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IMPACTO DE ALTERAÇÕES MOLECULARES NOS GENES *DNMT3A*, *IDH1*, *IDH2* E *TP53* NO PROGNÓSTICO DA LEUCEMIA MIELÓIDE AGUDA DO ADULTO

Recife
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Tese apresentada ao programa de Pós-graduação em Biologia Aplicada à Saúde da Universidade Federal de Pernambuco para obtenção do título de Doutor em Biologia Aplicada a Saúde pela Universidade Federal de Pernambuco.

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Aos pacientes com leucemia e suas famílias,
Dedico

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RESUMO

A leucemia mielóide aguda (LMA) é uma doença clonal dos precursores mielóides na medula óssea com aspectos clínicos e moleculares heterogêneos. Na última década, o uso do sequenciamento de nova geração identificou novas mutações em genes reguladores da metilação de DNA, tais como o *DNMT3a*, *IDH1* e *IDH2* em um grande percentual de pacientes com LMA. Ademais, tais abordagens revelaram ainda a relação entre alguns polimorfismos genéticos e a LMA, entre os quais, o polimorfismo Arg72Pro no gene *TP53* se destaca por reduzir a capacidade apoptótica da célula e por já ser associado a vários tipos de neoplasias. Apesar de grandes avanços na compreensão do papel biológico de tais alterações para a leucemogênese, sua relação com o comportamento clínico da LMA ainda não está bem estabelecida. Neste contexto, o presente estudo avaliou: **I)** o valor prognóstico de mutações dos genes *DNMT3a*, *IDH1* e *IDH2* em uma coorte brasileira de pacientes não selecionados (n=227) e em uma coorte independente disponibilizada pelo projeto "The Cancer Genome Atlas" (TCGA, n=180) e **II)** a associação do polimorfismo *TP53* Arg72Pro com o desenvolvimento e prognóstico da LMA (n=205). Não foi encontrada associação entre as mutações nos genes *IDHl*, *IDH2* e *DNMT3a* e sobrevida, quando avaliadas isoladamente. A análise do painel de mutações dos pacientes TCGA revelou que as mutações do tipo *DNMT3a* R882 apresentaram forte associação com as mutações nos genes *NPM1* e *FLT3* quando comparadas a *DNMT3a* não-R882 e *DNMT3a* selvagem. Ademais, foram observados dois perfis distintos de transcriptoma entre pacientes *DNMT3a* R882 mutados e coocorrência de *FLT3* ou *NPM1* e pacientes apenas com o *DNMT3a* R882 mutado. Uma vez que esses dados sugerem interação entre as mutações estudadas, uma variável composta intitulada R882/FLT3/NPM1 foi estabelecida, agrupando pacientes que apresentaram a combinação *DNMT3a* R882 + *FLT3* ou/e *NPM1*. A análise de sobrevida evidenciou um prognóstico marcadamente desfavorável, em ambas as coortes, para os pacientes incluídos na variável R882/FLT3/NPM1, tanto para sobrevida global quanto sobrevida livre de doença. Paralelamente, a análise do polimorfismo *TP53* Arg72Pro revelou que o alelo "Pro", quando em homozigose (modelo recessivo e codominante), foi associado com o desenvolvimento de LMA. Na análise de sobrevida, o genótipo Pro/Pro foi associado a uma sobrevida global superior, tanto na coorte inteira, quanto no grupo cariótipo normal. Dessa forma, o presente estudo demonstra que a análise conjunta das mutações no gene *DNMT3a* e suas mutações parceiras, *FLT3* e *NPM1* é mais eficaz na determinação de prognóstico que a análise de cada mutação isoladamente. Os presentes resultados trazem ainda o polimorfismo *TP53* Arg72Pro como um possível marcador de prognóstico para as LMAs de cariótipo normal.

Palavras-chave: Metilação do DNA. DNMT3a. IDH1. IDH2. TP53. LMA.

ABSTRACT

Acute myeloid leukemia (AML) is a clonal disorder of the myeloid precursors in bone marrow with heterogeneous clinical and molecular features. In the last decade, new generation sequencing techniques allowed the discovery of new mutations in genes involved in the DNA methylation. Additionally, these approaches brought new insights into the relation between several inhered genetic polymorphisms and AML, of which, the TP53 Arg72Pro polymorphism requires attention, due its reduced capacity to induce apoptosis and its association with other malignant disorders. Despite the advances in comprehending the mechanisms those molecular alterations in leukemogenesis, their clinical significance is still unestablished. Therefore, the current study aimed to evaluate I) the effect of *DNMT3a*, *IDH1* e *IDH2* mutations in a non-selected AML patients cohort from Brazil (n=227) and in an independent cohort from The Cancer Genome Atlas (TCGA, n=180) and II) the association of the *TP53* Arg72Pro polymorphism with the development of AML and its clinical outcome (n=205). There were no significant differences in survival regarding the mutational status of *IDHl*, *IDH2* e *DNMT3a* when individually analyzed. Analysis of the mutational landscape of TCGA patients revealed an enriched co-occurrence of *NPM1* and *FLT3* mutations in *DNMT3a* R882 mutated patients when compared to those carrying non-R882 *DNMT3a* mutations or wildtype *DNMT3a*. Furthermore, RNAseq analysis showed distinct patterns of PCA plots for patients carrying only *DNMT3a* R882 mutations when compared to those carrying both *DNMT3a* and *FLT3* or *NPM1*. Taking into consideration the marked interaction between these mutations, a new composite variable hereby identified as R882/FLT3/NPM1 was created, including *DNMT3a* R882 mutated patients with co-occurrence of *FLT3* and/or *NPM1*. Survival analysis showed that patients assigned to the R882/FLT3/NPM1 composite variable had poorer survival rates in both cohorts. Regarding the *TP53* Arg72Pro analysis, homozygosis for the proline-codifying allele (Pro)" was associated with the development of AML (recessive and codominant genetic models). Additionally, the Pro/Pro genotype was associated with longer overall survival both in the entire cohort and in the normal karyotype group, but no significant differences were observed in disease-free survival. The current study proposes a new prognostic model for DNMT3a-mutated AMLs based on the cooperative effect of *DNMT3a* R882, *FLT3* and *NPM1*. Such approach is not only more representative of the biological features of the disease, but was a more efficient predictor of survival than DNMT3a mutations alone. Furthermore, the current results also suggest that the *TP53* Arg72Pro polymorphism might be a useful marker for prognosis in normal karyotype AML.

