Renata Gonçalves de Resende

RELAÇÃO ENTRE POLIMORFISMOS GENÉTICOS E NÍVEIS DE CITOCINAS COM A DOENÇA DO ENXERTO CONTRA O HOSPEDEIRO AGUDA NO TRANSPLANTE ALOGÊNICO DE CÉLULAS-TRONCO HEMATOPOIÉTICAS

Belo Horizonte
Faculdade de Odontologia
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Dissertação apresentada ao colegiado de pós-graduação em Odontologia, da Faculdade de Odontologia da Universidade Federal de Minas Gerais como requisito parcial para a obtenção do grau de Mestre em Odontologia.

Área de concentração: Patologia Bucal

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“Agir, eis a inteligência verdadeira. Serei o que quiser. Mas tenho que querer o que for. O êxito está em ter êxito, e não em ter condições de êxito. Condições de palácio tem qualquer terra larga, mas onde estará o palácio se não o fizerem ali?”

Fernando Pessoa

Lista de abreviações e siglas

A: adenina

aGVHD: *acute graft-versus-host disease*

C: citosina

^{o}C: graus Celsius

$C_6H_{12}O_6$: glicose

$CaCl_2.2H_2O$: cloreto de cálcio

$CD4^+$: linfócito T $CD4^+$

$CD8^+$: linfócito T $CD8^+$

COEP: Comitê de Ética em Pesquisa

CTL: linfócitos T citotóxicos

DECH: doença do enxerto contra o hospedeiro

DECHa: doença do enxerto contra o hospedeiro aguda

DECHc: doença do enxerto contra o hospedeiro crônica

dl: decilitro

DNA: *deoxyribonucleic acid* (Ácido Desoxirribonucléico)

EDTA: ácido tetra etileno diamino

G: guanina

HCl: ácido clorídrico

GVHD: *graft-versus-host disease*

HLA: antígeno leucitário humano

HSCT: *hematopoietic stem cell transplantation*

IL1B: gene da citocina IL-1β

IL-1β: citocina interleucina-1β

IL6: gene da citocina IL-6

IL-6: citocina interleucina-6

IL10: gene da citocina IL-10

IL-10: citocina interleucina-10

IFNG: gene da citocina IFN-γ

IFN-γ: citocina interferon-γ

KCl: cloreto de potássio

Kb: quilobase

KH_2PO_4: hidrogenosfosfato de potássio

mg: miligrama

$MgSO_4$: sulfato de magnésio

ml: mililitros

mM: milimolar

NK: células natural killer

pb: pares de base

PCR: *polymerase chain reaction* – reação em cadeia da polimerase

pg: picograma

SNPs: *single nucleotide polymorphisms*

T: timina

TNFA: gene da citocina TNF-α

TNF-α: citocina fator de necrose tumoral-α

TCTH: transplante de células-tronco hematopoiéticas

UFMG: Universidade Federal de Minas Gerais

VNTRs: variable number of tandem repeat (número variável de repetições em tandem)

µl: microlitros

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Resumo

A doença do enxerto contra o hospedeiro aguda (DECHa) é uma das maiores causas de morbidade e mortalidade após o transplante alogênico de células-tronco hematopoiéticas (alo-TCTH). Polimorfismos genéticos estão associados a diferenças funcionais na expressão de citocinas, podendo levar a alterações no curso clínico da doença. Embora o conhecimento sobre a etiopatogenia da DECHa tenha sido consolidado nos últimos anos, poucos estudos mostram a contribuição de polimorfismos em genes de citocinas inflamatórias nos níveis desses mediadores e seu impacto na DECHa. Este estudo teve como objetivo investigar o impacto dos polimorfismos nos genes de *IL1B, IL6, IL10, IFNG* e *TNFA* nos níveis dessas citocinas, no sangue e na saliva, e a relação com a ocorrência e a gravidade da DECHa. Foram obtidas amostras de sangue e/ou raspado da mucosa bucal de 58 pacientes e seus doadores para análise dos polimorfismos nos genes de *IL1B, IL6, IL10, IFNG* e *TNFA* por meio de PCR. Amostras de sangue e saliva foram coletadas semanalmente sete dias antes do transplante (dia -7) até o dia 100 pós-transplante (dia +100) ou até antes do óbito dos pacientes, a fim de se avaliar os níveis das citocinas pelo método de ELISA. Os resultados mostraram que não houve associação entre a ocorrência da DECHa e os polimorfismos avaliados. Quando avaliada a ocorrência da doença em órgãos isolados, observou-se relação entre os genótipos do gene *TNFA* de doadores e a maior gravidade de DECHa no trato gastrointestinal e na pele. No que se refere à dosagem das citocinas, foi possível observar relação entre a presença de DECHa e altos níveis de IL-6, IL-10, no sangue, e IFN-γ, na saliva, e baixos níveis de IL-1β, na saliva. Além disso, altos níveis de IL-1β, na saliva, foram associados ao genótipo CC do gene *IL1B.* Níveis

aumentados de IL-6 e IL-10, na saliva, estavam associados ao fenótipo alto produtor dos genes *IL6*, dos doadores, e *IL10,* dos receptores, respectivamente. Associação também foi observada entre os níveis aumentados das citocinas IL-10 e IFN-γ, no sangue, e o fenótipo alto produtor dos genes *IL10* e *IFNG,* dos receptores, respectivamente. Por outro lado, os níveis de TNF-α no sangue e na saliva não mostraram relação com a ocorrência de DECHa ou com os genótipos de pacientes ou doadores. Os achados mostram a relevância do estudo genético dos pacientes e doadores para o entendimento da DECHa, assim como a dosagem das citocinas envolvidas no processo inflamatório pode representar uma valiosa ferramenta para a determinação da prevenção, evolução e tratamento da DECHa.

Palavras chaves: Transplante de células-tronco hematopoiéticas, doença do enxerto contra o hospedeiro, polimorfismos genéticos, citocinas

Association between cytokine gene polymorphisms and cytokine levels in acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation

Abstract

The graft versus host disease (GVHD) is a major cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (allo-HSCT). Genetic polymorphisms are associated with functional differences in the expression of cytokines and can lead to changes in the clinical course of disease. Although knowledge about the pathogenesis of the GVHD has been confirmed in recent years, few studies show the contribution of cytokine gene polymorphisms in the levels of these mediators and their impact on GVHD. This study aimed to investigate the impact of polymorphisms in the IL1B*, IL6, IL10, IFNG* and TNFA in levels of these cytokines in blood and saliva, and the relationship with the occurrence and severity of GVHD. We obtained blood samples and / or saliva of 58 patients and their donors for the analysis of polymorphisms in the *IL1B, IL6, IL10, IFNG* and *TNFA* using PCR. Blood samples and saliva were collected weekly seven days before transplantation (day -7) until day 100 post-transplant (day + 100) or even before the death of patients in order to assess the levels of cytokines by ELISA. The results showed no association between the occurrence of GVHD and polymorphisms evaluated. When we assessed the occurrence of disease in isolated organs, we observed relationship between donor *TNFA* genotypes and the greater severity of GVHD in the gut and skin. When the cytokine levels were determined, we observed association between the presence of GVHD and high levels of IL-6, in

blood and saliva, high IL-10 levels, in saliva, high IFN-γ levels, in saliva, and low levels of IL-1β, in blood. Furthermore, high levels of IL-1β in saliva were associated with CC genotype of the *IL1B* gene. Increased levels of IL-6 and IL-10 in saliva were associated with high producer phenotype of the *IL6* gene, in donors, and *IL10*, in recipients, respectively. Association was observed between high levels of the IFN-γ and IL-10, in blood, and high producer of the *IL10* and *IFNG* genes, in recipients, respectively. Moreover, levels of TNF-α in blood and saliva showed no association with the occurrence of GVHD or with the genotypes of patients or donors. Ours results shown the relevance of the genetic study of patients and donors for the understanding of GVHD, as well as cytokines levels could represent a valuable tool for the determination of the the evolution, prevention and treatment of the GVHD.

Keywords: Hematopoietic stem cell transplantation; Graft-versus-host disease; Polymorphisms; Cytokines

1. Introdução

1. Introdução

1.1 O Transplante de células-tronco hematopoiéticas

O transplante de células-tronco hematopoiéticas (TCTH) é indicado como medida terapêutica para diversas neoplasias hematológicas ou de outros tecidos, insuficiências medulares ou outros distúrbios congênitos da hematopoiese (Fonseca, 2000). O TCTH envolve a eliminação das células malignas com uma alta dose de quimioterapia, associada ou não a irradiação corpórea total, seguida pela infiltração de células normais mieloproliferativas (Deeg et al., 1996). A quimioterapia e/ou radioterapia pré-TCTH tem também como finalidade destruir o próprio sistema imune do paciente, reduzindo o risco de rejeição do enxerto (Dahllöf et al., 1997).

O transplante pode ser obtido de três fontes: singênica, a partir de um irmão gêmeo monozigoto; alogênica, de indivíduos diferentes, mas com o HLA compatível; ou autóloga, com células-tronco ou da medula óssea do próprio paciente, retiradas antes do transplante e posteriormente, reinfusionadas. As células progenitoras do TCTH podem ser obtidas da medula óssea aspirada da crista ilíaca; células-tronco periféricas (CTP), pelo uso de fatores estimuladores de colônia de granulócitos ou granulócitos-macrófagos (G-CSF ou GM-CSF), com posterior coleta no sangue periférico através de aférese; e também do sangue do cordão umbilical (Amos e Gordon, 1995).

Embora o TCTH represente a única possibilidade de cura para diversas doenças, o tratamento não é isento de complicações. Estas complicações podem ocorrer em todas as fases do transplante, frequentemente produzindo considerável morbidade. Pacientes submetidos ao TCTH podem desenvolver

complicações devido ao efeito da quimioterapia e da radioterapia, infecções devido à mielossupressão e a imunossupressão, e ao aparecimento da doença do enxerto contra o hospedeiro (DECH) (Bai et al., 1997).

1.2 A Doença do enxerto contra o hospedeiro

A DECH é uma doença imunologicamente mediada, sendo a mais importante complicação que se segue ao transplante alogênico de células-tronco hematopoiéticas (Sviland et al., 1991). A DECH pode ser em alguns casos, uma reação catastrófica e fatal em que as células imunocompetentes do enxerto reagem contra o tecido do hospedeiro (Bai et al., 1997). Esta reação ocorre, sobretudo após o transplante alogênico, embora raros casos de DECH em transplante autólogo já tenham sido descritos (Martin et al., 1995) . Estima-se que a DECH se desenvolve em 25 a 45% dos pacientes (Duncombe, 1997).

De acordo com a época de seu aparecimento, a DECH pode ser dividida em aguda e crônica. A forma aguda (DECHa) aparece até o dia +100 pós-TCTH. A forma crônica (DECHc) pode representar uma continuação de um caso previamente diagnosticado como DECHa, ou desenvolver-se mais de 100 dias após o TCTH, podendo vir a ocorrer vários anos após o transplante (Duncombe, 1997).

1.2.1 A Doença do enxerto contra o hospedeiro aguda

A DECHa envolve principalmente a pele, o fígado e o trato gastrointestinal. A primeira e mais comum manifestação da DECHa é a cutânea, que consiste em uma erupção maculopapular que pode começar em qualquer parte do corpo. O paciente pode queixar-se de prurido ou dor nas

áreas afetadas. As manifestações gastrointestinais incluem a diarréia, que pode tornar-se sanguinolenta, com presença de cólicas, náusea e vômito. Além disso, icterícia decorrente de hiperbilirrubinemia é a marca da doença em fígado (Jacobsohn e Vogelsang, 2007).

A DECHa é geralmente diagnosticada com base na apresentação clínica. Uma biópsia pode ser utilizada para confirmar o diagnóstico quando há suspeita de outras alterações, como infecção e reação a drogas. A biópsia da pele pode mostrar queratinócitos disceratóticos, exocitose linfocitária, necrose das células basais e depleção de células de Langerhans. Todos os resultados não estão necessariamente presentes em cada biópsia da pele e as características microscópicas não são patognomônicas em todos os casos (Esteban e Somlo, 1995). A endoscopia do trato gastrointestinal revela edema, descamação da mucosa e, algumas vezes, hemorragias. Tipicamente estes seriam encontrados no ceco, íleo e cólon, mas também pode envolver o trato gastrointestinal superior. A histopatologia revela necrose das células da cripta e focos de abscesso (Bone Marrow Transplantation Cambridge, 1994). A patologia do fígado com DECHa pode mostrar resposta inflamatória precoce com predomínio de linfócitos citotóxicos em ductos biliares e irregularidade desses ductos. As células epiteliais podem se apresentar aplainadas e com ausência de alguns núcleos (Bone Marrow Transplantation Cambridge, 1994).

Embora o diagnóstico da DECHa seja clínico, há outras condições que podem mimetizar ou coexistir com a doença, como a toxicidade de drogas e infecções. Por exemplo, os sintomas da infecção por citomegalovírus humano (HCMV) e colite (diarréia, dor abdominal) podem ser muito semelhantes aos da DECHa. Portanto, biópsia é recomendada para confirmar a suspeita clínica,

sempre que possível (Jacobsohn e Vogelsang, 2007). Finalmente, embora a definição de DECHa seja bem aceita envolvendo os três órgãos, não está claro se algumas complicações pós-transplante podem ser relacionadas ou realmente representam DECH. Por exemplo, alguns pacientes apresentam acúmulo pulmonar de células inflamatórias não relacionado à infecção cujo prognóstico é geralmente ruim. Resultados obtidos a partir de modelos animais sugerem que os mecanismos imunológicos que contribuem para a inflamação dos pulmões após o transplante podem ser similares aos responsáveis pela DECH (Jacobsohn e Vogelsang, 2007).

A fim de se determinar a gravidade da doença, Glucksberg e colaboradores (1974) propuseram um critério de gradação clínica da DECHa pós-TCTH, que consiste na determinação do estágio da doença em pele, fígado e trato gastrointestinal. A pele é avaliada de acordo com a percentagem do envolvimento da superfície corporal, no fígado a avaliação é determinada pelo volume da bilirrubina e no trato gastrointestinal pela quantidade de diarréia (Tabela 1). Utilizando esses critérios, um único grau é atribuído a cada paciente (Tabela 2).

Tabela 1: Estágio clínico da DECHa (Glucksberg et al., 1974).

Grau	Pele	Fígado	Trato gastrointestinal
0	Ausência de alterações	Bilirrubina < 2 mg/dl	Diarréia <500 ml/dia ou náusea persistente
1	Exantema maculopapular < 25% da superfície corporal	Bilirrubina entre 2-3 mg/dl	Diarréia 500-1000 ml/dia ou 280-555 ml/m^2/dia
2	Exantema maculopapular entre 25-50% da superfície corporal	Bilirrubina entre 3-6 mg/dl	Diarréia 500-1000 ml/dia ou 555-833 ml/m^2/dia
3	Eritroderma generalizado	Bilirrubina entre 3-6 mg/dl	Diarréia >1500 ml/dia ou >833ml/m^2/dia
4	Descamação e Bolhas	Bilirrubina > 15 mg/dl	Dor intensa- íleo paralítico

Tabela 2: Gradação clínica da DECHa (Glucksberg et al., 1974).

Grau	Estágio cutâneo	Estágio hepático	Estágio intestinal
I	1 a 2	0	0
II	3 ou 4	1 ou acima	1
III	0 a 3	2 ou acima	2 a 4
IV	4	4	0 a 4 + pele ou fígado

Em geral, os pacientes com grau III de DECHa apresentam 30% de chance de sobrevida a longo prazo e aqueles com grau IV tem menos de 5% chance de sobrevida a longo prazo. Pacientes sem DECHa ou com grau I-II tem acima de 80% de probabilidade de sobrevivência a longo prazo (Canh et al., 2005).

