenchyma and periductal tissue, with or without plasma cells, exocytosis of lymphocytes (without neutrophils) into lobular ducts and acini, and subsequent acinar destruction with fibrosis [11, 13-16]. In additon, the ducts of the minor salivary glands (MSG) become damaged, displaying epithelial cell apoptosis, ectasia, atrophy, and peripheral fibrosis [17].

Given the close similarities between cGVHD and autoimmune diseases, mainly primary biliary cirrhosis, and Sjogren's syndrome, a long-standing hypothesis has been postulated in that cGVHD results from pathogenic effects mediated by ‘‘autoimmune’’ T cells that recognize donor antigens or shared antigens of the donor and recipient rather than alloantigens of the recipient [1]. Primary biliary cirrhosis (PBC), such as cGVHD, is a multisystem disease, that involves lacrimal and salivary glands in 70-100% of patients. Damage to small intrahepatic bile ducts is characteristic of early PBC [18]. Of the patients with PBC, 58% to 93% have histologic abnormalities of the salivary glands, whereas 33% to 64% have abnormal sialometry or sialograms [19]. Although the clinical manifestations of salivary gland injury are similar in cGVHD and Sjögren syndrome (SS) [20], differences exist. SS and cGVHD share a lympho-plasmacytic peri-ductal and intralobular inflammatory infiltrate, with varying degrees of acinar atrophy and fibrosis. However, in SS, the peri-ductal cell infiltrate is more pronounced, and there is a predominance of CD4 versus CD8 T cells, whereas in cGVHD there is a predominance of CD8 T lymphocytes, with macrophages and plasma cells [21].

The liver is an exocrine glandular structure; similar to salivary and lacrimal glands, and has an acinar-ductular anatomy [18]. Both, liver and salivary glands have a common developmental endoderm origin and, express the HLA antigen, required for immune recognition [22, 23]. The ductal epithelium from salivary glands and bile ducts has a high density of histocompatibility complex antigens, so that, after HSCT, they become the target of the donor T cells [22, 23].

According to Duarte et al. [24], lobular inflammation and hepatocytes ballooning in liver samples, were considered favorable prognostic factors for non-relapse mortality and overall survival. Pavletic et al. [25] observed, on clinical grounds, that the impairment of the oral cavity was a factor in determining prognosis for survival in patients

with cGVHD. Considering that minor salivary gland samples are easily obtained and the significant frequency of oral and hepatic cGVHD, the impact as a predictor of treatment outcome for HSCT [24, 25], and the structural- and histological relationship between liver and salivary glands [18], the correlation between the histological findings of the salivary glands and liver in cGVHD may be of great importance.

Our goal was to compare mononuclear cells subsets in MSG and liver samples of patients with cGVHD after HSCT, and investigate whether there is any MSG histological change that might be predictive of the severity in the liver involvement by GVHD.

PATIENTS AND METHODS

The files of the Bone Marrow Transplantation unity of UNICAMP University Hospital during the period of 1994-2006 were searched, and 245 patients were found to be clinically diagnosed with cGVHD. Amongst these, 189 patients presented clinic signals suggestive of oral or liver GVHD; 49 patients shared oral and liver clinic symptoms/signals suggestive of cGVHD and had, at least, one MSG and liver biopsy sample collected for diagnosis purposes. Patients with other causes for liver dysfunction, and liver or MSG biopsies with insufficient amount of paraffin- embedded tissue for histological new cuts, were ruled out. After these evaluations, 36 patients were included in this study. They had undergone ablative HLA- matched- sibling HSCT. The patients' clinical data were retrieved from medical charts and are summarized in table 1.

The biopsy specimens were obtained on the same day that clinical signs and symptoms suggestive of cGVHD were detected. Liver biopsies were performed when laboratory abnormalities were suspected for cGVHD. The MSG samples were collected from the lower inner lip, approximately 10 mm beneath the vermilion border, through a 4 mm-punch, under local anesthesia. Liver biopsy was performed through percutaneous needle biopsy. The tissue specimens were fixed in 10% buffered formalin and paraffin-embedded for histological purposes.