Keywords: DNA methylation. DNMT3a. IDH1. IDH2. TP53. AML.

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LISTA DE ABREVIATURAS

AML	Acute miyeloid leukemia
ARG	arginine
ATP	adenosina trifosfato
CD	Cluster of differentiation
CI	Confidence interval
CN	Cariótipo normal
CR	Complete remission
D-2HG	D-2-hidroxiglutarato
DFS	Disease-free survival
DNA	Ácido desoxirribonucleico
DNMT3a	DNA methyltransferase 3a
EDTA	Ethylenediamine tetraacetic acid
FAB	Grupo Franco Americano Britânico
FLT3	Fms-Like Tirosine Kinase 3
HR	Hazard ratio
IDH1	Isocitrate dehydrogenase 1
IDH2	Isocitrate dehydrogenase 2
LMA	Leucemia mieloide aguda
LMC	Leucemia mieloide crônica
LPA	Leukemia promielocítica aguda
MBP	Methyl binding protein
MRC	Medical Research Council
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NCCN	National Comprehensive Cancer Network
NK	Normal Karyotype
NPM1	Nucleophosmin 1
OMS	Organização Mundial da Saúde
OS	Overall survival
PCA	Principal component analysis
PCR	Polymerase chain reaction

PRD	Proline-rich domain
PRO	Prolina
RC	Remissão completa
RFLP	Restriction fragment length polymorphism
SDS	dodecil sulfato sódico
TCGA	The Cancer Genoma Atlas
WBC	White blood cells
Wt	Wildtype
αKG	alfacetoglutarato

SUMÁRIO

1 INTRODUÇÃO

A leucemia mieloide aguda (LMA) constitui um grupo heterogêneo de neoplasias malignas do tecido hematopoiético que se caracteriza pelo acúmulo de células blásticas na medula óssea. Os blastos leucêmicos não conseguem passar pelo processo normal de maturação, sendo liberados no sangue periférico ainda em estágio indiferenciado disfuncional. Os principais sintomas derivam da anemia, neutropenia e trombocitopenia resultantes da carência de células efetivas e da invasão de órgãos como o baço, fígado, pele e cérebro pelas células malignas. A LMA é mais frequente em adultos, grupo no qual corresponde a cerca de 80% dos casos. No Brasil, não existe estimativa de incidência exclusiva para LMA, porém, são esperados mais de dez mil casos de leucemia aguda (incluindo outros subtipos) para o ano de 2019.

Em detrimento ao modelo clássico de subclassificação baseado em aspectos citomorfológicos proposto pelo Grupo Franco Americano Britânico (FAB), o atual modelo da Organização Mundial de Saúde (OMS), atualizado em 2016, se baseia principalmente nos aspectos moleculares da célula leucêmica. Tal forma de subclassificação permitiu o desenvolvimento de logaritmos para determinação do prognóstico, tais como o proposto pelo grupo de estudos European LeukemiaNet em 2010, com última revisão em 2017.

A gênese da LMA tem um caráter complexo e multifatorial, englobando fatores ambientais, representados principalmente pela exposição a genotóxicos, genéticos, tais como mutações e polimorfismos, e alterações epigenéticas, tais como metilação de DNA e acetilação de histonas. Nesse contexto, o recente avanço das técnicas de sequenciamento trouxe uma grande quantidade de informações à cerca de alterações genéticas antes desconhecidas na LMA, dentre as quais, podem-se destacar mutações nos moduladores da metilação do DNA e alterações germinativas (mutações herdadas e polimorfismos).

Apesar de a metilação de DNA aberrante já ser descrita como um fator importante para a leucemogênese há mais de uma década, sua origem era até então, desconhecida. Em 2011, entretanto, surgiu a primeira evidência capaz de explicar o surgimento desse fenômeno. Yan e colaboradores, ao realizarem sequenciamento de exoma em pacientes adultos com LMA, identificaram que 21% destes possuíam mutações *de novo* no gene DNA metiltransferase 3a, ou *DNMT3a,* o qual possui papel central na metilação do DNA. Essa observação colocou os moduladores epigenéticos em evidência no contexto da leucemogênese. Estudo posterior realizado pelo consórcio “The Cancer Genome Atlas” mostrou que 44% dos pacientes possuem ao menos uma mutação em genes envolvidos na regulação da metilação do DNA, tais como *DNMT3a, IDH1, IDH2* e *TET2*. Estudos com

modelos animais e culturas de células demonstram que tais alterações surgem em células-tronco normais, dando origem a um clone displásico, mas ainda de crescimento limitado. Em segundo momento, cooperam funcionalmente com outras mutações tardias, desencadeando o processo leucêmico propriamente dito.