O uso de doadores não relacionados, doadores incompatíveis, a idade do doador, doador do sexo feminino, a idade do receptor, o tipo de enxerto e determinados regimes condicionantes aumentam o risco do desenvolvimento da DECHa. Dada a relação atual de transplantes de doadores aparentados *versus* não-aparentados realizado anualmente (cerca de 2:1), espera se que cerca de 5.500 pacientes desenvolvam DECHa grau II-IV aguda por ano nos Estados Unidos (Jacobsohn e Vogelsang, 2007).

Atualmente, a profilaxia da DECHa é feita utilizando se uma combinação de ciclosporina ou tacrolimus com metotrexato, que propiciam um controle razoável da DECHa. Para os grupos de maior risco ou grupos que receberam enxertos não-convencionais (como doadores incompatíveis, os pacientes mais velhos, os regimes de intensidade reduzida, etc), alternativas são utilizadas. O micofenolato mofetil (MMF), é freqüentemente usado no transplante de intensidade reduzida por seu efeito na profilaxia da DECH, bem como na

promoção da “pega” do enxerto (Jacobsohn e Vogelsang, 2007). Para os pacientes que receberam enxertos incompatíveis, uma imunossupressão mais intensa é normalmente necessária. Métodos de depleção de células T, como globulina antitimócito, ATG, (Slavian et al., 1998) e alemtuzumab (Perez-Simon et al., 2002) tem sido utilizados para tentar reduzir a incidência de DECHa aguda. No entanto, estes métodos diminuem a ocorrência da DECH aguda, mas aumentam a incidência de infecção (devido ao atraso na reconstituição do sistema imune) e de recidiva (Jacobsohn e Vogelsang, 2007).

Depois que a DECHa ocorre, todas as fases de indução da doença estão ativas. O sucesso do tratamento, em última instância, consiste em interromper o processo. Para o controle da DECHa grau II-IV emprega se, normalmente, 2 ou 2,5 mg / kg / dia metilprednisolona (Jacobsohn e Vogelsang, 2007).

1.3 Os mediadores inflamatórios na DECH

Estudos apontam que a patogênese da DECHa relaciona-se a vias inflamatórias, além do aloreconhecimento de células T/HLA. A ativação dessa via ocorreria antes mesmo da infusão das células T doadoras e pode resultar da própria doença de base ou tratamentos prévios ao regime condicionante (Mulligham e Bardy, 2007; Mulligham e Petersdorf, 2006).

A importância da cascata de citocinas nas fases pré-condicionante e pós-transplante da DECH é atualmente bem estabelecida (Middleton et al., 1998). Segundo Mulligham e Petersdorf (2006), o desenvolvimento da DECH pode ser dividido em três estágios. Em um primeiro estágio, o regime de condicionamento, como irradiação ou quimioterapia, causa dano tecidual e

resulta na liberação de citocinas pró-inflamatórias, como TNF-α e IL-1, regulando a expressão de HLA e moléculas co-estimulatórias pelas células apresentadoras de antígenos, facilitando uma resposta alo-imune. Em seguida ocorre a ativação das células T do doador, levando a uma proliferação de linfócitos Th1, na presença de IL-12, com a liberação das citocinas IL-2 e IFN-γ, que por sua vez induzem a ativação de células T citotóxicas e células NK. Essas células, por meio de seus mediadores, levam aos efeitos celulares e inflamatórios, causando dano tecidual, eventos que caracterizam o último estágio.

Interleucina-1 (IL-1), Interleucina- 6 (IL-6), Interferon-γ (IFN- γ) e Fator de Necrose Tumoral-α (TNF-α) são citocinas pró-inflamatórias que estão envolvidas nas reações locais e sistêmicas. Essas citocinas têm sido relacionadas a várias reações adversas iniciais ao TCTH, atuando como mediadores e reguladores da DECH. Já a Interleucina-10 (IL-10) é uma citocina anti-inflamatória com funções antagônicas às citocinas produzidas pelas células Th1. IL-10 é capaz de bloquear a produção de IL-1, IL-6 e TNF-α, em resposta ao lipopolissacarídeo ou outro estímulo. No entanto, estudos questionam o efeito benéfico dessa citocina na DECH, já que alta produção de IL-10 pode inibir a resposta imune (Cavet et al. 2001).

Dessa forma, a habilidade das células doadoras e receptoras de produzir altos ou baixos níveis de citocinas pode influenciar os resultados pós-transplante (Middeleton et al., 1998). Muitos desses mediadores inflamatórios relacionados às vias de patogênese da DECH são codificados por genes polimórficos e hoje sabe se que esses polimorfismos influenciam nos efeitos pós-TCTH.

1.4 Polimorfismos genéticos

Polimorfismo genético é definido como a ocorrência de múltiplos alelos num *locus*, no qual pelo menos dois alelos aparecem com freqüências superiores a 1%. Os polimorfismos são utilizados como “marcadores” genéticos para distinguir diferentes formas de um gene em estudos de famílias (Miller et al., 2001).

Com as técnicas de DNA recombinante, tem sido possível detectar os polimorfismos que consistem, geralmente, em uma simples troca de base, deleções ou inserções casuais, ou na presença de números variáveis de cópias repetidas de um determinado fragmento de DNA (repetições em *tandem*). Uma das técnicas mais usadas para se detectar a ocorrência de polimorfismos gênicos envolve os polimorfismos de comprimento de fragmentos de restrição (RFLPs) (Botstein et al., 1980). Os RFLPs são polimorfismos de ponto que criam ou destroem sítios de restrição enzima-específicos. Como as enzimas de restrição têm sequências de reconhecimento específicas no DNA, as alterações na sequência do DNA genômico acarretam na criação ou na abolição de sítios de clivagem, alterando, desse modo, o tamanho de um ou mais fragmentos de DNA oriundos da ação da enzima de restrição (Otto et al., 1998).

A presença de polimorfismos em regiões promotoras pode gerar alterações na transcrição do gene e, quando ocorrem em éxons, pode alterar a sequência da proteína e, portanto, influenciar a função biológica da mesma (Taylor et al., 2004). Desta forma, a presença de polimorfismos genéticos pode acarretar alterações funcionais do gene, sendo neste caso denominados polimorfismos funcionais.

Polimorfismos funcionais, relacionados à diferença interindividual na síntese e secreção de proteínas, têm sido associados a doenças que têm uma patogênese inflamatória (Parkhill et al., 2000). A maioria dos trabalhos nesta área concentra-se em genes que codificam para citocinas e receptores importantes no processo inflamatório.

Vários estudos têm sido propostos com o objetivo de identificar marcadores genéticos específicos para as inúmeras doenças, enfatizando a busca de polimorfismos genéticos que possam interferir na susceptibilidade dos indivíduos a essas alterações. Alguns resultados podem ser contraditórios entre as populações. Isto deve se ao fato de que populações geográfica e etnicamente distintas terem sido avaliadas refletindo o “background” genético diferenciado entre elas, assim como a possível presença de fatores seletivos atuando em uma determinada população, o que provavelmente pode acarretar em resultados distintos.

1.5 Polimorfismos genéticos e a DECH

Polimorfismos nos genes das citocinas pró e anti-inflamatórias têm sido associadas a incidência e gravidade de DECH em pacientes submetidos a TCTH alogênico. Muitos estudos têm sido desenvolvidos objetivando verificar a presença de genes polimórficos e sua possível correlação com a patogenia da DECH (Dickinson et al., 2004; 2007; Markey et al., 2008). Outros estudos recentes têm avaliado o papel do polimorfismo genético, tanto em pacientes quanto em doadores, na incidência e na gravidade da DECH (Markey et al., 2008). De um modo geral, um gene pode ser considerado como um candidato ao estudo do impacto do polimorfismo na DECH, se os processos fisiológicos

determinados por ele estiverem associados com a presença ou gravidade da doença.

Trabalhos envolvendo polimorfismos em genes de citocinas e sua correlação com a DECH tem apresentado resultados relevantes. Klöger et al. (2000) verificaram associação entre o polimorfismo no gene *TNFd3* (TNFd3/d3) e DECHa, grau III e IV e *IL10* $^{-1064}$ com a DECHa severa. Outros trabalhos tem sido relatados na literatura associando polimorfismo também nos gene *TNF*, incluindo os haplotipos -1031T/-863C/ -857C e altos graus de severidade da DECHa (Mulligham e Bardy, 2007).

Outros grupos de pesquisa tem relacionado polimorfismo genético tanto do doador quanto do receptor a complicações pós-TCTH. Rocha et al. (2002) verificaram, em análise de 107 pacientes submetidos ao TCTH e seus doadores, que o polimorfismo genético tanto do receptor quanto do doador está envolvido no desenvolvimento da DECH. Esses autores constataram a influência do gene *IL-1Ra* (IL-IRN*2) como um polimorfismo genético protetor na DECHa. Os autores verificaram também a influência de polimorfismos dos genes *IL10* e *IL-1Ra* dos pacientes na incidência de DECH. Cavet et al. (2001), em trabalho semelhante, analisando 80 pacientes e doadores, verificaram que o receptor que possuía homozigose para o gene *IFNG* Íntron 1, alelo 3, estava relacionado ao desenvolvimento de DECHa. Neste estudo não foi verificada associação entre o gene *IFNG* Íntron ‘1 do doador e a DECHa. Ao analisarem o gene da citocina IL-6 no receptor, estes autores verificaram que o alelo *IL6*^{174}C associava-se a DECHa moderada, já o alelo *IL6*^{174}G estava associado se a maior incidência das formas aguda e crônica da doença.

Karabon et al. (2005) investigaram a associação do polimorfismo genético nos genes *IL6* e *IL10* em receptores e doadores de TCTH. Foram avaliados 93 pacientes e 74 doadores em várias fases após o transplante. Eles observaram que o alelo G dos pacientes e o genótipo GG do gene *IL6* (-174 G/C) do doador está associado ao desenvolvimento da DECHa. Outros estudos confirmam o papel do genótipo homozigoto *IL6* GG como fator associado à incidência de DECHa. A comparação entre a distribuição do genótipo *IL6* em pares doadores-receptores acumulam dois fatores desfavoráveis (alelo *IL6* G e genótipo doador *IL6* GG), o que resulta em alta freqüência em DECH. Nesse estudo, foi observado que a presença de *IL10* GCC/GCC em receptores está associada à diminuição do risco de DECH. Nenhuma associação foi encontrada em relação a polimorfismo nos genes dos doadores para IL-10.

Esses achados têm valor no que se refere ao entendimento da resposta imunológica do receptor, permitindo avanços na escolha do doador e estabelecendo novas condutas profiláticas e terapêuticas para a DECH.

1.6 Níveis de citocinas inflamatórias no sangue e DECH

Trabalhos estão sendo conduzidos a fim de se verificar a associação entre os níveis sanguíneos de citocinas envolvidas na patogênese da DECH e o aparecimento e gravidade da doença. Min et al. (2001) estudaram, em uma amostra de 52 pacientes, os níveis séricos das citocinas pró-inflamatórias IL-6, TNF-α, IL-8 e da citocina anti-inflamatória IL-10 por meio de ELISA sanduíche. Os autores analisaram as interações das citocinas ao mesmo tempo e constataram que os níveis séricos de IL-6 na semana +1 e IL-10 na semana +4 estão significativamente aumentados em pacientes que apresentavam DECHa,

o que sugere que a IL-6 seja a citocina mais importante no processo inflamatório inicial. Não foi verificada associação entre a ocorrência de DECH e os níveis de IL-8.

Hempel et al. (1999) demonstraram que altos níveis séricos de IL-10 estão associados a resultados fatais no TCTH. Imamura et al. (1994) observaram que a DECHa é induzida por interações sinérgicas entre IL-6,TNF-α e IFN- γ.

Em estudo envolvendo 13 pacientes brasileiros, Visentainer et al. (2003) observaram que os níveis séricos do Receptor solúvel de interleucina-2 (sIL-2R) e IL-10 desde a primeira semana até a 15ª semana pós-transplante eram significativamente maiores em pacientes que desenvolveram a DECHa do que naqueles que não haviam apresentado a doença. Nenhuma associação foi observada no que se refere à citocina TNF-α, embora tenha sido observado aumento nos níveis dessa citocina nas primeiras semanas pós-TCTH, o que é confirmado por outros autores (Remberger et al., 1995). A análise das citocinas IL-6 e IFN-γ também não revelou associação com DECHa.

Nota-se que inúmeros estudos apontam a relevância da citocina anti-inflamatória IL-10 na patogênese da DECHa. Alguns autores observam que na maioria das mortes pós-transplante, os níveis de IL-10 encontravam-se elevados.

Esses resultados reforçam a ideia de que um equilíbrio entre citocinas derivadas das células Th1 e Th2 pode ser importante na prevenção do desenvolvimento e no controle da DECH (Visentainer et al., 2003).

1.7 Níveis de citocinas inflamatórias na saliva e DECH

A patogênese da DECHa assemelha-se ao que é encontrado em uma variedade de processos, incluindo-se a mucosite pós-TCTH e doenças auto-imunes como o líquen plano bucal (Nicolatou-Galitis et al., 2001). Tais processos caracterizam-se por desordem inflamatória caracterizada por uma resposta imune, com acúmulo persistente de células inflamatórias e dano ao tecido (Rhodus et al., 2005).

Trabalhos tem sido propostos a fim de determinar os níveis das citocinas inflamatórias nos fluidos bucais em líquen plano bucal e em mucosite bucal após o TCTH. Rhodus et al. (2005) analisaram os níveis de TNF-α, IL-1-α e IL-6 na saliva de 13 pacientes diagnosticados com líquen plano oral empregando-se ELISA, tendo como grupo controle 13 pacientes saudáveis. Os autores observaram altos níveis dessas citocinas nos pacientes com líquen plano bucal, em relação ao grupo controle. Em outro estudo, 25 pacientes foram selecionados a fim de se determinar a relação entre os níveis salivares de TNF-α e a ocorrência de mucosite. Foi observado que, embora nenhuma relação estatísticamente significante tenha sido encontrada, a elevada concentração de TNF-α na saliva não estimulada no dia + 9 sugere que a saliva pode representar um método útil para detecção de marcadores pró-inflamatórios na mucosite bucal (Fall-Dickson et al., 2007).

Soma-se a isso, estudo recente aponta a relevância da análise dos níveis de citocinas inflamatórias na saliva e a ocorrência de DECHc bucal. Fall-Dickson et al. (2010) mostraram relação entre os níveis de IL-6 e IL-1α e a severidade da DECHc bucal.

Esses resultados apontam, em conjunto, que essas citocinas inflamatórias podem ser detectadas em fluidos bucais, com potencial de

prognóstico para monitoramento da atividade da doença e dessa forma contribuir para decisões terapêuticas.

Dessa forma, a dosagem dos níveis de citocinas na saliva pode também ser um valioso método para predizer a ocorrência da DECHa em paciente pós-transplante de células-tronco hematopoiéticas.

2. Objetivos

2. Objetivos

Objetivo geral

- Investigar a associação de polimorfismos funcionais de genes de citocinas mediadoras da inflamação em indivíduos submetidos ao TCTH e doadores e dos respectivos produtos destes genes no desenvolvimento da DECH aguda.

Objetivos específicos

- Avaliar a associação entre a ocorrência de polimorfismo nos genes das citocinas IL-1β, IL-6, IL-10, IFN-γ e TNF-α, em indivíduos submetidos ao TCTH, assim como nos doadores, com a ocorrência e gravidade da DECHa.