Histological study

Both, liver and MSG histological sections were stained with hematoxylin-eosin and Masson's trichrome for fibrosis evaluation. Liver samples were also stained for reticulin (to assess fibrosis and loss of architecture) and iron (Perl's staining).

All specimens were assessed for histological cGVHD diagnosis according to the National Institutes of Health (NIH) consensus pathology working group criteria (Shulman et al., 2006), to wit: 1- no GVHD, 2- possible GVHD, 3- consistent with GVHD (equivalent to "favor," "suggestive of," or "probable"), or 4- definite (unequivocal) GVHD.

Immunohistochemical study

Four micrometer- thick sections were obtained, dewaxed and rehydrated. The primary monoclonal antibodies used were: CD4, clone OPD4 at a 1:100 dilution; CD8, clone C8/144B, at a 1:200 dilution; CD45, clone 2B11+PD7/26, at a 1:500 dilution; CD68, clone KP1, at a 1:700 dilution; CD138, clone MI15, at a 1:200 dilution, all from DAKO (Dako Cytomation, Carpinteria, CA, USA). The epitope retrieval was performed through a steamer with citrate buffer. The EnVision polymer (Dako Cytomation) was used as a reaction amplifier, and staining followed the supplier's recommendation. Visualization of the antibody complex was achieved using 3,3-diaminobenzidine tetrahydrochloride (Sigma, St Louis, Missouri, USA) according to the manufacturer's instructions. Sections were counterstained with Mayer hematoxylin. Unsatisfactory sections were excluded and the procedures were repeated. Appropriate negative and positive controls were included in each essay.

The quantitative analysis for each antibody was accomplished as follows: digital images were obtained through a Power Shot A630 Canon camera connected to a Nikon Eclipse E200 microscope, under a 40x objective. Regarding liver samples, the number of captured microscopic fields ranged with the size and amount of portal tracts by liver samples. Thus, enough fields were photographed in order to sufficiently analyze 10 complete portal tracts. For MSG samples, 10 blindly and randomly fields were captured. Digital images were analyzed through Imagelab 2000® (Image analysis system, Diracom, Sao Paulo, Brazil); the software was used for cell quantification and portal tracts area measurements. The mean number of the immunomarked cells/mm^2 by portal tract in liver or by specimen in MSG was used for statistical analysis.

Statistical Analysis

The main tests applied were the Spearman correlation and multiple regressions for modeling the relationship between a scalar variable (NIH consensus schema) and MSG subset cells. Statistical significance level was set at $p<0.05$. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) for Windows 14.0.

RESULTS

Seventy two biopsy samples were analyzed (36 MSG and 36 liver specimens). The biopsy day post-HSCT ranged from 77 to 2380 for liver (median 257) and from 77 to 1681 (median 189) for MSG. Thus, for one same patient, the day MSG and liver biopsies were collected, did not necessarily coincide. Twenty-one patients had GSM biopsy collected before liver biopsy, considering these biopsies, the difference between the day post-HSCT of GSM and liver sample collect ranged from 5 to 308 days (median 112). Seventy two percent of cases had a previous clinical diagnosis of oral cGVHD at the time of liver biopsy. Regarding skin, eyes and gastrointestinal tract, only 25, 22 and 3% of cases, respectively, had a previous clinical diagnosis of cGVHD at the time of liver biopsy (Table 2).

Positive correlation was found between CD4+ ($r=85\%$, $p<0.0001$), CD8+ ($r=60\%$, $p= 0.002$), and CD45+ ($r= 46\%$, $p=0.01$) cells/mm^2 in MSG and liver biopsy samples (Table 3 and Figure 1).

Liver NIH diagnoses 3 or 4 were correlated with serum levels > 2 times above the upper normal limit of ALT ($r=47\%$, $p=0.0004$), AST ($r=33\%$, $p = 0.05$) and AP ($r=38\%$, $p=0.02$). Through multiple regression test, having NIH schema as dependent variable, a predictive value of CD45+ and CD8+ cells/mm^2 was found in MSG for NIH liver GVHD diagnosis ($p= 0.01$ and $p= 0.02$, respectively), taking the model of class 3 or 4 of the NIH schema as a reference, to wit: when the number of CD45 positive-cells was higher than 1000/mm^2, the chance of the specimen to be part of NIH critical group was higher whereas for CD8, the fewer the cells, the greater the specimen chance to be in the NIH schema ≥ 3.