Concomitantemente à descoberta das mutações nos moduladores epigenéticos, vários autores vêm relatando uma anteriormente desconhecida relação entre o *background* genômico, ou seja, as características germinativas do indivíduo, e o desenvolvimento da LMA. Em 2016, tal avanço culminou na criação de uma nova entidade que engloba as LMAs com alterações germinativas na classificação das leucemias agudas proposta pela OMS. Neste âmbito, alguns polimorfismos genéticos em genes envolvidos no ciclo celular, apoptose e metabolização de drogas, parecem atuar no contexto de leucemogênese, bem como na resposta celular à quimioterapia.

Dentre os polimorfismos estudados, destaca-se o *rs*1042522, que ocasiona a troca do aminoácido arginina por prolina no códon 72 do gene *TP53*, comprometendo parcialmente a função pró-apoptótica da proteína codificada. Esse polimorfismo é amplamente estudado em tumores sólidos e portadores homozigotos da variante 72P parecem ter maior probabilidade de desenvolver o câncer colorretal, de mama, pulmão e linfoma.

Apesar de as mutações nos genes *DNMT3a*, *IDH1*, *IDH2* e *TET2* e do polimorfismo *TP53* R72P serem bem caracterizados do ponto de vista funcional, seu valor prognóstico ainda não está definido. Os resultados disponíveis são divergentes: enquanto alguns autores observam impacto prognóstico na coorte inteira ou em subgrupos específicos, outros não observam um impacto significativo. Dessa forma, se fazem necessárias outras abordagens na investigação dessas mutações, que incluam esforços no sentido de investigar esses marcadores em outras populações, analisando coortes de pacientes não selecionados, melhor representando a realidade vivida nos serviços de onco-hematologia.

1.1 OBJETIVOS

1.1.1 Geral:

Investigar o valor prognóstico de mutações nos genes *DNMT3a*, *IDH1* e *IDH2* e do polimorfismo *TP53* R72P em pacientes com LMA do adulto.

1.1.2 Específicos:

- Determinar a frequência das mutações nos genes *DNMT3a*, *IDH1* e *IDH2* e do polimorfismo *TP53* R72P em pacientes brasileiros com LMA do adulto;

- Analisar a possível associação das alterações pesquisadas com o prognóstico e com características clínicas e biológicas dos pacientes;

- Validar os resultados em coorte independente através do uso de banco de dados públicos;

- Estabelecer um modelo prognóstico que tenha como base a interação entre mutações nos genes *DNMT3a*, *FLT3* e *NPM1*.

- Analisar, em estudo caso-controle a associação do polimorfismo *TP53* R72P com o risco de desenvolvimento da LMA.

2 REVISÃO DE LITERATURA

2.1 LEUCEMIA MIELOIDE AGUDA

A leucemia mieloide aguda (LMA) constitui um grupo heterogêneo de neoplasias malignas do tecido hematopoiético que se caracteriza pelo acúmulo de células blásticas na medula óssea. Os blastos leucêmicos não conseguem passar pelo processo normal de maturação, sendo liberados no sangue periférico ainda em estágio indiferenciado e disfuncional. Os principais sintomas derivam da anemia, neutropenia e trombocitopenia resultantes da carência de células efetivas e da invasão de órgãos, como baço, fígado, linfonodo e cérebro, pelas células malignas.

LMA é o tipo de leucemia aguda mais frequente no adulto, grupo no qual corresponde a cerca de 80% dos casos (DÖHNER, 2015). No Brasil, não existe especificamente uma estimativa de incidência de LMA, porém, são esperados mais de dez mil casos de leucemia aguda para cada ano do biênio 2018-2019 (INCA, 2018). Apesar de rara, quando comparada a outros cânceres, a LMA tem uma evolução rápida, silenciosa e apresenta uma alta taxa de letalidade. Os poucos dados disponíveis sobre a LMA no Brasil são alarmantes quando comparados aos países desenvolvidos, com sobrevida estimada em cinco anos de 20% (CAPRA *et al.*, 2007; PAGNANO *et al.*, 2000) e 40%, respectivamente (BURNETT *et al.*, 2011; MOORE, 2005; PETERSDORF, 2007). A gênese da LMA tem um caráter extremamente multifatorial, englobando fatores ambientais, tais como exposição a genotóxicos, genéticos, tais como mutações e polimorfismos, além de alterações epigenéticas, tais como metilação de DNA e acetilação de histonas (DÖHNER, 2010).