-Avaliar a associação entre os níveis das citocinas IL-1-β, IL-6, IL-10, IFN-γ e TNF-α, no sangue e na saliva, nos pacientes submetidos ao TCTH com a ocorrência da DECHa.

-Avaliar a associação entre a ocorrência de polimorfismos funcionais nos genes das citocinas IL-1-β, IL-6, IL-10, IFN-γ e TNF-α, em indivíduos submetidos ao TCTH e nos doadores, com os níveis de citocinas, no sangue e na saliva.

3. Artigo I

Title: Investigation of functional *IL10* gene polymorphism and IL-10 levels in acute graft-versus-host disease*

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Abstract

Background: Graft-versus-host disease (GVHD) represents a major complication in allogeneic hematopoietic stem cell transplant (allo-HSCT) recipients. Although studies have been conducted concerning the investigation of cytokine polymorphisms in the development of acute GVHD (aGVHD), the contribution of recipients and donors as regards cytokine levels has not yet been thoroughly assessed.

Objective: The aim of this study was to investigate the impact of *IL10* polymorphisms on cytokine levels in blood and saliva, in addition to the occurrence and severity of aGVHD.

Methods: Fifty-eight consecutive allo-HSCT recipients and their donors were included in this prospective study. Saliva and/or blood samples were obtained from recipients and donors to determine *IL10* polymorphisms. The IL-10 levels in the blood and saliva were also assessed. The samples were collected from seven days before transplant (day -7) to 100 days after allo-HSCT (day +100), once a week or until the death of recipient.

Results: No association was found between recipient and donor *IL-10* polymorphism and IL-10 levels in the saliva with aGVHD. In contrast, IL-10 levels in the blood were associated with the occurrence of aGVHD. The high producer phenotype in the recipient was also associated with high levels of IL-10 in the blood and saliva.

Conclusion: Although *IL10* polymorphisms were not associated with the occurrence and severity of aGVHD, the genetic background of the recipient did in fact influence the production of cytokine. Furthermore, as IL-10 levels in the

blood were associated with the disease development, this parameter may well be a useful predictor of aGVHD development.

Keywords: Hematopoietic stem cell transplant; Graft-versus-host disease; Acute Graft-versus-host disease; Polymorphisms; Cytokines

Introduction

Hematopoietic stem cell transplant (HSCT) is currently an important curative treatment for many patients with malignant and non-malignant hematological diseases. Graft-versus-host disease (GVHD) develops in many allogeneic HSTC recipients and is one of the main factors affecting the success of transplants, as its occurrence is associated with high morbidity and mortality rates [1-4]. GVHD occurs as result of complex immunological interactions between host cells and transplanted donor cells. Acute GVHD (aGVHD) most commonly begins within 100 days after allo-HSCT and is characterized by lymphocyte infiltration and epithelial cell apoptosis within target organs, typically in the gastrointestinal tract, in the liver, and on the skin. aGVHD is clinically ranked from grade I to grade IV according to the extension of organ damage [2,4,5].

Cytokines play an important role in the pathological damage of aGVHD, influencing two out of the three stages of aGVHD development (the so-called “cytokine storm”), as described by Ferrara and Yanik [6] : In the step 1, the conditioning regimen leads to the secretion of inflammatory cytokines, *e.g.* tumor necrosis factor-α (TNF- α) and interleukin-1 (IL-1). In step 2, donor T cells proliferate and secrete IL-2 and interferon-γ (IFN-γ). In step 3, activated cytotoxic T lymphocytes (CTL) and natural killer (NK) cells induce target tissue through cell-mediated cytotoxicity.

Cytokine interleukin 10 (IL-10) is produced by a variety of different cells, of both hematopoietic and non-hematopoietic lineages. IL-10 is usually regarded as potent suppressor of the immune responses and hence it is thought to be useful

in preventing GVHD. However, it has also been shown to have some immunostimulatory effects [7, 8].

In addition to immunological stimulus, it is well-known that cytokine production also remains under genetic control due to the polymorphisms of several cytokine genes. Allelic variants of cytokine genes are typically related to either a higher or lower production of cytokines [7]. Previous studies have shown an association between genetic variations in cytokine-related genes in recipients and donors and the occurrence of aGVHD [9-11]. However, the impact of genetic polymorphisms on cytokine levels in blood and saliva were not assessed in these subjects. The aim of the present study was to investigate the impact of *IL10* polymorphisms on cytokine levels in blood and saliva as well as to test the hypothesis if both were in fact associated with the occurrence and severity of aGVHD.

Methods

Subjects and sample collection

Fifty-eight consecutive allo-HSCT recipients and related donors from Hospital das Clínicas at Universidade Federal de Minas Gerais (HC-UFMG), between October 2006 and October 2008, were deemed eligible and included in this prospective study. Recipients were conditioned for allo-HSCT according to the specific protocols from the Stem Cell Transplant Unit at HC-UFMG and varied according to the type of the disease, disease status and the previous treatment at the time of transplantation. Cyclosporin, in combination with methotrexate or mycophenolate mofetil, was used for GVHD prophylaxis, whereas 2 mg/kg of methylprednisolone, in combination with Cyclosporin, was used for GVHD

treatment. Clinical data from patients and donors are described in Table 1. Saliva and blood samples were collected from recipients and donors one week before the HSTC and were submitted for DNA analysis. Saliva samples were collected using cotton swabs on the floor of the mouth, tongue, and labial and buccal normal oral mucosa of the HSCT subjects. Sites with localized injuries were not included. The cotton swabs were removed with a sterile cytobrush, placed immediately in sterile tubes containing 500 µl of Krebs buffer (NaCl 20%, KCl 2%, CaCl2.2H2O 2%, MgSO4, KH2PO4, and C6H12O6), and stored at -20℃ until processing. Peripheral blood (4 ml) was collected in vacutainer tubes containing EDTA and stored at -70℃ until processing.

To determine the cytokine levels, one blood and one saliva sample were obtained once a week from seven days before transplant (day -7) to 100 days after allo-HSCT (day +100). The saliva sample was colleted in Salivette tubes (Sarstedt AG & Co, Numbrecht, Germany) according to the manufacturer's instructions. The saliva samples were subsequently diluted (1:1) in a PBS solution containing protease inhibitors (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA, and 0.01 mg/mL aprotinin A) and 0.05% Tween-20 and subsequently stored at -20^{o}C until analysis. The serum samples were obtained from venous blood samples and were centrifuged within 2 hours after blood collection and stored at -20℃.

The total protein content in the saliva was determined using the Bradford Reagent (Sigma, Saint Louis, MO, USA) and the BSA standard (Fermentas Life Sciences, Vilnius, Lithuania). The total protein content was used to correct the IL-10 values for each sample.

DNA isolation

Total genomic DNA was extracted from saliva and blood samples using QIAamp DNA Blood Mini Kit (Quiagen, Valencia, CA, USA) in accordance with to the manufacturer's instructions. The final elution of saliva and blood DNA was performed in 50 µl of a specific AE buffer from the Kit and stored at -20°C until use.

IL-10 gene polymorphism analysis

Recipient and donor *IL10* G > A at -1082 polymorphism was assessed by means of polymerase chain reaction (PCR) amplification and digestion. The sequences of PCR primers were 5' CCAAGACAACACTACTAAGGCTCCTTT 3' and 5' GCTTCTTATATGCTAGTCAGGTA 3', with an expected PCR product size of 377 pb, as previously described by Koch et al. [13] The PCR was carried out in a total volume of 50 µ, containing approximately 400 ng of DNA, primers (20 pmol/reaction), and 25 µ of Pre-mix buffer (Phoneutria Biotecnologia, Belo Horizonte, Brazil). According to the manufacturer, the Pre-mix buffer contained 50 mMKCl, 10 mM Tris–HCl pH 8.4, 0.1% Triton X-100, 1.5 mMMgCl2, deoxynucleoside triphosphates, and 1.25 units of Taq DNA polymerase. The conditions for amplification consisted of 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 54°C for 35 s, and 72°C for 30 s. The run was terminated by final elongation at 72°C for 5 min. In all steps, the lid temperature was 103°C. The products all contained 5U XagI of the restriction enzyme (Promega, Madison, WI, USA) at 37°C for 4h and obtained 280+97 bp for the AA genotype, 280+253+97+27 bp for the GA genotype, and 253+97+27 bp for the GG

genotype. Visualization of the product was performed in a 6.5% polyacrylamide gel electrophoresis stained with silver.

Detection of IL-10 levels

Cytokine concentration was determined by means of a quantitative sandwich ELISA technique using a Human IL-10 DuoSet Kit (DY217B, R&D Systems, Minneapolis, MN, USA).

Acute GVHD grades

All patients were classified from grade I to grade IV for acute GVHD, as described by Glucksberg et al. [5].

Statistical analysis

Univariate analyses were performed using the Chi-Square, Mann-Whitney and Friedman tests within an SPSS software (SPSS Inc., version 16.0, Chicago, IL). A p value ≤ 0.05 was considered statistically significant.

Results

Clinical outcomes

Acute GVHD could be observed in 27 patients. All patients were classified from grade 0 to grade IV for acute GVHD: 38 subjects were within grades 0-I (65.5%), whereas grades II-IV were observed in 20 individuals (34.5%).

IL10 gene polymorphisms in recipient and donor of allo-HSCT and *aGVHD*

Results of recipient and donor *IL10* gene polymorphism, their prevalence and corresponding phenotypes, and the impact of the polymorphisms on the incidence and severity of aGVHD are presented in table 2. According to previous studies [13] in addition to due to the low number of subjects with the GG genotype, the individuals were grouped into low (AA genotype) and high producer phenotypes (GA and GG genotypes). The frequencies of the gene polymorphisms found were similar to those reported in white population [9]. In the present study we did not find association between recipient and donor *IL10* polymorphism and the occurrence or severity of aGVHD.

IL-10 levels

Tables 3 and 4 summarize the mean, minimum, and maximum levels of blood and saliva IL-10 levels during the 100 days following allo-HSCT. In each week, the number of valid samples analyzed suffered variations according to the availability of the samples and depending on the complications which arise from the post-HSCT treatment of the individuals. Week 1 represents day -7 before transplant.

IL-10 levels in blood and saliva and the incidence of aGVHD

An increased level of IL-10 was observed in the blood of patients with aGVHD (mean 504.05 pg/ml) compared to individuals without aGVHD (mean 275.50 pg/ml) ($p= 0.01$). No association was found between IL-10 levels in saliva and the development of aGVHD. As there was not enough valid cases for proceeding the Friedman test in all weeks, it was used for the weeks 1 to 8 in blood (patients without aGVHD) and for the weeks 1 to 11 (patients with

aGVHD). In the saliva, the analysis was done for the weeks 1 to 16 (patients without aGVHD) and for the weeks 1 to 7 (patients with aGVHD). No statistical association was found in blood IL-10 levels in patients without and with aGVHD and in saliva IL-10 levels in patients without and with aGVHD (Fig. 1 and Fig. 2).

IL-10 levels in blood and saliva and IL10 polymorphisms in recipients of HSCT

IL-10 blood levels were associated with *IL10* polymorphism in recipients at week 7, where an increased level of this cytokine in the high producer phenotype can be observed (Table 3). An increased level of IL-10 cytokine in the saliva was also found in recipients with the IL-10 high producer phenotype at week 12 (Table 4).

IL-10 levels in blood and saliva and IL-10 polymorphisms in donors of HSCT

Donor genotypes were analyzed according to IL-10 levels in the blood and saliva of recipients during the first 100 days following HSCT. The donor *IL10* genotype was not associated with IL-10 levels in blood or saliva (data not shown).

IL-10 levels in blood and saliva according to date of diagnosis of aGVHD

Samples were divided into the following groups: From 30 days to 07 days before, day of diagnosis, and 30 days after the diagnosis of aGVHD. The median of IL-10 blood and saliva levels among these periods were not statistically different (data not shown).

Discussion

The importance of the cytokine cascade in the different phases of GVHD is well-established. Earlier studies have shown that the genetic make-up of recipients and donors can influence the success or failure of HSCT, and it has been suggested that the existence of high or low cytokine production is considered a significant risk factor associated with transplant results. Some authors have reported that genetic polymorphisms can in fact be responsible for phenotypic variations and transplant results, including aGVHD [14, 15].

IL-10 is a key immunomodulatory cytokine that is produced by B cells, regulatory T-cells, monocytes, and dendritic cells [16]. It plays an important role in the downregulation of TNF and Th1 cytokine production, as well as in the inhibition of T-cell proliferation. *In vitro* results show that administration of IL-10 decreases alloreactivity of T cells in mixed lymphocyte culture assays [17]. However, one *in vivo* study showed that IL-10 exacerbates GVHD at higher levels but provides protection within physiological doses [12]. Although the role of IL-10 cytokine in the pathogenesis of GVHD is not well-established, it does appear to have an impact on the fatal outcome of patients after transplants [2]. The gene of IL-10 cytokine maps to chromosome 1 (1q31-32) and is highly polymorphic, presenting several microsatellites and five SNPs that form haplotypes associated with high (GCC), intermediate (ATA), and low (ACC) IL-10 production [3]. Nucleotide variations in genes that encode this molecule may affect the transcription or translation of the gene, the secretion or the function of the correspondent protein and may play an important role in the pathogenesis of GVHD [18].

One of the aims of the present study was to determine whether recipient and donor *IL10* (-1082 G/A) gene polymorphism contributes to the development and severity of aGVHD after allo-HSCT. The present study found no association between recipient and donor *IL10* polymorphism and aGVHD development and severity. Other studies involving *IL10* polymorphisms and transplant outcomes showed divergent results. The low producer (ACC) haplotype in recipient was associated with severe acute GVHD grades III-IV in ciclosporin alone [14] and ciclosporin plus MTX-treated [19] HLA-matched sibling HSCT cohorts. The intermediated producer *IL10* haplotype (ATA) was confirmed to play a role in severe aGVHD in two large cohorts, where almost 1000 patients were analyzed [18]. Kögler et al. [20] studied HLA-mismatched cord blood transplant and did not find association between *IL10* gene polymorphism with GVHD. Middleton et al [9] reported that the alleles of the *IL10* -1064 promoter region microsatellite polymorphism possess greater numbers of dinucleotide (CA) repeats that are associated with more severity of GVHD. However, other authors reported an association between high producer *IL10* haplotype (GCC) and the occurrence of grade 2 to 4 aGVHD [21]. In addition, the donor *IL10* genotype has also been associated with a lower risk of aGVHD. Socié et al. [21] reported that the presence of donor *IL10* GCC/GCC homozygous genotype protects the patients from aGVHD. Other authors reported that the presence of high number of CA repeats in the donors was associated with an increased risk for aGVHD [14], while Karabon et al. [22] did not find significant association between *IL10* polymorphisms in donors and the incidence of acute GVHD. These discrepant findings may be partly explained by the differences in transplant conditioning regimens and initial release of cytokine by the recipient [9, 14]. We are aware

that the relatively small numbers of patients in our study do not allow a definitive conclusion regarding the impact of cytokine genotype-associated GVHD risk. Thus, more studies are necessary to confirm our data.

Measurements of cytokine levels in the serum of patients with GVHD have been used to investigate the impact of these cytokines on the development and course of the disease. To assess the impact of the *IL10* polymorphism on cytokine levels of HSCT patients, blood and saliva levels of IL-10 were analyzed, attempting to associate it with the occurrence of aGVDH. It could be observed that the donor IL-10 genotype did not influence IL-10 levels in blood and saliva; however, recipients with a high producer phenotype did show higher levels of cytokine in the blood and saliva. It could also be observed that high IL-10 levels in the blood were associated with the presence of the aGVHD. Hempel et al. [23] reported that the high IL-10 serum levels in patients in post-HSCT treatment were significantly associated with a fatal outcome. Similar findings were reported by Min et al. [24], where high IL-10 levels in the blood at week +4 after the transplant were associated with fever, severe stomatitis, and aGVHD. Although high IL-10 production can lead to immunodeficiency, and consequently to the development of complications after HSCT [6], its increased production may be an attempt to suppress the various inflammatory cytokines that tend to increase during the leukocyte recovery phase [25]. Therefore, an increased level of IL-10 is a negative indicator of the cytokine storm that mediates aGVHD development.