DISCUSSION

Chronic GVHD resembles autoimmune and other immunologic disorders, such as scleroderma, Sjögren syndrome, primary biliary cirrhosis, and bronchiolitis obliterans [1, 3]. Skin, mouth, liver, eyes and gut, in decreasing order, are the most frequently involved sites [26]. In our cohort, we observed that in 72% of the cases, the diagnosis of oral cGVHD had been suggested before liver biopsy was collected. This draws attention to the role of the mouth as a herald site to the development of the disease. Although the skin is considered to be the most commonly affected organ [26], only 25% of cases had a previous diagnosis of cGVHD in skin at the time of liver biopsy. In fact, 72% to 83% of patients with cGVHD showed oral involvement [11]. Our data indicate that the clinical changes resulting from cGVHD in the oral cavity occur earlier or, at least, simultaneously, to clinical and laboratorial liver changes.

The pathogenesis of cGVHD is poorly understood [2, 12]. Donor-derived immunocompetent T cells have been shown to react against host tissue after allo-HSCT, directly or through exaggerated inflammatory responses [27, 28]. Current concepts regarding the pathogenesis of cGVHD include: 1- the persistence of alloreactive T cells, 2- a Th1–Th2 shift of the cellular immune response (cGVHD seems to be mediated prominently by the Th2 cytokine response), 3- a failure of control by regulatory T cells and/or impaired negative selection of T cells in the thymus, 4- replacement of antigen presenting cells (APC) of the host by APCs of the donor leading to indirect antigen presentation of allo-antigens, 5- an increasing role of B cells producing auto- and allo-antibodies against the host, and 6- nonspecific mechanisms of chronic inflammation leading to fibrosis of involved organs.

Some studies have reported that salivary gland changes are histologically characterized by the presence of lymphocyte infiltrate, with slight predominance of CD8+ versus CD4+ T cells, in the glandular parenchyma and periductal tissue [3, 14, 17]. With respect to liver, in a murine model for GVHD, the percentage of CD4+ cells (29%) was seen to be 3 times that of CD8+ cells (11%) and 30% of the cells were positive for Mac-1, a differentiation marker of macrophages, large granular lymphocytes, and natural killer cells [30]. In our samples there was a predominance of CD8+ T cells over CD4+ T cells in both

organs, liver and MSG. Despite the small size of our sample, it was possible to compare mononuclear cells subsets in both organs (MSG and liver) and to screen MSG histological changes predictive for liver GVHD diagnosis. We found a positive correlation between lymphocytes subsets in liver and MSG. The strongest correlation was related to CD4+ T cells (85%), however CD8+ T cells were also correlated (60%). No correlation was found for macrophages (CD68+ cells) population.

The primary target of liver GVHD is the bile duct epithelium in different species of experimental animals [17]. The bile ducts are destroyed by an immune-mediated reaction, directed against the histocompatibility antigens. The small intrahepatic bile ducts are the most affected, as they contain the highest density of histocompatibility complex antigens [18]. Similarly, in MSG cGVHD, ducts appear to be the first target [14], possibly due to their high expression of histocompatibility antigens as well as the accessibility of the salivary glands to pathogenic lymphocytes [23].

Takahashi et al [31] pointed to the participation of CD4+ and CD8+ T lymphocytes in the destructive processes of intrahepatic bile ducts by observation of these cells either in proximity of- or within the degenerated cholangioepithelium. We found an important participation of CD4+ and CD8+ T cells in the liver inflammatory infiltrate; however it was not possible to discern their specific targets due to the high cellular density throughout the portal tract in most cases. On the other hand, within MSG samples, a cellular CD4+ and CD8+ infiltrate was observed around ducts, nevertheless, in most ducts, the cells that infiltrated the epithelium were the CD8 + T cells . Therefore, the involvement of CD8+ T cells in the damage of the salivary ducts appears to be more significant than that of CD4+ cells.