2.1.1 Diagnóstico e classificação

O diagnóstico clínico da LMA é feito a partir da observação do quadro agudo, caracterizado por anemia, sangramentos e febre. Pode haver infiltração leucêmica em órgãos diversos, causando hepatomegalia, esplenomegalia, hipertrofia gengival (principalmente quando existe um componente monocítico), dor óssea, infiltração cutânea e comprometimento do sistema nervoso central (DÖHNER *et al.*, 2017). O principal critério diagnóstico é o percentual mínimo de 20% de blastos na medula óssea e/ou no sangue periférico. Ao hemograma, apresenta-se mais comumente como uma anemia normocítica/normocrômica, sendo comum a presença de policromasia e até de eritroblastos circulantes, acompanhada de plaquetopenia e neutropenia. A contagem de leucócitos pode variar entre baixa, normal ou elevada e o percentual de blastos frequentemente está acima de 20%, mas também podem estar ausentes no sangue periférico (DÖHNER, 2017). No mielograma, observa-se infiltração

medular por blastos leucêmicos, com redução na quantidade de células de outras linhagens, podendo ainda haver displasia. Também é comum haver alterações hemostásicas; como o alargamento do tempo de protrombina e tromboplastina parcial ativada, redução do fibrinogênio e aumento do D-dímero. Outro achado frequente é o aumento sérico da enzima lactato desidrogenase (KALAYCIO *et al.*, 2007)(THOMAS *et al.*, 1999).

Devido à grande heterogeneidade clínica e biológica da LMA, fez-se necessária a criação de um sistema de subclassificação da doença. O primeiro a ser amplamente aceito foi o proposto pelo grupo Franco-Americano-Britânico (FAB) em 1976. A FAB levou em consideração principalmente critérios citomorfológicos, imunofenotípicos e citoquímicos e subdividiu a LMA em oito subtipos: M0 a M7. Entretanto, com os avanços nos campos da citogenética e biologia molecular, observou-se que estes novos parâmetros eram de grande importância clínica e biológica para a doença. Com a compreensão que as alterações moleculares conferem aspectos particulares aos clones leucêmicos e distinguem entidades dentro do heterogêneo diagnóstico de LMA, a OMS criou em 2001, com revisões em 2008 e 2016, uma nova classificação (Quadro 1) (ARBER *et al.*, 2016). A criação dos critérios diagnósticos da OMS trouxe uma nova forma de compreender as leucemias agudas, visto que pela primeira vez, achados moleculares sobrepujaram critérios diagnósticos tradicionais. A presença de achados citogenéticos característicos da LMA tais como as translocações CBFB-MIH11, RUNX1-RUN1T1 e PML-RARa são suficientes para determinar o diagnóstico da doença, podendo inclusive se sobrepor ao principal e clássico critério diagnóstico: a contagem de blastos medulares superior a 20% (DÖHNER *et al.*, 2017).

Quadro 1. Classificação da LMA segundo a OMS.

Leucemia Mielóide Aguda e Neoplasias de Células Precursoras Relacionadas
- LMA com anormalidades genéticas recorrentes
LMA com t(8;21)(q22;q22.1); RUNX1-RUNX1T1
LMA com inv(16)(p13.1q22) ou t(16;16)(p13.1;q22); CBFB-MYH11
LPA com PML-RARA
LMA com t(9;11)(p21.3;q23.3); MLLT3-KMT2A
LMA com t(6;9)(p23;q34.1); DEK-NUP214
LMA com inv(3)(q21.3q26.2) ou t(3;3)(q21.3;q26.2); GATA2, MECOM
LMA (megacarioblástica) com t(1;22)(p13.3;q13.3); RBM15-MKL1
Entidade provisória: LMA com BCR-ABL1
LMA com mutação NPM1
LMA com mutação bialélica de CEBPA
Entidade provisória: LMA com mutação RUNX1
- LMA com alterações mielodisplásicas relacionadas
- Neoplasia Mieloides relacionadas com terapia
- LMA não classificáveis
LMA com mínima diferenciação
LMA sem maturação
LMA com maturação
Leucemia Mielomonocítica Aguda
Leucemia Monoblástica e Leucemia Monocítica Aguda
Leucemia Eritroide Pura
Leucemia Megacarioblástica Aguda
Leucemia Basofílica Aguda
Panmielose aguda com mielofibrose
- Sarcoma mieloide
- Proliferações mieloides relacionadas com síndrome de Down
Mielopoese anormal transitória (MAT)
Leucemia mielóide associada à síndrome de Down
- Neoplasias de células dendríticas plasmocitóides blásticas

Fonte: Adaptado de Arber *et al*, 2016

2.1.2 Mutações nos genes *FLT3* e *NPM1*

O gene *FLT3 (*Fms-Like Tirosine Kinase 3) é um receptor transmembrana composto por cinco domínios extracelulares do tipo imunoglobulina (responsáveis pela ligação ao seu ligante, FL), um domínio transmembrana; domínio justamembrana; dois domínios quinase e um domínio de inserção de quinases. Geralmente, é expresso em percussores hematopoiéticos e em situações normais, desencadeia uma cascata de reações importantes para promover a proliferação e diferenciação celular (DAVER *et al.*, 2019). Mutações nesse gene são

frequentemente encontradas em pacientes com LMA (em torno de 25%), sendo a mutação mais comum a duplicação *in tandem* no domínio justamembrana (*FLT3*-ITD), apesar de mutações pontuais no domínio tirosinaquinase (códon D835) também serem encontradas em menor frequência (em torno de 12% das mutações no *FLT3*). Ambos os tipos de mutação resultam na ativação constitutiva do *FLT3*, independente da atuação de seu ligante.

A mutação *FLT3*-ITD tem grande importância do estabelecimento do prognóstico da LMA, principalmente nos pacientes com cariótipo normal, conferindo um prognóstico mais reservado (DOHNER, 2017; DONNELL *et al.*, 2017; ZHANG *et al.*, 2019). Tendo em vista sua alta frequência em pacientes com LMA, vários inibidores seletivos do *FLT3* vêm sendo desenvolvidos e alguns já possuem autorização para uso clínico, tal como o sorafenib (DAVER, 2019).