Conclusion

Although *IL10* polymorphisms were not associated with the occurrence and severity of aGVHD, the genetic background of the recipient does in fact influence the production of cytokines. In addition, as IL-10 levels in the blood were associated with disease development, this parameter may well be a useful predictor of aGVHD development.

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Tables

Table 1- Clinical characteristics of alo-HSCT patients and donors (n=58)

Parameteres	**Total (n=58)**
Recipient median age in years (range)	31.5 (5 - 56)
Female gender	25 (43.2%)
Primary Disease	
- Malignant	
- Chronic Myeloid Leukemia	9 (15.5%)
- Acute Myeloid Leukemia	15 (26%)
- Acute lymphoid leukemia	5 (8.6%)
- Non-Hodgkin's Lymphoma	4 (6.9%)
- Hodgkin's Lymphoma	3 (5.1 %)
- Other malignancies*	4 (6.9%)
- Bone Marrow Failure Syndrome**	18 (31%)
HLA match	
- HLA matched related	52 (89.6%)
- HLA matched unrelated	4 (6.8%)
- HLA mismatched related	2 (3.6%)
Donor median age in years (range)	35.4 (6-69)
Donor Female gender	21(36.3%)
Conditioning regimen	
-BU/CY	20 (34.5%)
-CY+/-ATG or Alemtuzumab	14 (24.2%)
-BU + FLU +/- Alemtuzumab	9 (15.5%)
-CY+FLU+/-Alemzutumab	8 (13.8%)
-MEL+FLU+/-Campath	5 (8.6%)
-Others***	2 (3.4%)
Ethnic group	Brazilian mixed population
Source of stem	
- Bone marrow	32 (55.2%)
- Peripheral blood stem cells	25 (43.1%)
- Umbilical cord blood	1 (1.7%)

*Myelodisplastic Syndrome (n=1) Myelofibrosis (n=1) Multiple Mieloma (n=2)
** Paroxysmal noctural hemaglobinuria (n=2) Severe Aplastic Anemia (n=14) Fanconi anemia (n=2)
*** BU/MEL (n=1) Cytarabine/Campath/FLUD (n=1)
BU busulfan; CY: cyclophosphamide; FLUD: fludarabine; MEL: melphalan; ATG: antithymoglobulins

Table 2 - Association between recipient and donor cytokine genotypes and occurrence and grade of aGVHD (n=58)

Gene	Genotype	Associated phenotype	*n (%)*	GVHD A	GVHD P	*p**	GVHD 0-I	GVHD II-IV	*p**
IL10 (-1082) (P)	AA	Low	22 (37.9)	12	10	NS	16	06	NS
	GA/GG	High	36 (62.1)	19	17		22	14	
IL10 (-1082) (D)	AA	Low	02 (3.4)	01	01	NS	01	01	NS
	GA/GG	High	56 (96.6)	30	26		37	17	

NS: not significant P: present A: absent * Chi-Square Test P: patient D: donor

Table 3: Association between blood IL-10 levels and recipient *IL10* genotypes (n=58)

Weeks	Genotype	Associated phenotype	n valid	IL-10 Levels (pg/ml) Mean	Minimum	Maximum	*p**
1	AA	Low	19	283.39	0.00	2954.94	-
	GA/GG	High	24	589.08	0.00	7265.10	
2	AA	Low	19	256.39	0.00	2954.94	NS
	GA/GG	High	26	633.11	0.00	7265.10	
3	AA	Low	21	287.89	0.00	1825.88	NS
	GA/GG	High	24	486.48	0.00	5809.48	
4	AA	Low	22	212.99	0.00	1477.94	NS
	GA/GG	High	23	457.44	0.00	4954.05	
5	AA	Low	14	197.57	0.00	671.61	NS
	GA/GG	High	28	445.06	0.00	5296.79	
6	AA	Low	15	140.99	0.00	1065.20	NS
	GA/GG	High	20	759.45	0.00	5954.02	
7	AA	Low	18	210.05	0.00	941.77	0.03
	GA/GG	High	20	232.14	0.00	2915.15	
8	AA	Low	16	142.44	0.00	730.33	NS
	GA/GG	High	25	269.90	0.00	1793.38	
9	AA	Low	14	50.72	0.00	257.46	NS
	GA/GG	High	21	199.31	0.00	1352.23	
10	AA	Low	16	118.67	0.00	756.29	NS
	GA/GG	High	21	263.67	0.00	2837.37	
11	AA	Low	19	130.98	0.00	954.70	NS
	GA/GG	High	20	196.06	0.00	1959.88	
12	AA	Low	12	56.23	0.00	250.70	NS
	GA/GG	High	18	250.70	0.00	1808.37	
13	AA	Low	13	189.84	0.00	1387.26	NS
	GA/GG	High	18	194.28	0.00	1978.28	
14	AA	Low	12	46.95	0.00	305.70	NS
	GA/GG	High	14	211.17	0.00	2180.92	
15	AA	Low	14	119.19	0.00	768.36	NS
	GA/GG	High	10	376.87	0.00	1991.56	
16	AA	Low	14	62.28	0.00	752.80	NS
	GA/GG	High	10	294.47	0.00	1374.33	

NS: not significant * Mann Whitney test

Table 4: Association between saliva IL-10 levels and recipient *IL10* genotypes (n=58)

Weeks	Genotype	Associated phenotype	n valid	IL-10 Levels (pg/mg protein) Mean	Minimum	Maximum	*p**
1	AA	Low	16	503.17	0.00	7528.51	-
	GA/GG	High	22	117.58	0.00	816.89	
2	AA	Low	16	1041.67	0.00	11439.56	NS
	GA/GG	High	21	143.41	0.00	1614.31	
3	AA	Low	15	517.36	0.00	6578.82	NS
	GA/GG	High	21	84.79	0.00	529.02	
4	AA	Low	14	387.56	0.00	2581.22	NS
	GA/GG	High	15	54.08	0.00	271.55	
5	AA	Low	13	146.66	0.00	866.15	NS
	GA/GG	High	16	181.41	0.00	1245.73	
6	AA	Low	13	205.75	0.00	955.43	NS
	GA/GG	High	18	127.81	0.00	482.41	
7	AA	Low	12	100.21	0.00	341.40	NS
	GA/GG	High	15	205.19	0.00	901.46	
8	AA	Low	10	92.03	0.00	428.93	NS
	GA/GG	High	16	156.79	0.00	558.11	
9	AA	Low	14	63.76	0.00	228.03	NS
	GA/GG	High	15	847.05	0.00	8204.74	
10	AA	Low	12	99.99	0.00	326.71	NS
	GA/GG	High	18	243.78	0.00	932.58	
11	AA	Low	12	470.62	0.00	4105.29	NS
	GA/GG	High	16	184.29	0.00	1064.35	
12	AA	Low	09	21.02	0.00	74.61	0.03
	GA/GG	High	14	279.65	0.00	1903.60	
13	AA	Low	10	33.09	0.00	104.66	NS
	GA/GG	High	17	176.77	0.00	1011.25	
14	AA	Low	09	152.94	0.00	591.96	NS
	GA/GG	High	18	134.80	0.00	683.12	
15	AA	Low	08	60.10	0.00	268.89	NS
	GA/GG	High	13	168.94	0.00	556.15	
16	AA	Low	07	72.94	0.00	343.69	NS
	GA/GG	High	16	131.76	0.00	406.19	

NS: not significant * Mann Whitney test

Figures

Figure 1

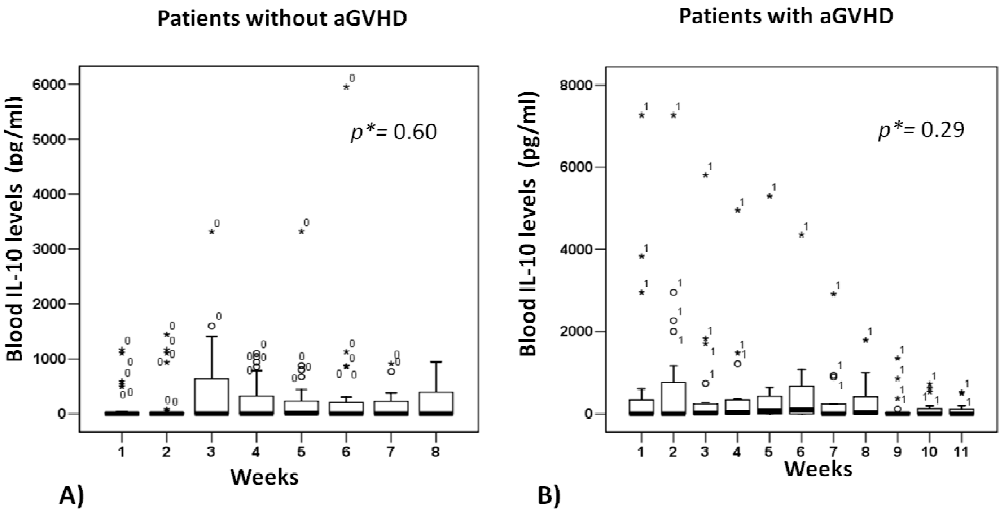

Fig. 1- IL-10 levels in the blood during the weeks after HSCT in patients who develop aGVDH (A) and in patients who did not develop aGVHD (B) (* Friedman test).

Figure 2

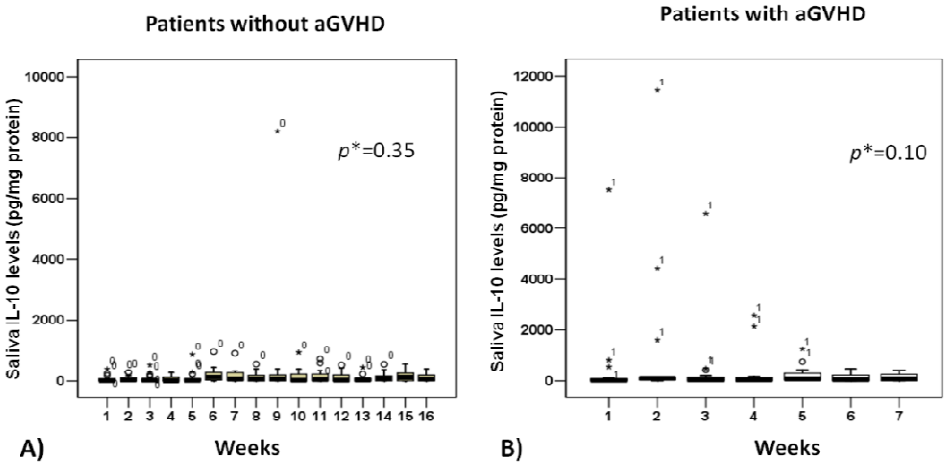

Fig. 2- IL-10 levels in the saliva during the weeks after HSCT in patients who develop aGVDH (A) and in patients who did not develop aGVHD (B) (* Friedman test).

4. Artigo II

Title: Association between pro-inflammatory cytokine gene polymorphisms and cytokine levels in acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation

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Abstract

Graft-versus-host disease (GVHD) is a major cause of morbidity and mortality in allogeneic hematopoietic stem cell transplant (allo-HSCT) recipients. Cytokine gene polymorphisms are associated with functional differences in cytokine expression and can alter the clinical course of GVHD. Although the understanding of GVHD pathophysiology has significantly improved, few studies are currently available to evaluate the contribution of recipient and donor gene polymorphism at cytokine levels and its association with GVHD. The objective of this study was to investigate the association of recipients and donors *IL1B, IL6, IFNG* and *TNFA* gene polymorphisms regarding occurrence and severity of acute GVHD (aGVHD) and their impact on cytokine levels in blood and saliva. Fifty-eight consecutive allo-HSCT recipients and their donors were prospectively studied. Saliva and/or blood samples were obtained from recipients and donors to determine *IL1B, IL6, IFNG*, and *TNFA* gene polymorphisms. Cytokine levels in blood and saliva were also assessed by ELISA. Samples were collected weekly from seven days before transplant (day -7) to 100 days after allo-HSCT (day +100) or until death. No association was found between recipient and donor *IL1B, IL6, IFNG*, and *TNFA* gene polymorphism with aGVHD. However, an association between the donor *TNFA GA/AA* genotypes and an increased severity of aGVHD in the gut and skin could be observed. In contrast to TNF-α level, IL-1β, IL-6, and IFN-γ levels were associated with occurrence of aGVHD as well as with recipient or donor genotypes. Although *IL1B, IL6, IFNG*, and *TNFA* polymorphisms were not associated with occurrence and severity of aGVHD, *TNFA GA/AA* donor

genotype is in fact related to aGVHD activity in isolated organs. In addition, genetic background of recipients and donors directly influenced the production of cytokines. Finally, as IL-1β, IL-6, and IFN-γ levels were associated with aGVHD development, these parameters may well be a useful predictor.

Keywords: Hematopoietic stem cell transplantation; Graft-versus-host disease; Polymorphisms; Cytokines

Introduction

Allogeneic hematopoietic stem-cell transplantation (allo-HSCT) is a well-established curative treatment for many hematological malignancies and some non-malignant disorders [1, 2]. Complications, such as infections, recurrence of malignancy, and graft-versus-host disease (GVHD) are still major obstacles to success [3]. GVHD occurs as a result of complex immunological interactions between the host and transplanted donor cells. The overall grade of acute GVHD (aGVHD) ranges from I to IV in severity and predicts the clinical course of the disease [4].

Cytokines are important mediators and regulators of GVHD [5]. Conditioning regimens damage host cells, with release of proinflammatory cytokines. Gastrointestinal mucosal injury allows bacterial lipopolysaccharide to stimulate the secretion of tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) by monocytes [6, 7]. Subsequently, donor T cells proliferate and secrete IL-2 and interferon-γ (IFN-γ). Finally, cytokine activated cytotoxic T lymphocytes (CTL) and natural killer (NK) cells induce damage in target tissues through cell-mediated cytotoxicity [5].

Prior studies have already demonstrated the association between cytokine polymorphisms and occurrence of aGVHD [2]. However, the effect of these polymorphisms on cytokine levels in blood and saliva were not prospectively addressed. In some circumstances, an association found between a polymorphism and a disease may be well-explained by linkage disequilibrium of different genes. Therefore, studying the relationship between gene polymorphisms and cytokine levels is important to understand the real contribution of genotypes and phenotypes in pathogenesis of GVHD. Thus, the

purpose of this study was to evaluate the association between polymorphisms and cytokine levels in blood and saliva and their relationship with the development of aGVHD in allo-HSCT patients.

Methods

Subjects and sample collection

Fifty-eight consecutive allo-HSCT recipients and related donors from Hospital das Clinicas - Universidade Federal de Minas Gerais (HC-UFMG), between October 2006 and October 2008, were included in this prospective study. Recipients were conditioned for allo-HSCT according to specific protocols from the Stem Cell Transplant Unit at HC-UFMG. Cyclosporin, in combination with methotrexate or mycophenolate mofetil, was used for GVHD prophylaxis, whereas 2 mg/kg of methylprednisolone in combination with cyclosporin were used for GVHD treatment. Saliva and/or blood samples were collected from recipients and donors one week before allo-HSTC and DNA was extracted for analysis. Saliva samples were collected using cotton swabs on the floor of the mouth, tongue, and labial and buccal normal oral mucosa of HSCT subjects; placed immediately in sterile tubes containing 500 µl of Krebs buffer (NaCl 20%, KCl 2%, CaCl22H2O 2%, MgSO4, KH2PO4, and C6H12O6); and stored at -20°C until processing. Sites with localized injuries were not included. Peripheral blood (4 ml) was collected in vacutainer tubes containing EDTA and stored at -70°C until processing.