The relationship between CD8 and CD45 positive cells in MSG and the liver GVHD diagnosis (through NIH schema) suggests that the growing number of CD45+ cells indicate an active and progressive inflammatory status culminating with the involvement of other organs. In contrast CD8+ T cells in MSG, in this process stage, have an inverse participation in the diagnosis of liver GVHD.

The correlation of NIH schema with serum levels of ALT, AST and AP > 2 times above the upper normal limit confirmed the association of NIH schema for

histopathological diagnosis of liver GVHD with the clinical abnormalities on which the decision to collect liver biopsy was based.

Briefly, the cell density of CD8+ and CD45+ lymphocytes subsets in MSG samples had a predictive value for GVHD diagnosis in liver, which points to the importance of a histological evaluation of MSG. There is a certain risk in obtaining liver biopsies, especially in patients who have been submitted to HSCT, however this does not happen with MSG. Thus, salivary glands biopsies could help estimate hepatic changes in cGVHD patients.

CONCLUSION

Liver mononuclear infiltrates mirror those of MSG in cGVHD. Considering the set of clinical and laboratory findings, MSG biopsies which are easily obtained, could help estimate hepatic changes in cGVHD patients.

CONFLICT OF INTERESTS

The authors declare that they have no conflicting interests.

ACKNOWLEDGEMENTS

The authors thank Mr. Adilson Abilio Piaza for his help in taking photomicrographs. This paper was supported by the Coordination for Higher Level Graduates Improvement (CAPES) and São Paulo Research Fundation (FAPESP).

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TABLES

Table 1. Patients' (N=36) data

Variable	N (%)
Age, median (range)	29.5 (12-59)
Male sex	21 (58)
Disease	
CML	24 (66.7)
ALL	4 (11)
SAA	3 (8.3)
AML	2 (5.6)
MDS	2 (5.6)
PNH	1 (2.8)
Conditioning regimen	
BU + CY	31 (86.1)
BU + VP-16 + CY	3 (8.3)
CY + TBI	1 (2.8)
CY	1 (2.8)
Graf	
Bone marrow	21 (58.3)
Peripheral blood	15 (41.7)
After donor lymphocyte infusion	5 (13.9)
GVHD prophylaxis	
CsA/MTX	32 (88.9)
CsA/Corticosteroid	4 (11.1)
Mean Follow-up, median (range) *	122 (8-180)

ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; AML, acute myelogenous leukemia; SAA, severe aplastic anemia; PNH, paroxysmal nocturnal hemoglobinuria; VP-16, Etoposide; BU, Busulfan; CY, cyclophosphamide; TBI, total body irradiation; CsA, Cyclosporin A; MTX, Methotrexate.

* Follow-up in months.

Table 2. Clinical signs of cGVHD in oral cavity, gastrointestinal tract, skin, and eyes at the time of liver biopsy obtaining.

Target organ	Frequency (%)
Skin	9 (25)
Oral cavity	26 (72)
Eyes	8 (22)
Gastrointestinal tract	1 (3)

Table 3. Correlation between liver and minor salivary glands mononuclear subsets.

	Correlation (p)	Liver*	Salivary Gland*
CD4	85% (< 0.0001)	1516.92 (± 936)	203.28 (± 294)
CD8	60% (0.002)	2397.61 (± 1358)	488.05 (± 436)
CD68	NS	963.08 (± 629)	259.33 (± 242)
CD45	46% (0.01)	2915.45 (± 2017)	667 (± 772)
CD138	NS	362.12 (± 485)	307.36 (± 271)

Pearson's correlation; NS, not significant; *Mean (standard deviation) of cells/mm^2