As alterações genéticas mais comuns em pacientes adultos com LMA são as mutações no gene *NPM1*, estando presentes em aproximadamente um terço dos casos. Esse gene codifica a nucleofosmina (NPM), uma proteína presente predominantemente no nucléolo, mas que transita entre o núcleo e citoplasma. Ela é uma chaperona, atuando em processos como o transporte de partículas pré-ribossômicas através da membrana nuclear, na duplicação do centrossomo e na regulação do ciclo celular (MEDINGER, 2016). As mutações no *NPM1* podem ser pequenas inserções ou deleções que alteram o quadro de leitura na tradução da proteína, que por sua vez, perde a capacidade de se concentrar no núcleo, migrando para o citoplasma (*NPM1*c+), perdendo a sua função normal (FALINI *et al.*, 2011). Na ausência de mutações no *FLT3* em pacientes de cariótipo normal, mutações no *NPM1* são associadas a um prognóstico favorável e boa taxa de resposta à quimioterapia de indução (TALATI, 2017).

2.1.3 Fatores prognósticos na LMA

Os fatores prognósticos podem ser subdivididos entre aqueles relacionados à condição de saúde geral do paciente e aqueles relacionados aos aspectos biológicos do clone leucêmico. Os fatores do primeiro grupo são particularmente relevantes na predição da mortalidade relacionada aos efeitos adversos do tratamento e torna-se mais importante com o aumento da idade do paciente, enquanto que os do segundo grupo estão relacionados à resistência à quimioterapia e ao risco de recaídas (DOHNER *et al.*, 2010). A idade avançada está entre os fatores prognósticos mais relevantes, pois está intimamente associada à incidência de diversas comorbidades, principalmente cardiopatias, doenças renais e diabetes, que fragilizam o paciente, agravando os efeitos da quimioterapia. Ademais, alterações moleculares de mau prognóstico são mais comumente encontradas em pacientes idosos, elevando assim, o risco de

resultados desfavoráveis (ALMEIDA, 2016; APPELBAUM *et al.*, 2006; JULIUSSON *et al.*, 2009; THEIN, 2014).

A hiperleucocitose (geralmente considerada como contagem de leucócitos superior a 100 x 10^9/L no sangue periférico) tem sido associada a um prognóstico desfavorável devido à elevada taxa de morte precoce (frequentemente causada por leucoestase pulmonar) e ao alto risco de recaída (GREENWOOD *et al.*, 2006). No que concerne o imunofenótipo do blasto leucêmico, a expressão aberrante do antígeno CD56 é associada a um prognóstico adverso, e vem sendo utilizada na clínica para acompanhamento dos pacientes submetidos à quimioterapia (COELHO-SILVA *et al.*, 2017).

Alterações citogenéticas, estruturais ou numéricas, foram identificadas e classificadas em três grupos: prognóstico favorável, que inclui a t(8;21)(q22;q22), inv(16)(p13;q22), t(16;16)(p13;q22) ou t(15;17)(q22;q21); prognóstico intermediário, que inclui pacientes com cariótipo normal (LMA-CN) e portadores de alguma anormalidade citogenética não enquadrada nas demais categorias; prognóstico adverso, que inclui inv(3)(q21;q26)/t(3;3)(q21;q26), translocações balanceadas envolvendo 11q23, t(6;9)(p23;q34), t(1;22)(p13;q13) e cariótipo complexo (DOHNER *et al*., 2017).

O grupo de LMA-CN engloba a maior parte dos pacientes (em torno de 50%) e por falta de uma classificação mais refinada, foram por muito tempo classificados como risco intermediário. Entretanto, pacientes com CN apresentam uma heterogeneidade clínica considerável, o que sugere que ali haveria mais de um grupo prognóstico não distinguíveis por citogenética (IVEY *et al.*, 2016). Com o avanço das técnicas de biologia molecular, constatou-se uma alta incidência de mutações, tais como nos genes *FLT3*, *NPM1* e *CEBPA*, raramente encontradas em pacientes com anomalias cromossômicas. A descoberta dessas mutações foi importante não só para a compreensão dos aspectos moleculares da LMA-CN, mas permitiu também a estratificação desse grupo (ANDERSSON *et al.*, 2004; VELLOSO *et al.*, 2011; ROCKOVA *et al.*, 2011). Nesse contexto, o grupo de estudos European LeukemiaNet, em 2010 (Quadro 2), propôs um logaritmo de estratificação levando em consideração os dados citogenéticos e moleculares, em detrimento do sistema Medical Research Council (MRC), que incluía apenas os achados citogenéticos (DÖHNER *et al.*, 2010).

Quadro 2. Estratificação de risco sugerida pelo grupo LeukemiaNet - 2010. *Definido como três ou mais alterações cromossômicas na ausência de uma das translocações ou inversões recorrentes designados pela OMS, isto é, t(15;17), t(8;21), inv(16) ou t(16;16), t(9;11), t(v;11)(v;q23), t(6;9), inv(3) ou t(3;3).