To determine cytokine levels, one blood and one saliva sample were obtained once a week from seven days before transplant (day -7) to 100 days after allo-HSCT (day +100). Saliva samples were collected in Salivette® tubes (Sarstedt

AG & Co, Numbrecht, Germany) according to manufacturer instructions. Saliva samples were subsequently diluted (1:1) in a PBS solution containing protease inhibitors (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA, and 0.01 mg/mL aprotinin A) and 0.05% Tween-20 and subsequently stored at -20^{o}C until analysis. Serum samples were obtained from venous blood samples centrifuged within 2 hours after blood collection and stored at -20°C.

Total protein content in saliva was determined using the Bradford Reagent (Sigma, Saint Louis, MO, USA) and BSA standard (Fermentas Life Sciences, Vilnius, Lithuania). Total protein content was used to correct cytokine values for saliva samples.

DNA isolation

Total genomic DNA was extracted from the saliva and blood samples using QIAamp DNA Blood Mini Kit (Quiagen, Valencia, CA, USA) according to manufacturer instructions. The final elution of the saliva and blood DNA was performed in 50 µl of a specific AE buffer from the Kit and stored at -20°C until use.

Cytokine gene polymorphism analysis

Recipient/Donor genotypes for *IL1B* (+3954), *IL-6* (-174), and *TNFA* (-308) polymorphisms were analyzed by polymerase chain reaction (PCR) amplification and digestion, as previously described [8, 9]. *IFNG* (+874) polymorphisms were assessed by PCR amplification, using allele specific primers, according to Awad et al., 1999 [10]. The products were viewed in a

6.5% polyacrylamide gel electrophoresis stained with silver. Sequences of the PCR primers and restriction enzymes used are listed in Table 2.

Individuals were grouped into low and high producer phenotypes predicted for these cytokines according to their genotypes, as previously described [11, 12, 13].

Cytokine analysis

Cytokine concentration was determined by means of a quantitative sandwich ELISA technique using the following Kits: Human IL-1β DuoSet (DY201); Human IL-6 DuoSet (DY206); Human IFN-γ DuoSet (DY285); Human TNF-α DuoSet (DY210) (R&D Systems, Minneapolis, MN, USA).

Acute GVHD grades

Acute GVHD was clinically diagnosed (with or without histopathological confirmation) by the assistant physician. All patients were staged for each isolated aGVHD organ involvement (gut, skin, and liver) and classified from grade I to grade IV for acute GVHD, as previously described by Glucksberg et al. (1974) [4].

Statistical analysis

Univariate analyses were performed using the Fisher exact or Chi-square tests for categorical variables; the Mann-Whitney test was used for continuous variables, using SPSS software (SPSS Inc., version 17.0, Chicago, IL). A p value ≤ 0.05 was considered statistically significant.

Results

Clinical outcomes

Clinical data from patients and donors are described in Table 1**.** Acute GVHD was observed in 27 patients. Thirty-eight patients were within grades 0-I (65.5%), whereas grades II-IV were observed in 20 individuals (34.5%). Patients were also classified for aGVHD organ involvement: 38 subjects were within stage 0-1 in gut (65.5%), 49 in skin (34, 5%), and 53 in liver (91.4%), whereas 20 individuals were within stage 2-4 in gut (34. 5%), 9 in skin (15.5%), and 5 in liver (8.6%).

Impact of cytokine gene polymorphisms on aGVHD in patients and donors of allo-HSCT

Results of donor and recipient gene polymorphisms, their prevalence, and corresponding phenotypes, as well as the impact of polymorphisms on the incidence and severity of aGVHD, are shown in table 3. Neither incidence nor severity grade of acute GVHD were associated with *IL1B, IL6, IFNG*, and *TNFA gene* polymorphisms. However, an association between donor TNFA *GA/GG* genotypes and an increased severity of aGVHD in the gut and skin could be observed (Table 4).

Cytokine levels

Figures 1, 2, 3, 4 and 5 shows levels of IL-1β, IL-6, IFN-γ and TNF-α , in blood or saliva, during the 100 days following allo-HSCT. Week 1 represents day -7 before transplant. Only statistically significant data were shown. Where no

statistically significant data could be found, the data were omitted and indicated in the text as “data not shown”.

IL-1β levels, aGVHD,* and *polymorphism

IL-1β levels in blood and saliva and incidence of aGVHD

Figure 1A and 2A shows the kinetics of IL-1β levels during the weeks after HSCT according to presence or absence of aGVHD .No association could be observed between IL-1β levels in blood and aGVHD (Figure 1A). A significant association was observed between a decreased IL-1β level in saliva at weeks 4, 6, and 16 after allo-HSCT and presence of aGVHD (Figure 2A). While median levels of IL-1β in patients without aGVHD in these weeks were 64.49 pg/mg protein, 24.32 pg/mg protein, and 27.39 pg/mg protein, the levels in individuals with aGVHD were 19.13 pg/mg protein, 3.70 pg/mg protein, and 15.20pg/mg protein ($p= 0.03$; 0.02; 0.01, respectively).

IL-1β levels in blood and saliva and IL-1β polymorphisms in recipients and donors of HSCT

No association was found between recipients *IL1B* genotypes and IL-1β levels in blood (data not shown). A significant association between the recipient *IL1B* genotypes and the IL-1β level in saliva could be observed at weeks 8, 13, and 16, where high IL-1β levels were related to *CC* genotype that is described as a low producer phenotype (Figure 3). While median levels of IL-1β in patients who presented *CC* genotypes in these weeks were 26.77 pg/mg protein, 25.97 pg/mg protein, and 31.72 pg/mg protein, the median levels in individuals with

CT/TT genotypes, reported as high producer phenotype, were 5.15 pg/mg protein, 8.92 pg/mg protein, and 15.93 pg/mg protein (p= 0.05; 0.01; 0.05, respectively). IL-1β levels in blood and saliva were not associated with *IL1B* polymorphisms in donors (data not shown).

IL-6 levels, aGVHD,* and *polymorphism

IL-6 levels in blood and saliva and incidence of aGVHD

Figures 1B and 2B shows the kinetics of IL-6 levels during the weeks after HSCT, according to presence or absence of aGVHD. A significant association was observed between increased levels of IL-6 in blood at weeks 4, 5, and 12 after allo-HSCT and the presence of aGVHD (Figure 1B). Median levels of IL-6 in patients without aGVHD during these periods were 22.01 pg/ml, 29.55 pg/ml, and 7.50 pg/ml, respectively, while levels in patients with aGVHD were 68.80 pg/ml, 48.71 pg/ml, and 44.49 pg/ml (p= 0.03; 0.05; 0.01, respectively). Significant association was also observed between an increased IL-6 level in saliva at weeks 3, 5, 6, 10 and 11 after allo-HSCT and the presence of aGVHD (Figure 2B). While median levels of IL-6 in patients without aGVHD in these weeks were 2.50 pg/mg protein, 0.00 pg/mg protein, 0.00 pg/mg protein, 0.00 pg/mg protein and 0.00 pg/mg protein, the levels in individuals with aGVHD were 85.87 pg/mg protein, 9.96 pg/mg protein, and 5.11 pg/mg protein, 5.96 pg/mg protein and 14.50 pg/mg protein (p= 0.01; 0.05; 0.03, 0.01 and 0.05, respectively).

IL-6 levels in blood and saliva and IL-6 polymorphisms in recipients and donors of HSCT

IL-6 levels in blood and saliva were not associated with *IL6* polymorphism in recipients (data not shown). No association was found between donor *IL6* genotypes and IL-6 levels in blood in patients (data not shown). A significantly increased level of IL-6 in saliva of patients was found in donors with *IL6 CG/GG* genotypes, which predict high producer phenotype, at weeks 6, 7 and 11(Figure 4). While median levels of IL-6 in patients who presented low producer phenotype (*CC* genotypes) in these weeks were 0.00 pg/mg protein, 0.00 pg/mg protein, and 0.00 pg/mg protein, the median levels in individuals with high producer phenotype (*CG/GG* genotypes) were 2.32 pg/mg protein, 13.00 pg/mg protein, and 4.58 pg/mg protein (p= 0.05; 0.03; 0.02, respectively).

IFN-γ levels, aGVHD,* and *polymorphism

IFN-γ levels in blood and saliva and incidence of aGVHD

Figures 1C and 2C shows the kinetics of IFN-γ levels during the weeks after HSCT, according to the presence or absence of aGVHD .No association was observed between blood levels of IFN-γ and aGVHD (Figure 1C). A significant association was observed between increased IFN-γ levels in saliva and presence of aGVHD at weeks 10, 11, 12, 14, and 16 after allo-HSCT (Figure 2C). The median levels of IFN-γ in patients without aGVHD during these periods were 2.01 pg/mg protein, 0.00 pg/mg protein, 0.00 pg/mg protein, 0.00 pg/mg protein, and 0.00 pg/mg protein, respectively, whereas levels in individuals with aGVHD were 38.22 pg/mg protein, 38.43 pg/mg protein, 40.78 pg/mg protein, 26.89 pg/mg protein, and 26.27 pg/mg protein (p=0.02; <0.001; 0.01; 0.01; 0.02, respectively).

IFN-γ levels in blood and saliva and IFN-γ polymorphisms in recipients and donors of HSCT

Significant association between recipients *IFNG* genotypes and IFN-γ levels in blood could be observed at week 4 where high IFN-γ levels with *IFNG AT/TT* genotype, associated with high producer phenotype (Figure 4). While median levels of IFN-γ in patients who presented low producer phenotype (*AA* genotype) in these weeks were 0.00 pg/ml, the median levels in individuals with high producer phenotype (*AT/TT* genotypes) were 538.89 pg/ml (p= 0.03). No association was found between patients *IFNG* genotypes and IFN-γ levels in saliva (data not shown). IFN-γ levels in blood and in saliva were not associated with *IFNG* polymorphisms in donors (data not shown).

TNF-α levels, aGVHD,* and *polymorphism

TNF-α levels in blood and saliva and incidence of aGVHD

Figures 1D and 2D shows the time period of TNF-α levels according to presence or absence of aGVHD, where an increase of TNF- α levels in blood and saliva during the first weeks after HSCT could be noticed in patients with aGVHD. No association could be observed between blood or saliva TNF-α levels and aGVHD (Figures 1D and 2D).

TNF-α levels in blood and saliva and TNF-α polymorphisms in recipients and donors of HSCT

No association was also found between recipient and donor *TNFA* genotypes and TNF-α levels in the blood and saliva (data not shown).

IL-1β, IL-6, IFN-γ, and TNF-α levels in blood and saliva according to the time of diagnosis of aGVHD

Samples were divided into the following groups: seven days days before aGVHD diagnosis, on day of aGVHD diagnosis, and 30 days after aGVHD diagnosis. IL-1β, IL-6, IFN-γ, and TNF-α values in the blood and saliva were compared during these periods and proved not to be statistically different (data not shown).

Discussion

The importance of cytokine cascade on the different phases of GVHD is well-established. Acute GVHD, which is associated with lesions of various organs or tissues, is mainly caused by donor T lymphocytes present in grafts. These cells recognize antigenic disparities between donor and recipient. In recipients, toxicity of conditioning regimens tends to result in significant host injury, with inflammatory cytokines being released in both serum and tissues [6, 14]. Stimulation of mature donor T cells within an environment of increased surface cell expression of leukocyte adhesion molecules and HLA molecules commonly leads to the recruitment and activation of additional mononuclear effectors cells from the donor, which amplifies the inflammatory response [15].

Both host tissue and donor cells contribute to generation of pro-inflammatory cytokines, including IL-1β, IL-6, IFN-γ, and TNF-α, which are related to the occurrence of aGVHD. Previous studies have indicated that genetic factors from

recipients and donors, such as cytokine polymorphisms, are associated with incidence and severity of aGVHD [16, 17]. However, their influence on phenotypes (i.e. cytokine levels) after HSCT has yet to be explored in depth. In a recent paper, the present research group reported that recipients with IL-10 high producer phenotypes presented higher IL-10 levels in both blood and saliva and that high blood IL-10 levels were associated with the occurrence of aGVHD [18].

While some studies have already addressed the impact of pro-inflammatory cytokine polymorphisms or cytokine levels in aGVHD development, the present study aimed to investigate the impact of genotypes on cytokine levels and to evaluate the association between them and aGVHD occurrence and severity.

IL-1β is one of the ten members of *IL1* family. Binding of IL-1 to its receptor leads to induction of various genes, including other cytokines like IL-6, involved in the inflammatory response [1]. IL-1β is released before allo-HSCT due to the conditioning regimen and is involved in tissue damage [19]. Recent reports have shown that *IL1B* gene polymorphisms were associated with GVHD outcomes [19, 20]. Lin et al [21] reported a weak association between donor or recipient *IL1B* genotypes and the occurrence of GVHD. Our findings demonstrated no association between *IL1B* gene polymorphisms and the occurrence and severity of aGVHD. When IL-1β levels were assessed, however, it was found that lower levels of IL-1β were in fact associated with occurrence of GVHD. Furthermore, kinetics of this cytokine show low IL-1β levels nearly all the time in patients with aGVHD in both blood and saliva (Fig 1A). In addition, surprisingly, higher saliva levels of IL-1β were associated with a recipient *CC* genotypes, previously described as a low producer phenotype.

These unexpected results may well be explained by variations in the initial release of cytokines by recipient after the conditioning regimen ("cytokine storm"). IL-1 production might also be affected by levels of its specific antagonist (IL-1RA) [22]. A previous study showed that polymorphism of IL-1Ra gene showed an association with the severity of acute GVHD [23].

IL-6 is a cytokine that plays a role in immune, inflammatory, and acute phase responses. It is produced by numerous cell types, induces the development of cytotoxic T cells, and plays a major role in the pathogenesis of GVHD [5]. Functional polymorphism in the *IL6* gene has been extensively studied and the presence of the *IL6 G* allele in recipients of HSCT have been associated with increased IL-6 production and acute GVHD [17, 24, 25, 26]. An association between *IL6* gene polymorphisms and occurrence and severity of aGVHD was not found. In contrast, measurements of IL-6 levels showed that this cytokine appeared to be one of the most important cytokines associated with aGVHD in this study. Increased IL-6 levels were seen in blood and saliva in association with aGVHD, confirming previous studies [27, 28, 29]. Moreover, the increase of IL-6 in patients with aGVHD was observed during the initial weeks of the transplant, as previously described in literature [27, 30]. Finally, increased levels of IL-6 were associated with the *CG/GC* genotypes, which predict high producer phenotype.

IFN-γ is a pleiotropic cytokine with potent pro-inflammatory actions in innate and adaptative immune response and plays a complex role in GVHD with organ-specific pathogenic and protective effects [23]. The most studied polymorphism in *IFNG* gene is located within the first intron of this gene, a $(CA)_n$ microsatellite polymorphism. There are two common alleles: allele 2 and allele 3. Cavet et. al

[31] found a strong association between allele 3 homozygosity and a more severe aGVHD incidence when compared to other genotypes. In this study, *IFNG* gene polymorphism was associated with neither occurrence nor severity of aGVHD. However, IFN-γ levels do play an important role in aGVHD development, where high salivary levels of this cytokine were associated with disease development. Moreover, IFN-γ levels increased in the first and last weeks analyzed in patients who develop aGVHD. This finding is in accordance with other authors who have reported increased levels of IFN-γ in blood of patients with aGVHD [27, 32, 33]. We also found correspondence between the *IFNG* high genotype and the high producer of IFN-γ.