FIGURES

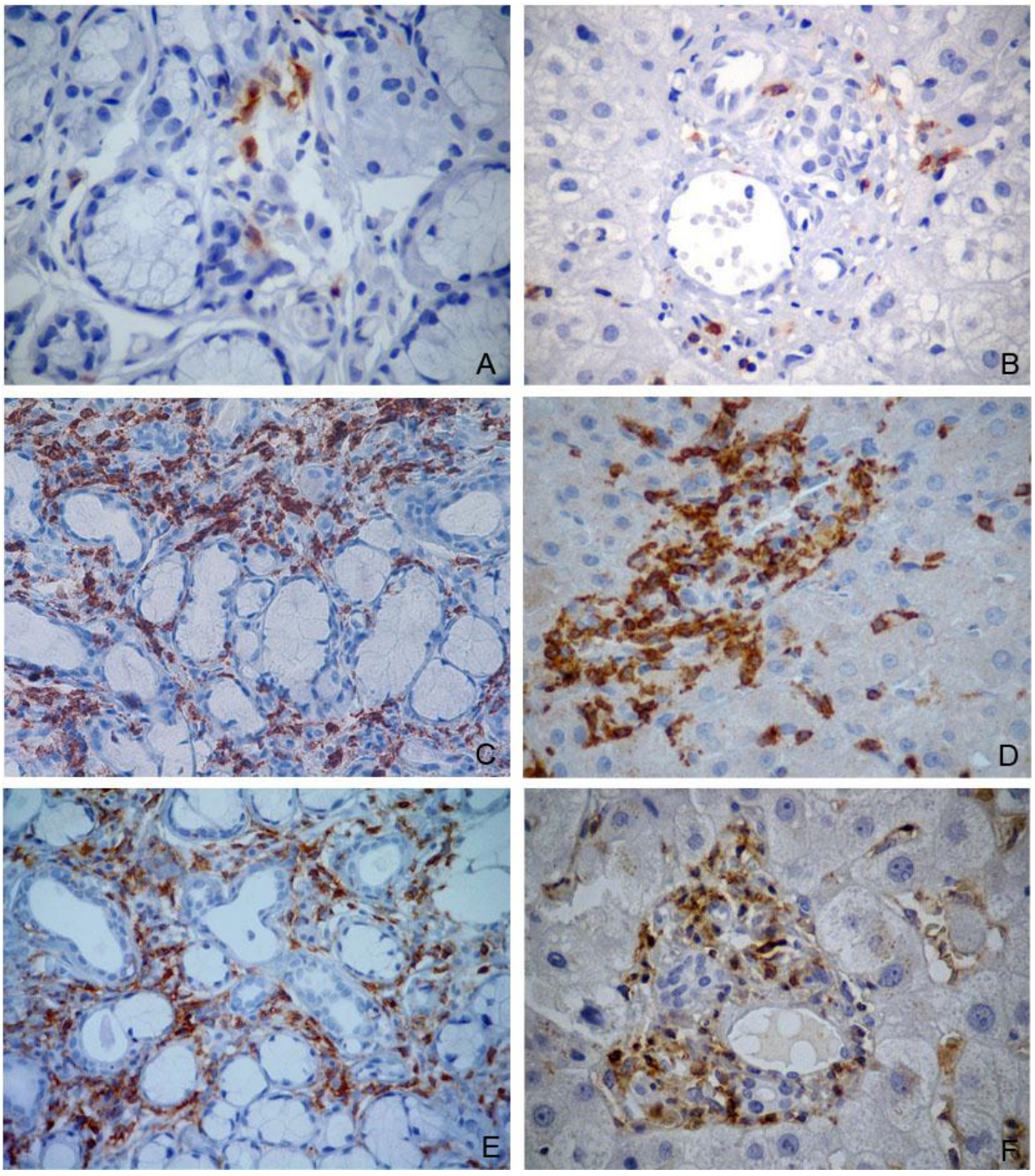

Figure 1. Immunostained sections of minor salivary glands (MSG) and liver of cGVHD patients. A: MSG section immunostained for CD4; B: Liver section immunostained for CD4; C: MSG section immunostained for CD8; D: Liver section immunostained for CD8; E: MSG section immunostained for CD45; F: Liver section immunostained for CD45. Original magnification x400.

CONCLUSÃO

1) Os aspectos histológicos em glândulas salivares menores (GSM) e fígado apresentam correlação entre si.