Grupo genético	**Subtipo**
Favorável	t(8;21)(q22;q22); *RUNX1-RUNX1T1*
	inv(16)(p13.1;q22) ou t(16;16)(p13.1;q22); *CBFB-MYH11*
	NPM1 mutado sem *FLT3*-ITD (cariótipo normal)
	CEBPA mutado (cariótipo normal)
Intermediário I	Outros cariótipos normais não especificados no grupo favorável
Intermediário II	t(9;11)(p22;q23); *MLLT3-MLL*
	Anormalidades citogenéticas não classificadas como favoráveis ou adversas
Adverso	inv(3)(q21;q26.2) ou t(3;3)(q21;q26.2); *RPN1-EVI1*
	t(6;9)(p23;q34); *DEK-NUP214*
	t(v;11)(v;q23); *MLL* rearranjado
	-5 ou del(5q); -7; abnl(17p); cariótipo complexo*

Fonte: Adaptado de DOHNER *et al.*, 2010.

Atualmente, alterações moleculares, como as mutações nos genes *DNMT3a, IDH1*, *IDH2, TP53,* vêm sendo amplamente estudadas como potenciais marcadores prognósticos. Além de mutações, outros parâmetros como carga alélica, expressão gênica e presença de polimorfismos também são cada vez mais alvo de estudos clínicos (DÖHNER *et al.*, 2017).

2.2 METILAÇÃO DO DNA FISIOLÓGICA E NO CÂNCER

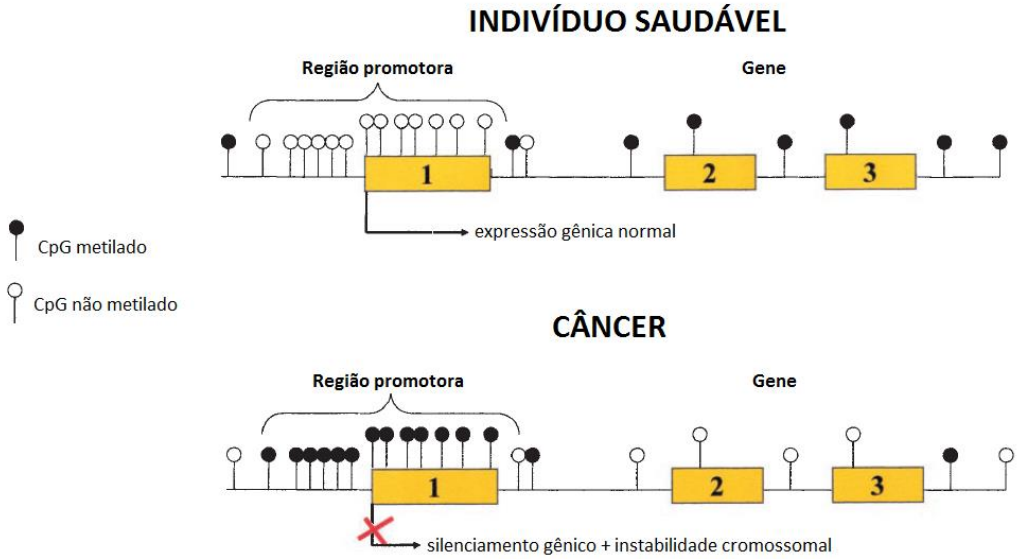
A adição do grupamento metil ao carbono 5 da citosina precedente a uma guanina é catalisada por uma família de enzimas chamadas DNA metil transferases (DNMT), sendo conhecidos atualmente cinco membros: DNMT1, DNMT2, DNMT3a, DNMT3b e DNMT3L, que diferem em suas funções no sentido de metilar de novo, ou metilar fitas recém-criadas na duplicação do DNA (RACEVSKIS *et al.*, 2012). Acredita-se que a metilação das citosinas atrai as proteínas ligadoras do radical metil (MBP), proteínas capazes de formar um *cluster* ao redor da região hipermetilada do DNA, criando uma barreira física ao acesso de fatores de transcrição. O processo funciona como talvez o mais influente mecanismo de regulação gênica em eucariotos e ocorre preferencialmente nas chamadas ilhas CpG, regiões ricas em

citosinas seguidas de guanina, mais frequentemente encontradas nas regiões promotoras de diversos genes (SCHOOFS, 2014).

A metilação do DNA é um processo fisiológico e atua na regulação da expressão gênica durante a diferenciação celular e tecidual. Entretanto, a hipermetilação aberrante em genes supressores de tumor é frequentemente observada na maioria das doenças malignas humanas, inclusive na LMA. Quando em genes supressores tumorais, o fenômeno pode ter efeito semelhante ao das mutações clássicas, resultando na disfunção do gene envolvido (SCHOOFS, 2014).

De forma inversa ao padrão encontrado em células normais, se observa hipometilação no corpo do gene e hipermetilação nas ilhas CpG de regiões promotoras no DNA de células cancerosas (Figura 1) (ESTELLER, 2005). A literatura descreve metilação de regiões promotoras em diversos genes supressores de tumor em pacientes com LMA, tais como os genes *p15, p16, Rb, TP73, DAPK, MDR1, ER e MGMT* (WAINSCOAT, 2004). Um outro achado curioso na LMA, é a frequente hipometilação dos genes dos *clusters* gênicos HOXA e HOXB. Essa hipometilação resulta no aumento da expressão desses genes, que por sua vez, inibem o processo de diferenciação mieloide, que caracteriza a LMA (QU *et al.*, 2014).

Figura 1. Metilação de DNA no câncer. Os pontos brancos representam dinucleotídeos CpG não-metilados enquanto os pretos, dinucleotídeos CpG metilados. Na primeira situação a transcrição do gene ocorre normalmente; na segunda, mostra o silenciamento gênico provocado pela metilação do DNA.