TNF-α is a multifunctional pro-inflammatory cytokine, produced by monocytes and macrophages, which up-regulates adhesion and MHC molecule expression, induces cell death, and plays a major role in GVHD immune response. In addition, this cytokine is involved in the etiology of aGVHD in gut and skin. [1, 14] *TNFA* polymorphisms have been implicated in the development and incidence of aGVHD [1, 16, 34, 35]. Polymorphism at a -308 position has been associated with increased TNF-α production *in vitro* [11]. Data from the present study showed no association between occurrence and severity of aGVHD with *TNFA* polymorphism; however, an association between donor *TNFA GA/AA* genotypes and an increased severity of aGVHD in gut and skin could be observed. In addition, no association was found between TNF-α levels in blood and saliva and occurrence of aGVHD or recipient and donor genotypes. These results can be partially explained by differences in recipient transplantation conditioning regimens and MHC compatibility, according to Xun et al. [6]. However, kinetics profile of this cytokine showed an increased

production of TNF-α in the first weeks after transplant, after conditioning regimen [35, 36]. Accordingly, some studies showed that increased levels of blood TNF-α were associated with the occurrence and severity of aGVHD [36, 37].

In summary, this study shows, for the first time, an association between cytokine polymorphisms, cytokine production and development of aGVHD in specific organs. The genetic background of recipients and donors proved to influence the production of cytokine. As IL-1β, IL-6, and IFN-γ levels were associated with aGVHD development, these parameters may well be a useful predictor of aGVHD occurrence.

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Tables

Table 1- Clinical and Demographic Characteristics of the patients and donors

Parameteres	**Total (n=58) Median (range) or n (%)**
Recipient age (years)	31.5 (5 - 56)
Female gender	25 (43.2%)
Primary Disease	
- Malignant	
- Chronic Myeloid Leukemia	9 (15.5%)
- Acute Myeloid Leukemia	15 (26%)
- Acute lymphoid leukemia	5 (8.6%)
- Non-Hodgkin's Lymphoma	4 (6.9%)
- Hodgkin's Lymphoma	3 (5.1 %)
- Other malignancies*	4 (6.9%)
- Bone Marrow Failure Syndrome**	18 (31%)
HLA match	
- HLA matched related	52 (89.6%)
- HLA matched unrelated	4 (6.8%)
- HLA mismatched related	2 (3.6%)
Donor age (years)	35.4 (6-69)
Donor Female gender	21(36.3%)
Conditioning regimen	
-BU/CY	20 (34.5%)
-CY+/-ATG or Alemtuzumab	14 (24.2%)
-BU + FLU +/- Alemtuzumab	9 (15.5%)
-CY+FLU+/-Alemzutumab	8 (13.8%)
-MEL+FLU+/-Campath	5 (8.6%)
-Others***	2 (3.4%)
Ethnic group	Brazilian mixed population
Source of stem	
- Bone marrow	32 (55.2%)
- Peripheral blood stem cells	25 (43.1%)
- Umbilical cord blood	1 (1.7%)

*Myelodisplastic Syndrome (n=1) Myelofibrosis (n=1) Multiple Mieloma (n=2)
** Paroxysmal noctural hemaglobinuria (n=2) Severe Aplastic Anemia (n=14) Fanconi anemia (n=2)
*** BU/MEL (n=1) Cytarabine/FLU/Campath (n=1)
BU busulfan; CY: cyclophosphamide; FLU: fludarabine; MEL: melphalan; ATG: antithymoglobulin

Table 2 – Primers sequences and restriction enzymes used for the polymerase chain reaction amplification of polymorphic sites of *IL1B, IL6, IFNG* and *TNFA* genes

Genes	Locus	Primers sequences	Base pairs (bp)	Restriction enzymes	Products of digestion	Reference
IL1B	+3954 (C/T)	5' CTC AGG TGT CCT CGA AGA AAT CAA A 3' 5' GCT TTT TTG CTG TGA GTC CCG 3'	194	*TaqI* (37ºC/4h)	TT=182 + 12pb CT=182+97+85bp CC=97+ 85+12bp	KORNMAN et al., 1997
IL6	-174 (G/C)	5`CAGAAGAACTCAGATGAC TG 3` 5`GTGGGGCTGATTGGAAAC C 3`	431	*Hsp92II* (37ºC/12h)	CC=229+122+51 +29 GC=229+173+12 2+51+29bp *GG=229+173+29 bp*	KLEIN *et al.*, 2001
INFG	+874 (A/T)	5`TTCTTACAACACAAAATCA AATCT-`3 (allele T) 5`TTCTTACAACACAAAATCA AATCA-`3 (allele A) 5`TCAACAAAGCTGATACTC CA-`3 (common)	239			AWAD et al.,1999
TNFA	-308 (G/A)	5'AGGCAATAGGTT TTGAGGGCCAT 3' 5'TCCTCCCTGCTCCGATTC CG 3'	107	*NcoI (37ºC/12h)*	AA=107bp GA=107+87+20bp GG=87+20bp	*KORNMAN et al., 1997*

Table 3 - Association between recipient and donor cytokine genotypes and occurrence and grade of aGVHD (n=58)

	Gene	Genotype	*n (%)*	Associated phenotype	GVHD A	GVHD P	*p*	GVHD 0-I	GVHD II-IV	*p*
Recipient	*IL1B* (+3954)	CC	33(56.9)	Low	17	16	NS[b]	20	13	NS[b]
		TT/CT	25(43.1)	High	14	11		18	7	
	IL6 (-174)	CC	4 (6.9)	Low	3	1	NS[a]	3	1	NS[a]
		GG/GC	54(93.1)	High	28	26		35	19	
	IFNG (+874)	AT/AA	48(82.8)	Low	26	22	NS[a]	33	15	NS[a]
		TT	10(17.2)	High	5	5		5	5	
	TNFA (-308)	GG	3 (5.1)	Low	3	0	NS[a]	3	0	NS[a]
		GA/AA	55(94.9)	High	28	27		35	20	
Donor	*IL1B* (+3954)	CC	26(44.9)	Low	15	11	NS[b]	19	7	NS[a]
		TT/CT	32(55.1)	High	16	16		19	13	
	IL6 (-174)	CC	10(17.2)	Low	6	4	NS[b]	7	3	NS[a]
		GG/GC	48(82.8)	High	25	23		31	17	
	IFNG (+874)	AT/AA	42(72.5)	Low	22	20	NS[b]	28	14	NS[b]
		TT	16(27.5)	High	9	7		10	6	
	TNFA (-308)	GG	38(65.5)	Low	21	17	NS[b]	26	12	NS[b]
		GA/AA	20(34.5)	High	10	10		12	8	

NS: not significant P: present A: absent [a] Fisher Test [b] Qui-square test

Table 4 - Association between recipient and donor cytokine genotypes and occurrence and stage of aGVHD according organ involvement (n=58)

	Gene	Genotype	Associated	GVHD Gut		*p*	GVHD Skin		*p*	GVHD Liver		*p*
			phenotype	0-1	2-4		0-1	2-4		0-1	2-4	
	IL1B	CC	Low	22	11	NS[b]	28	5	NS[a]	22	3	NS[a]
	(+3954)	TT/CT	High	16	9		21	4		31	2	
	IL6	CC	Low	3	1	NS[a]	4	0	NS[a]	4	0	NS[a]
Recipient	(-174)	GG/GC	High	35	19		45	9		49	5	
	IFNG	AT/AA	Low	32	16	NS[a]	41	7	NS[a]	44	4	NS[a]
	(+874)	TT	High	6	4		8	2		9	1	
	TNFA	GG	Low	2	1	NS[a]	3	0	NS[a]	3	0	NS[a]
	(-308)	GA/AA	High	36	19		46	9			5	
	IL1B	CC	Low	19	7	NS[a]	27	5	NS[a]	31	2	NS[a]
	(+3954)	TT/CT	High	19	13		22	4		22	3	
	IL6	CC	Low	32	16	NS[a]	9	1	NS[a]	44	4	NS[a]
Donor	(-174)	GG/GC	High	6	4		40	8		9	1	
	IFNG	AT/AA	Low	29	13	NS[b]	9	8	NS[a]	38	4	NS[a]
	(+874)	TT	High	9	7		15	1		15	1	
	TNFA	GG	Low	32	6	**<0.001**[a]	35	3	**0.03**[a]	36	2	NS[a]
	(-308)	GA/AA	High	6	14		14	6		17	3	

NS: not significant P: present A: absent [a] Fisher Test [b] Qui-square test

Figures

Figure 1

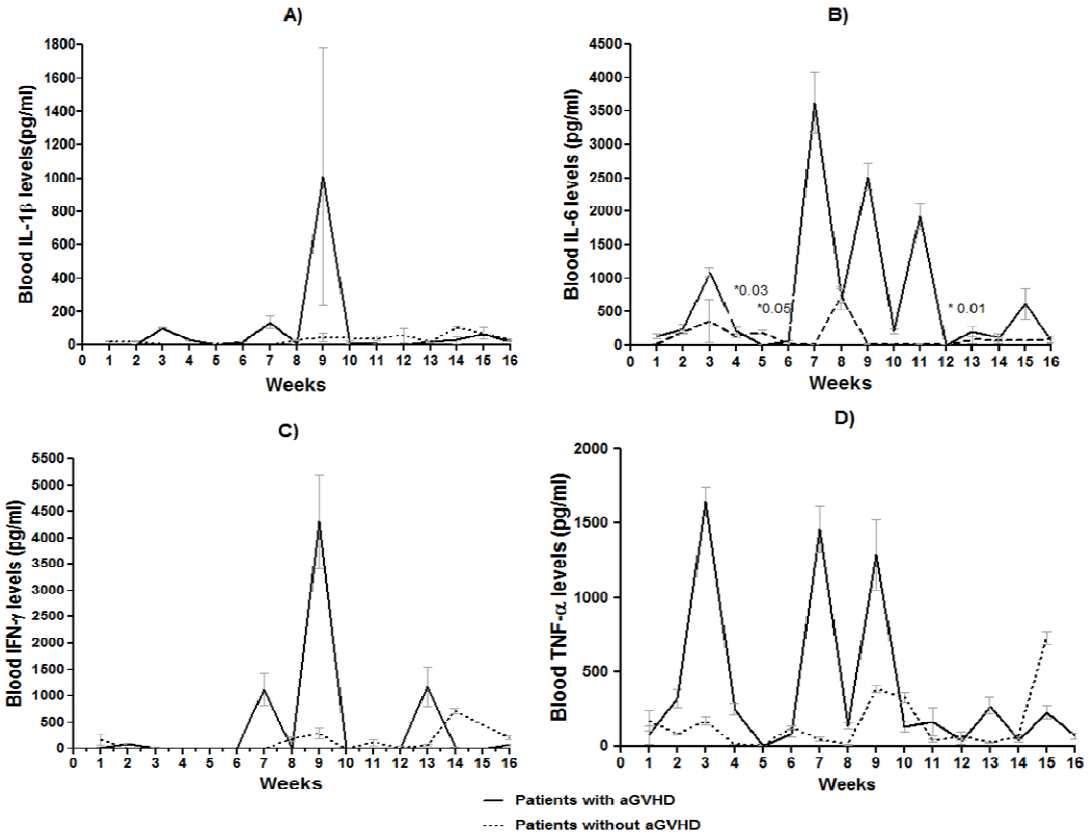

Figure 1 - Kinetics of IL-1β (A), IL-6 (B), INF-γ (C) and TNF-α (D) levels in blood, in patients who developed aGVHD and in patients who did not developed aGVHD. *Mann Whitney test

Figure 2

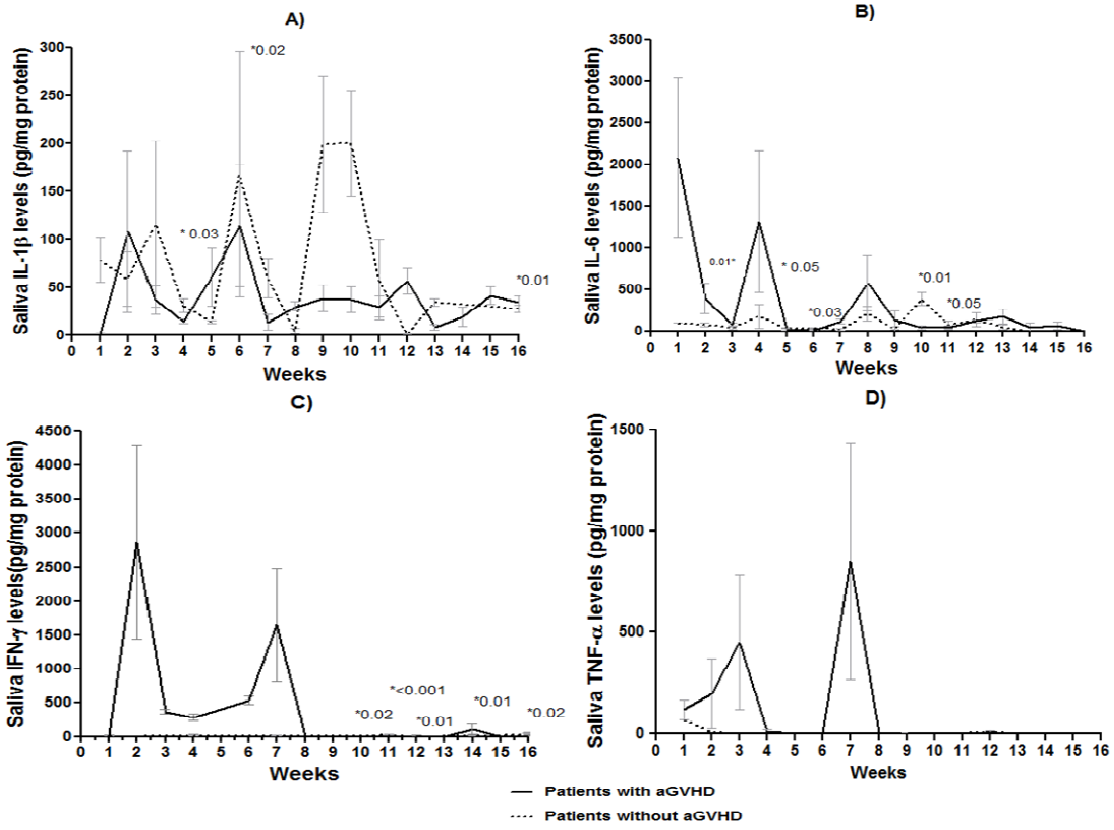

Figure 2 - Kinetics of IL-1β (A), IL-6 (B), INF-γ (C) and TNF-α (D) levels in saliva, in patients who developed aGVHD and in patients who did not developed aGVHD. *Mann Whitney test