- ✓ Há correlação positiva entre a quantia de linfócitos T $CD4^+$, $CD8^+$ e $CD45^+/mm^2$ em glândulas salivares e fígado.

2) A variação de linfócitos T $CD8^+$ e $CD45^+/mm^2$ em biópsias de glândulas salivares menores sinalizam o diagnóstico de DECHc em fígado.

3) Comparando os três grupos de pacientes (com DECHc, sem DECH e pacientes que nunca foram tratados pelo TCTH) concluímos quanto à:

- ✓ Subpopulação de células inflamatórias - As GSM de pacientes com DECHc apresentam aumento do número de linfócitos T $CD4^+$, $CD8^+$, $CD45RO^+$ e leucócitos $CD45^+/mm^2$. O fígado de pacientes com DECHc apresentou aumento do número de linfócitos T $CD8^+$ e $CD45RO^+/mm^2$ por espaço porta.
- ✓ Área acinar produtora de muco - A perda de ácinos funcionais das GSM se inicia com o condicionamento pré-transplante e pode ser uma causa da xerostomia observada nos pacientes pós-TCTH.

4) Achados histológicos tanto em glândulas salivares menores quanto em fígado tem correlação com alterações clínicas e podem apresentar valor prognóstico para o paciente com DECHc.

- ✓ A presença de degeneração dos ácinos com fibrose intersticial e dilatação ductal e/ou de infiltrado linfocítico ao redor dos ductos em glândulas salivares menores tem influencia no desenvolvimento da DECHc extensa. Não houve correlação dos achados histológicos em fígado com a forma extensa da DECHc.
- ✓ O diagnóstico histopatológico da DECH em fígado tem correlação com alterações dos níveis séricos das enzimas hepáticas ALT, AST e FA.

- ✓ Os achados histológicos, “linfócitos ao redor e permeando os ductos” em glândulas salivares menores; “colestase canalicular” e a “presença de infiltrado inflamatório lobular composto por células polimorfonucleares além de linfócitos”, no fígado; demostraram ter valor prognóstico, em relação à sobrevida global do paciente.

Apesar das atuais restrições quanto à indicação da biópsia de mucosa oral e GSM no diagnóstico da DECHc, os dados aqui apresentados mostram a importância das alterações histológicas em glândulas salivares menores na representação do acometimento da DECHc em outros órgãos com maior dificuldade de acesso, como o fígado.

A validação dos dados aqui encontrados em um número maior de amostras, pode melhorar a compreensão da patogênese da DECHc oral e sua correlação com o envolvimento em outros órgãos.

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CEP, 18/02/03
(Grupo III)

PARECER PROJETO: N° 598/2002

I-IDENTIFICAÇÃO:

PROJETO: **"ANÁLISE COMPARATIVA DAS ALTERAÇÕES MICRÓSCOPICAS DE GLÂNDULAS SALIVARES MENORES E DE DUCTOS BIBLIARES INTRA-HEPÁTICOS NA GVHDee"**
PESQUISADOR RESPONSÁVEL: Andresa Borges Soares
INSTITUIÇÃO: Depto. de Anatomia Patológica/FCM/UNICAMP
APRESENTAÇÃO AO CEP: 19/12/2002

II - OBJETIVOS

Verificar uma possível correlação entre as perdas acinares e ductuais das glândulas salivares menores, com as alterações em ductos biliares intra-hepáticos.

III - SUMÁRIO

Será realizado um estudo descritivo, retrospectivo, analisando tecido hepático e de mucosa labial de 30 biópsias hepáticas e 30 biópsias de mucosa oral de sujeitos que realizaram transplante de medula óssea alogênico no Serviço de Transplante de Medula Óssea da FCM/UNICAMP. Os sujeitos já são submetidos de rotina a esses dois procedimentos, segundo o protocolo de atenção a esse tipo de paciente no serviço. Os blocos de parafina estocados no Serviço de Anatomia Patológica serão submetidos apenas a uma nova avaliação, através das técnicas de imunohistoquímica utilizando anticorpos primários monoclonais.