Fonte: Adaptado de ESTELLER, 2005.

2.2.1 Mutações nos genes reguladores da metilação do DNA

Apesar de a metilação aberrante de diversos genes em pacientes com LMA já ser estudada há mais de uma década, sua causa primária era até então desconhecida. A primeira evidência que explicasse esse fenômeno foi descrita por Yan e colaboradores, que ao realizar sequenciamento de exoma em pacientes com LMA, identificou que 21% destes possuíam mutações *de novo* no gene *DNMT3a* (YAN *et al.*, 2011). Em consequência, vários outros estudos vêm demonstrando que essa mutação não só atua na gênese da LMA, mas pode também interferir em seu curso clínico (LOGHAVI *et al.*, 2014; SHLUSH *et al.*, 2014; XIE *et al.*, 2015). Estudos posteriores identificaram mutações em outros genes também envolvidos na metilação do DNA, tais como *IDH1, IDH2* e *TET2* (PAPAEMMANUIL *et al.*, 2016). Um estudo posterior realizado pelo consorcio "The Cancer Genome Atlas" mostrou que 44% dos pacientes possuem ao menos uma mutação em genes envolvidos na regulação da metilação do DNA (TCGA NETWORK, 2013).

2.2.2 Mutações no gene *DNMT3A*: definição e aspectos funcionais

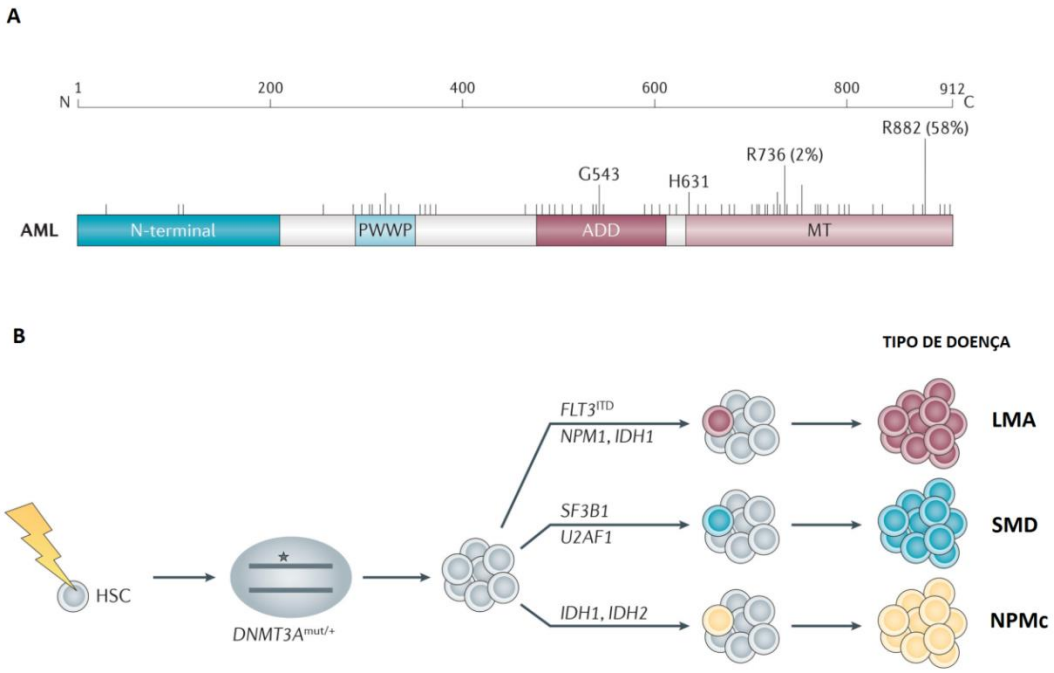
A enzima DNA metiltransferase 3A é uma proteína de 139 kDa, codificada por um gene de 23 exons localizado no cromossomo 2p23 (YANG, 2015). A *DNMT3a* possui três principais domínios: o PWWP (prolina-triptofano), ADD (ATRX, DNMT3, DNMT3L) e domínio catalítico. Os dois primeiros são responsáveis pela ancoragem da enzima em resíduos de lisina nas histonas (H3K4) e regulação negativa da atividade enzimática, enquanto que o último possui o sítio ativo, promovendo a metilação do DNA (Figura 2A) (YANG; 2015). Apesar de já serem descritas mais de 90 mutações ao longo do gene *DNMT3a*, existe um *hotspot* no códon 882 que abriga 60% das mutações encontradas em pacientes com LMA (METZELER *et al.*, 2017; IM, 2014; SINGH, 2012). As mutações no *DNMT3a,* apesar de ainda não completamente compreendidas, parecem ter como consequência função aberrante, com redução da metilação global e concomitante hipermetilação das regiões promotoras (LOBERG *et al.*, 2019; JEONG *et al*, 2018; YAN *et al*, 2011).

Dentre os vários eventos necessários para a malignização de precursores mieloides, o surgimento de mutações no *DNMT3a* parece estar entre os primeiros a ocorrer e, não sendo suficiente para causar transformação maligna, quando ocorre isoladamente (POTTER *et al.*, 2018; SHLUSH *et al.*, 2014). Em suporte a essa teoria, ao avaliar 2.530 idosos saudáveis (55 a 101 anos), um estudo recente observou uma taxa de 13% de indivíduos portadores da mutação no *DNMT3a* sem fenótipo leucêmico, caracterizando apenas um quadro de hematopoese clonal em estágio inicial. Os indivíduos mutados tiveram um risco até dez vezes

maior de desenvolvimento de LMA em relação àqueles sem a mutação (BUSCARLET *et al.*, 2017; GENOVESE *et al.*, 2014).