Figure 3

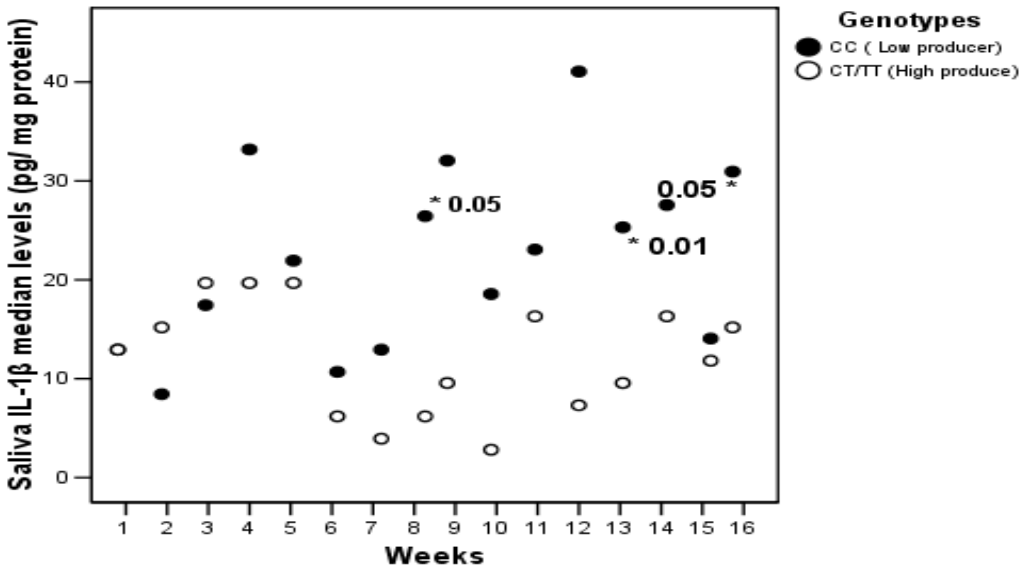

Figure 3 – Association between IL-1β levels in saliva and *IL1B* recipient genotypes during the weeks after HSCT. *Mann Whitney test

Figure 4

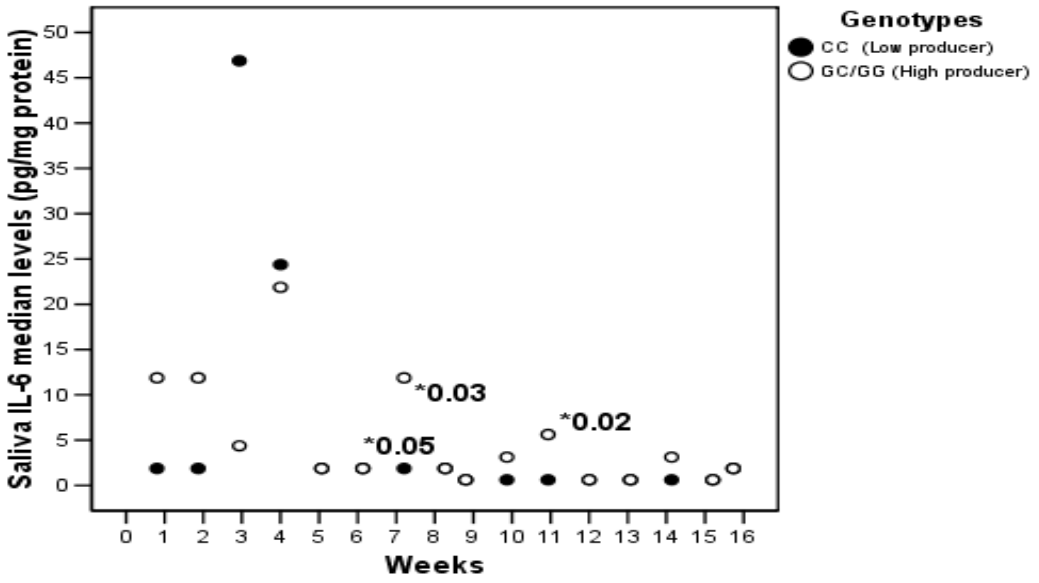

Figure 4 – Association between IL-6 levels in saliva and *IL6* donor genotypes during the weeks after HSCT. *Mann Whitney test

Figure 5

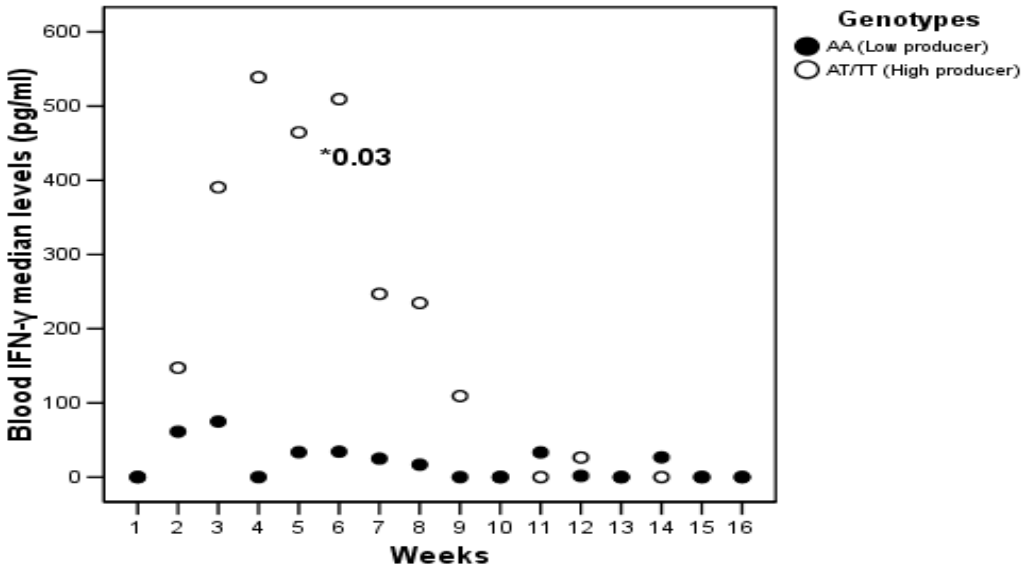

Figure 5 – Association between INF-γ levels in saliva and *IFNG* recipient genotypes during the weeks after HSCT. *Mann Whitney test

5. Considerações Finais

5. Considerações finais

Os elementos essenciais da doença do enxerto contra o hospedeiro (DECH) foram inicialmente definidos por Billingham (1966). Nesses últimos 44 anos, inúmeros trabalhos se empenharam na tentativa de determinar e confirmar o papel dos linfócitos T advindos do doador na etiopatogenia da doença (Antin e Ferrara 1992). Atualmente, a importância da cascata de citocinas após o condicionamento e durante a DECH pós-transplante está bem estabelecida. A resposta imune da DECHa ocorre em duas fases, uma aferente e uma eferente. Na fase aferente da doença, as células T CD4+ e CD8+ reagem a aloantígenos classe I e II do hospedeiro na superfície das células apresentadoras de antígenos (APC). O regime condicionante inicia a resposta imune pelo dano aos tecidos do hospedeiro, como mucosa intestinal, fígado e outros, com a indução da liberação de citocinas. Na fase eferente da DECHa, acredita-se que as células T ativadas produzam um cascata de citocinas. Esses mediadores recrutariam e ativariam células efetoras, como macrófafos e células “natural killer” (NK), que reagem tanto com os tecidos do doador como do hospedeiro (Reddy e Ferrara, 2003).

A DECHa ainda representa uma das principais complicações pós-TCTH, cuja incidência varia de 30-80% e pode ser fatal em 50% dos casos (Dickinson et al., 2004). Vários estudos investigam o impacto de polimorfismos genéticos em complicações decorrentes do TCTH (Dickinson et al., 2007).

Polimorfismos nos genes de citocinas ocorrem dentro das sequencias regulatórias 5` ou 3` dos genes e pode alterar a transcrição e consequentemente a produção das citocinas. Dentro de uma população

normal, alto ou baixo produtores de citocinas existem naturalmente devido ao polimorfismo genético inerente (Dickinson et al., 2007).

Dessa forma, nos últimos dez anos tem sido investigado o potencial dos genótipos na determinação da DECHa. Os estudos de associação entre polimorfismos genéticos e a doenças têm se mostrado uma importante ferramenta não só para investigar a etiologia das doenças, mas também para estabelecimento do prognóstico e tratamentos a serem instituídos (Dickinson et al., 2004). Soma-se a isso, a relevância do estudo do fenótipo dessas citocinas no desenvolvimento da DECHa. Sabe-se que o dano tecidual da DECH pode ser mediado pela resposta imunológica e muitos estudos demonstram o aumento dos níveis sanguíneos de várias citocinas durante a DECH (Remberger et al., 2003).

Tendo em vista que poucos trabalhos se dedicaram a associar os polimorfismos das citocinas inflamatórias dos pacientes e doadores bem como avaliar o impacto destes genótipos na produção de citocinas e ocorrência da aDECH, este trabalho teve como finalidade associar polimorfismos genéticos de várias citocinas envolvidas na etiopatogenia da DECHa aos níveis dessas citocinas durante o pós-transplante e relacionar à ocorrência e gravidade da doença, em pacientes brasileiros.

Não foram notadas diferenças estaticamente significativas entre os polimorfismos avaliados e a DECHa, o que não pode ser confirmado por outros trabalhos que mostram associação entre a DECH e polimorfismos nos genes de *ILIB* (Cullup et al., 2001; MacMillan et al., 2003), *IL6* (Moore et al., 1993; Cavet et al., 1999; Sócie et al., 2001), *IL10* (Sócie et al., 2001; Karabon et al., 2005) *IFNG* (Cavet et al., 2001) e *TNFA* (Middeleton et al., 1998; Cavet et al.,

1999). Estes achados podem estar parcialmente explicados pelo relativo pequeno número de amostras na população avaliada. No entanto, foi possível observar relação entre o polimorfismo no gene de *TNFA* e a maior gravidade da doença em trato gastrointestinal e em pele. Nota-se ainda, relação entre os níveis de IL-1β, IL-6, IL-10 e IFN-γ e a ocorrência de DECHa, o que confirma por outros estudos da literatura (Piguet et al., 1987; Remberger et al., 2001). Além disso, pode-se observar associaçaão entre o genótipo alto produtor de *IL-6, IL10* e *IFNG* e os níveis dessas citocinas, em sangue e na saliva, e associação entre o genótipo baixo produtor de *IL1B* aos altos níveis da citocina na saliva.

Diante do exposto, estudos que avaliem o impacto dos polimorfismos em genes de citocinas inflamatórias na produção desses mediadores e no desenvolvimento da DECH são importantes e podem ser instrumentos para identificar pacientes com risco aumentado para o desenvolvimento da DECHa, e com isso pode facilitar uma possível prevenção e tratamento da doença. Somado a isso, uma importante aplicação deste estudo deve ser considerada: a relevância do uso da saliva como auxiliar no entendimento do desenvolvimento e em um possível diagnóstico precoce da DECHa, por tratar-se de uma forma menos invasiva e também representativa da cinética das citocinas no paciente pós-TCTH.

6. Conclusões

6. Conclusões

Por meio deste estudo podemos concluir:

- Não existe associação entre a os polimorfismos dos genes de *ILIB, IL6, IL10, IFNG* e *TNFA* e a ocorrência e a gravidade da DECHa.

- Existe associação entre o polimorfismo no gene de *TNFA* dos doadores e a maior gravidade de DECHa no trato gastrointestinal e na pele. Não se observa relação entre os polimorfismos de *ILIB, IL6,* e *IFNG* e a ocorrência de DECH em órgãos isolados.

- Níveis aumentados de IL-6, no sangue e na saliva, IL-10, no sangue, e IFN-γ, na saliva, estão relacionados ao desenvolvimento de DECHa.

- A diminuição de IL-1β na saliva está associada com a ocorrência de DECHa.

- Níveis aumentados de IL-1β, na saliva, estão associados ao genótipo CC do gene *IL1B* dos receptores.

- Níveis aumentados de IL-6 e IL-10, na saliva, estão associados ao fenótipo alto produtor dos genes *IL6* dos doadores e *IL10* dos receptores, respectivamente.

- Níveis aumentados de IL-10 e IFN-γ, no sangue, estão associados ao fenótipo alto produtor dos genes *IL10 e IFNG* dos receptores, respectivamente.

7. Referências Bibliográficas

7. Referências bibliográficas

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8. Anexos

ANEXO A

Universidade Federal de Minas Gerais
Faculdade de Odontologia

TERMO DE ESCLARECIMENTO

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (PACIENTE)

Esse documento tem como finalidade propor a sua participação no projeto de pesquisa "RELAÇÃO ENTRE POLIMORFISMOS GENÉTICOS DOS DOADORES E RECEPTORES DE TRANSPLANTE ALOGÊNICO DE CÉLULAS-TRONCO HEMATOPOIÉTICAS COM A DOSAGEM DAS CITOCINAS IL-1α, TNFα, IL-6, IFN-γ E IL-10 E A ASSOCIAÇÃO COM A DOENÇA DO ENXERTO CONTRA O HOSPEDEIRO".

Nos pacientes que fizeram o transplante de medula óssea, também conhecido como transplante de células tronco hematopoiéticas (TCTH), a doença do enxerto contra o hospedeiro (DECH) é muito freqüente, podendo causar várias complicações no tratamento e até mesmo, em alguns casos, a morte após o transplante. A doença do enxerto contra o hospedeiro é uma reação imunológica das células do doador contra o organismo do paciente.

Este projeto de pesquisa tem como objetivo estudar algumas substâncias relacionadas à esta reação imunológica, presentes no organismo, que podem levar ao aparecimento da DECH ou complicar essa doença em pacientes após o transplante. Para esse estudo, será necessário coletar saliva com um tubo especial e também realizar uma coleta do seu sangue (6 ml), semanalmente até o dia + 100 e após 1 ano, para que sejam realizados exames de análise dessas substâncias. Esses exames servirão para identificar a presença dessas substâncias inflamatórias no sangue e na saliva. Nossa idéia é que existem variações nos níveis dessas substâncias na saliva e no sangue dos pacientes. Além disso, será importante também estudar se as células do doador também podem fazer uma quantidade maior ou menor destas substâncias após o transplante. Estas variações podem servir para prever o aparecimento da DECH.

Este estudo não oferecerá riscos à sua saúde, uma vez que o sangue já é colhido de rotina para realização de outros exames durante o transplante e a coleta da saliva também não causará qualquer risco à sua saúde, pois o material é descartável. As amostras coletadas apenas serão utilizadas para a finalidade do projeto e serão descartadas após o estudo. Por outro lado, esta pesquisa nos ajudará a entender a DECH, e no futuro permitir que o aparecimento dessa doença seja descoberto antecipadamente, contribuindo para uma possível prevenção e tratamento individualizado. Portanto, melhorando a qualidade de vida do paciente, diminuindo os desconfortos que a DECH causa.

TERMO DE LIVRE CONSENTIMENTO

Li e entendi as informações fornecidas. Tive a oportunidade de fazer perguntas e todas as minhas dúvidas foram respondidas a contento. Autorizo a realização das coletas de sangue e saliva para este projeto de pesquisa. Permito também a utilização dos dados para divulgação e ensino, respeitando sempre meu direito de não ser identificado.

Este formulário está sendo assinado voluntariamente por mim. Em qualquer momento da pesquisa, posso retirar este consentimento, havendo a continuação normal do tratamento.

Local: _____ Data: ___/___/_____

_____ _____
NOME DO PACIENTE NOME DO RESPONSÁVEL

_____
_____
ASSINATURA DO PACIENTE ASSINATURA DO RESPONSÁVEL

DOCUMENTO APRESENTADO: _____ N.º:_____

Pesquisador: **Renata Gonçalves de Resende** Telefone: (31) 34092477
Orientador: **Prof. Dr. Ricardo Santiago Gomez** Telefone: (31)34902477

Este projeto foi aprovado pelo Comitê de Ética em Pesquisa da UFMG (COEP). Em caso de dúvida, você pode ligar para o **COEP** através do número (31) 3409-9364.

Universidade Federal de Minas Gerais
Faculdade de Odontologia

TERMO DE ESCLARECIMENTO

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (DOADOR)

Esse documento tem como finalidade propor a sua participação no projeto de pesquisa "RELAÇÃO ENTRE POLIMORFISMOS GENÉTICOS DOS DOADORES E RECEPTORES DE TRANSPLANTE ALOGÊNICO DE CÉLULAS-TRONCO HEMATOPOIÉTICAS COM A DOSAGEM DAS CITOCINAS IL-1α, TNFα, IL-6, IFN-γ E IL-10 E A ASSOCIAÇÃO COM A DOENÇA DO ENXERTO CONTRA O HOSPEDEIRO".