IV - COMENTÁRIOS DOS RELATORES

Trata-se de estudo onde será examinada biópsias de fragmentos de mucosa oral e de fígado, de pacientes submetidos ao transplante de medula óssea. Será um estudo retrospectivo, sem contato com os pacientes, onde será analisado apenas o material já emblocado no Departamento de Anatomia Patológica. A justificativa para a realização da pesquisa é a possibilidade de se obter um diagnóstico precoce da doença no fígado, antecedendo, dessa forma a introdução de medicamentos e aumentando a sobrevida dos pacientes. O protocolo é bem estruturado. Não há riscos para os pacientes. O termo de consentimento pode ser dispensado face a peculiaridade do projeto.

V - PARECER DO CEP

O Comitê de Ética em Pesquisa da Faculdade de Ciências Médicas da UNICAMP, após acatar os pareceres dos membros-relatores previamente designados para o presente caso e atendendo todos os dispositivos das Resoluções 196/96 e 251/97, assim como todos os anexos incluídos na Pesquisa, resolve aprovar sem restrições o Protocolo de Pesquisa supracitado.

VI - INFORMAÇÕES COMPLEMENTARES

O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado (Res. CNS 196/96 – Item IV.1.f) e deve receber uma cópia do Termo de Consentimento Livre e Esclarecido, na íntegra, por ele assinado (Item IV.2.d).

Pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado e descontinuar o estudo somente após análise das razões da descontinuidade pelo CEP que o aprovou (Res. CNS Item III.1.z), exceto quando perceber risco ou dano não previsto ao sujeito participante ou quando constatar a superioridade do regime oferecido a um dos grupos de pesquisa (Item V.3.).

O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (Res. CNS Item V.4.). É papel do pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e enviar notificação ao CEP e à Agência Nacional de Vigilância Sanitária – ANVISA – junto com seu posicionamento.

Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. Em caso de projeto do Grupo I ou II apresentados anteriormente à ANVISA, o pesquisador ou patrocinador deve enviá-las também à mesma junto com o parecer aprovatório do CEP, para serem juntadas ao protocolo inicial (Res. 251/97, Item III.2.e)

Relatórios parciais e final devem ser apresentados ao CEP, de acordo com os prazos estabelecidos na Resolução CNS-MS 196/96.

VII - DATA DA REUNIÃO

Homologado na II Reunião Ordinária do CEP/FCM, em 18 de fevereiro de 2003.

Prof. Dr. Sebastião Araújo
PRESIDENTE do COMITÊ DE ÉTICA EM PESQUISA
FCM / UNICAMP

CEP, 22/08/06.
(**PARECER PROJETO:** N° 598/2002)

PARECER

I-IDENTIFICAÇÃO:

PROJETO: **"ANÁLISE COMPARATIVA DAS ALTERAÇÕES MICROSCÓPICAS DE GLÂNDULAS SALIVARES MENORES E DE DUCTOS BILIARES INTRA-HEPÁTICOS NA GVHDc"**

PESQUISADOR RESPONSÁVEL: Andresa borges Soares

II - PARECER DO CEP

O Comitê de Ética em Pesquisa da Faculdade de Ciências Médicas da UNICAMP tomou ciência e aprovou a alteração do título do estudo já aprovado para **"ANÁLISE COMBINADA DAS ALTERAÇÕES MICROSCÓPICAS DE GLÂNDULAS SALIVARES MENORES E FÍGADO APÓS O TRANSPLANTE ALOGÊNICO DE MEDULA ÓSSEA"**, bem como o investigador principal que passa a ser **Tânia Cristina Benetti**, referente ao protocolo de pesquisa supracitado.

O conteúdo e as conclusões aqui apresentados são de responsabilidade exclusiva do CEP/FCM/UNICAMP e não representam a opinião da Universidade Estadual de Campinas nem a comprometem.

Homologado na VIII Reunião Ordinária do CEP/FCM, em 22 de agosto de 2006.

Profa. Dra. Carmen Silvia Bertuzzo
PRESIDENTE DO COMITÊ DE ÉTICA EM PESQUISA
FCM / UNICAMP

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