Nos pacientes que fizeram o transplante de medula óssea, também conhecido como transplante de células tronco hematopoiéticas (TCTH), a doença do enxerto contra o hospedeiro (DECH) é muito freqüente, podendo causar várias complicações no tratamento e até mesmo, em alguns casos, a morte após o transplante. A doença do enxerto contra o hospedeiro é uma reação imunológica das células do doador contra o organismo do paciente.

Este projeto de pesquisa tem como objetivo estudar algumas substâncias relacionadas à esta reação imunológica, presentes no organismo, que podem levar ao aparecimento da DECH ou complicar essa doença em pacientes após o transplante. Para esse estudo, será preciso realizar uma coleta do seu sangue (3 ml), para que sejam realizados exames de análise. Esses exames servirão para identificar a presença dessas substâncias inflamatórias no seu sangue. Nossa idéia é de que variações nas formas dessas substâncias em paciente e doadores, assim como a presença delas no sangue dos pacientes possam servir para prever o aparecimento da DECH.

Este estudo não oferecerá riscos à sua saúde, uma vez que o sangue será colhido somente uma vez e o material é descartável. A amostra obtida será utilizada para a finalidade do projeto e será descartada após o estudo. Por outro lado, esta pesquisa nos ajudará a entender a DECH, e no futuro permitir que o aparecimento da doença seja descoberto antecipadamente, contribuindo para uma possível prevenção e tratamento individualizado. Portanto, melhorando a qualidade de vida do paciente, diminuindo os desconfortos que a DECH causa.

TERMO DE LIVRE CONSENTIMENTO

Li e entendi as informações fornecidas. Tive a oportunidade de fazer perguntas e todas as minhas dúvidas foram respondidas a contento. Autorizo a realização da coleta de sangue para este projeto de pesquisa. Permito também a utilização dos dados para divulgação e ensino, respeitando sempre meu direito de não ser identificado.

Este formulário está sendo assinado voluntariamente por mim. Em qualquer momento da pesquisa, posso retirar este consentimento, havendo a continuação normal do tratamento.

Local:_____ Data: ___/___/_____

_____ _____
NOME DO DOADOR NOME DO RESPONSÁVEL

_____ _____
ASSINATURA DO DOADOR ASSINATURA DO RESPONSÁVEL

DOCUMENTO APRESENTADO: _____ N.º:_____

Pesquisador: **Renata Gonçalves de Resende** Telefone: (31) 34092477
Orientador: **Prof. Dr. Ricardo Santiago Gomez** Telefone: (31)34092477

Este projeto foi aprovado pelo Comitê de Ética em Pesquisa da UFMG (COEP). Em caso de dúvida, você pode ligar para o **COEP** através do número (31) 3409-9364.

ANEXO B

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM PESQUISA - COEP

Parecer nº. ETIC 613/07

Interessado(a): Prof. Ricardo Santiago Gomez
DCPCO
Faculdade de Odontologia - UFMG

DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP aprovou, no dia 07 de março de 2008, após atendidas as solicitações de diligência, o projeto de pesquisa intitulado **"Relação entre polimorfismos genéticos dos doadores e receptores de transplante alogênico de células-tronco hematopoiéticas com a dosagem das citocinas IL-*a*, TNF-*a*, IL-6, IFN*y* e IL-10 e a associação com a doença do enxerto contra o hospedeiro"** bem como o Termo de Consentimento Livre e Esclarecido.

O relatório final ou parcial deverá ser encaminhado ao COEP um ano após o início do projeto.

Profa. Maria Teresa Marques Amaral
Coordenadora do COEP-UFMG

Av. Pres. Antonio Carlos, 6627 – Unidade Administrativa II - 2º andar – Sala 2005 – Cep31270-901 – BH-MG
Telefax: (031) 3409-4592 - e-mail: coep@prpq.ufmg.br

Universidade Federal de Minas Gerais
Hospital das Clínicas
Diretoria de Ensino, Pesquisa e Extensão - DEPE
UFMG

Belo Horizonte, 11 de abril de 2008.

PROCESSO Nº 221/07

TÍTULO: "RELAÇÃO ENTRE POLIMORFISMOS GENÉTICOS DOS DOADORES E RECEPTORES DE TRANSPLANTE ALOGÊNICOD DE CÉLULAS-TRONCO HEMATOPOIÉTICAS COM A DOSAGEM DAS CITOCINAS IL-1A, TNFA, IFN-Y E IL-10 E A ASSOCIAÇÃO COM A DOENÇA DO EXNXERTO CONTRA O HOSPEDEIRO".

Sr(a) Pesquisador(a):

Reportando-nos ao projeto de pesquisa acima referenciado, considerando sua concordância com o parecer da Comissão de Avaliação Econômico-financeira de Projetos de Pesquisa do HC e a aprovação pelo COEP/UFMG em 07/03/2008 , esta Diretoria aprova seu desenvolvimento no âmbito institucional.
Solicitamos enviar à DEPE ***relatório*** parcial ou final, após um ano.

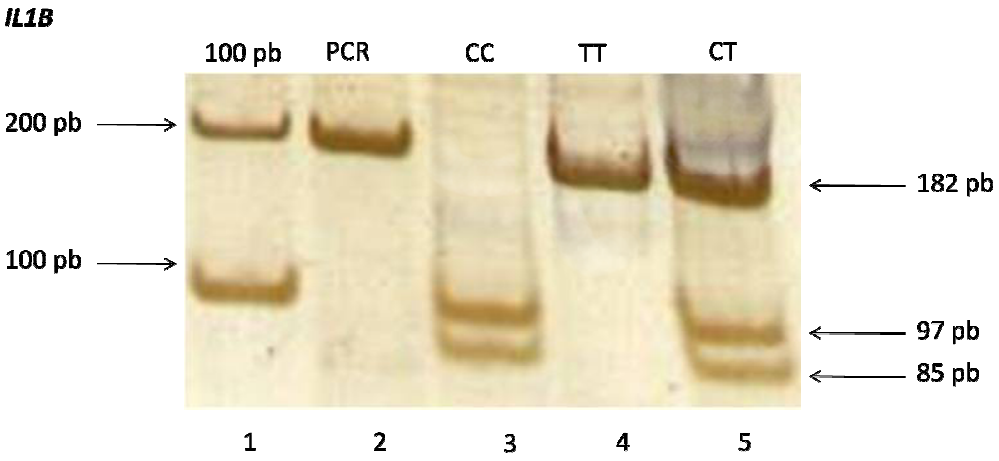
Atenciosamente,

PROF. HENRIQUE VITOR LEITE
Diretor da DEPE/HC-UFMG

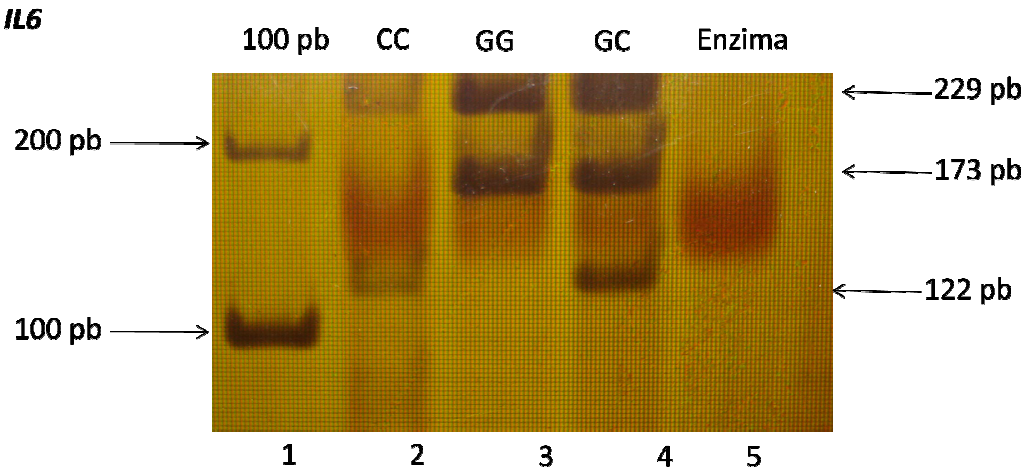
Ào Sr.
Prof. Ricardo Santiago Gomez
Depto.Odontologia
Faculdade de Odontologia-UFMG

CGC: 17.217.985/0034-72 - Av. Prof. Alfredo Balena, 110 – 1º andar
Bairro Santa Efigênia – CEP 30130-100 – Belo Horizonte - MG
Telefone: (31) 3409-9379 – 3409-9375 - FAX: (31) 3409-9380 - depe@ hc.ufmg.br

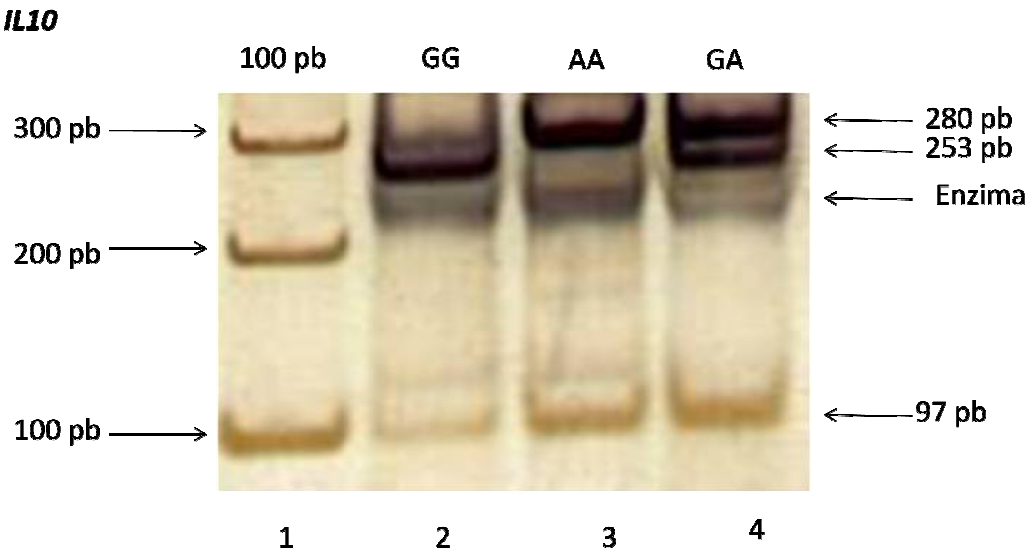
ANEXO C



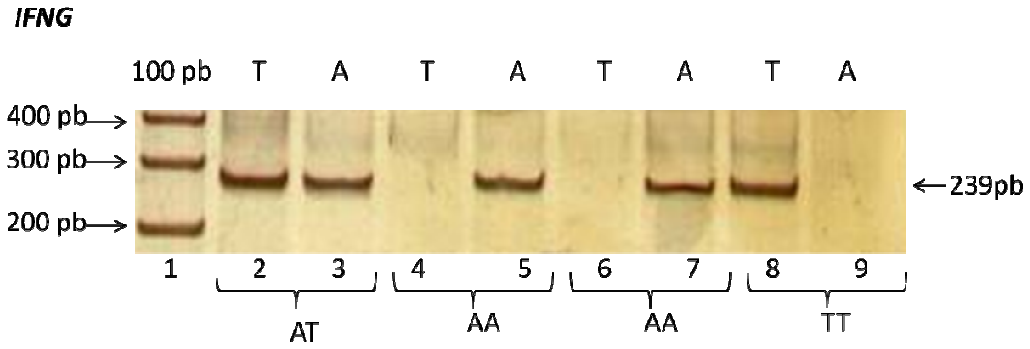
Eletroforese em gel de poliacrilamida a 6,5% referente às reações de digestão enzimática dos produtos de PCR para detecção do polimorfismo (C/T), *lócus* +3954, no gene *IL1B.* Canaleta 1: padrão de peso molecular de 100 pb, canaleta 2: banda de 194 pb referente ao produto de PCR; canaleta 3: bandas de 97 pb+ 85 pb referentes ao paciente com genótipo CC; canaleta 4: banda de 182 pb referente ao paciente com genótipo TT; canaleta 5: bandas de 182 pb+ 97 pb+ 85 pb, referentes ao paciente com genótipo CT.



Eletroforese em gel de poliacrilamida a 6,5% referente às reações de digestão enzimática dos produtos de PCR para detecção do polimorfismo (G/C), *locus* -174 no gene *IL6.* Canaleta 1: padrão de peso molecular de 100 pb, canaleta 2: bandas de 229 pb + 122 pb referentes ao paciente com genótipo CC; canaleta 3: banda de 229 pb + 173 pb referentes ao paciente com genótipo GG; canaleta 4: bandas de 229 pb + 173 pb + 122 pb, referentes ao paciente com genótipo GC; canaleta 5: mancha referente a enzima *Hsp92II*.

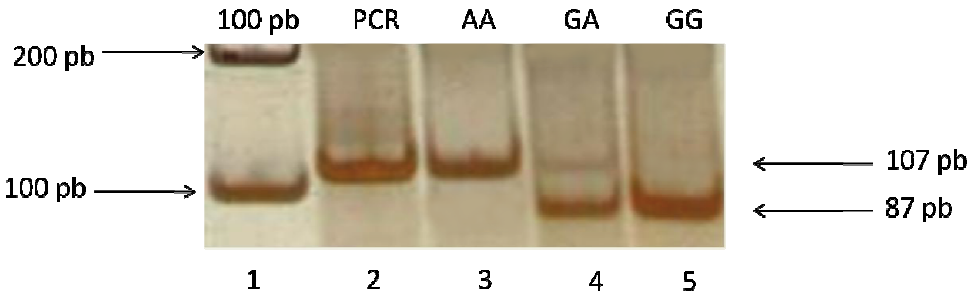


Eletroforese em gel de poliacrilamida a 6,5% referente às reações de digestão enzimática dos produtos de PCR para detecção do polimorfismo (G/A), *locus* -1082 no gene *IL10.* Canaleta 1: padrão de peso molecular de 100 pb, canaleta 2: bandas de 253 pb +97 pb referentes ao paciente com genótipo GG; canaleta 3: banda de 280 pb + 97 pb referentes ao paciente com genótipo AA; canaleta 4: bandas de 280 pb+ 253 pb+ 97 pb, referentes ao paciente com genótipo GA.



Eletroforese em gel de poliacrilamida a 6,5% representando produtos de PCR para o gene *IFNG.* Canaleta 1: padrão de peso molecular de 100 pb; canaleta 2 e 3: bandas correspondentes aos alelos alelos T e A, referentes ao paciente com genótipo AT; canaleta 4 e 5: banda correspondente ao alelo A referente ao paciente com genótipo AA; canaleta 6 e 7: banda correspondente ao alelo A referente ao paciente com genótipo AA; canaleta 8 e 9: banda correspondente ao alelo T referente ao paciente com genótipo TT.

TNFA



Eletroforese em gel de poliacrilamida a 6,5% referente às reações de digestão enzimática dos produtos de PCR para detecção do polimorfismo (G/A), *locus* -308 no gene *TNFA.* Canaleta 1: padrão de peso molecular de 100 pb, canaleta 2: banda de 107 pb referente ao produto de PCR; canaleta 3: bandas de 107 pb referente ao paciente com genótipo AA; canaleta 4: bandas de 107 pb + 87 pb referentes ao paciente com genótipo GA; canaleta 5: banda 87 pb, referente ao paciente com genótipo GG.