Devido ao fato de ser encontrada frequentemente associada a mutações nos genes *FLT3* e *NPM1*, (TCGA NETWORK, 2013) a combinação dessas alterações foi foco de diversos estudos clínicos e em modelos animais. Estudos em modelos murinos condicionais demonstram que as mutações no gene *DNMT3a* causam expansão clonal sem repercussão clínica, funcionando como um primeiro "*hit*". Após o surgimento de uma segunda mutação em genes como o *FLT3* e *NPM1*, ocorre um desencadeamento rápido, com alta penetrância, da LMA (Figura 2B) (GURYANOVA *et al.*, 2017; LOBERG *et al.*, 2019; MEYER *et al.*, 2016; YANG *et al.*, 2016). Análise de transcriptoma e metiloma em pacientes com LMA portadores de mutações concomitantes nos genes *DNMTA3a*, *FLT3* e *NPM1* mostram que essas mutações interagem funcionalmente entre si, formando uma entidade a parte, com perfil molecular distinto das demais (RIBEIRO, 2013; TCGA NETWORK, 2013; YANG, 2016). Apesar da evidente interação, pouco ainda se sabe sobre o mecanismo biológico envolvido nessa combinação de mutações.

Figura 2. Mutações no gene *DNMT3a* em pacientes com LMA. Distribuição e tipo das mutações encontradas no gene *DNMT3a* no contexto e pacientes com LMA do adulto (A). Expansão clonal pré-maligna causada pela mutação no *DNMT3a* que só desencadeia a doença propriamente dita apos o surgimento de uma segunda mutação (B) .



Adaptado de YANG 2015.

2.2.2.1. *Significado clínico das mutações no DNMT3A*

As mutações no gene *DNMT3a* vêm sendo amplamente estudadas como possíveis marcadores de prognóstico em pacientes com LMA, entretanto, a literatura é conflitante e os resultados devem ser avaliados com cautela. Apesar de muitos estudos não observarem diferença nos índices de remissão completa, a sobrevida dos pacientes que possuem a mutação é frequentemente inferior (GURYANOVA *et al.*, 2017; IVEY *et al.*, 2016; KUMAR *et al.*, 2018; PAPAEMMANUIL *et al.*, 2016; THOL *et al.*, 2010; TIE *et al.*, 2014). Tal achado sugere que, apesar de a sensibilidade a quimioterápicos da célula leucêmica não ser afetada pela mutação, a mesma é também encontrada em clones pré-leucêmicos que escapam ao tratamento e eventualmente se expandem, causando uma recaída (SCHUURHUIS *et al.*, 2018).

Em contrapartida, outros estudos não observaram diferenças de sobrevida significativas (GAIDZIK, V I *et al.*, 2017; GAIDZIK *et al.*, 2013; SCHMALBROCK *et al.*, 2018). Alguns autores observaram impacto prognóstico apenas em grupos específicos da coorte, quando restringidos a idade ou grupo de risco citogenético. É provável que a diversidade de resultados ocorra devido a: I) uso de diferentes protocolos terapêuticos, II) diferenças nas características clínicas e gerais da coorte estudada e III) modelo de análise dos resultados, levando em consideração outras mutações associadas, cariograma e etc (ROLLER, 2013; FIGUEROA *et al.*, 2010; MARCUCCI *et al.*, 2015). Um ponto ainda não esclarecido é a diferença entre mutações em diferentes sítios da enzima. Em pacientes com LMA, aproximadamente 60% das mutações do gene *DNMT3a* ocorrem no códon 882, localizado no domínio catalítico e as demais ocorrem de forma distribuída em toda a sequência do gene. Guryanova e colaboradores (2017) observaram que somente as mutações no códon R882 estavam associadas à presença de doença residual mínima. Já os resultados obtidos por Marcucci e colaboradores (2015) sugerem que as mutações não-R882 têm valor prognóstico apenas em pacientes jovens, enquanto as mutações R882 têm valor prognóstico em pacientes idosos. Um estudo realizado em uma grande coorte européia evidenciou distintos perfis moleculares entre esses dois grupos de mutações. As mutações no códon R882 foram fortemente associadas às mutações no *NPM1* e *FLT3* e ao cariótipo normal, enquanto as não-R882 apresentavam menor co-ocorrência dessas alterações e foram encontradas em pacientes com translocações balanceadas. (GAIDZIK *et al.*, 2013). Curiosamente, dois estudos identificaram a presença de mutações no gene *DNMT3a* em 10% a 13% de indivíduos saudáveis, entretanto, apenas 9% a 12% dessas mutações eram no códon R882 (BUSCARLET *et al.*, 2017; GENOVESE *et al.*, 2014). Dessa forma, o tema ainda é

controverso, se fazendo necessários mais estudos que comparem clinicamente as diferentes mutações no gene *DNMT3a*.

Como abordado anteriormente, LMAs com o perfil *DNMT3A*/*NPM1*/*FLT3* mutados representam uma entidade com características biológicas bem definidas (METZELER *et al.*, 2018). O fato de as mutações não-R882 não se associarem ao perfil *FLT3+*/*NPM1+* sugere que essas mutações repercutem de forma distinta na função enzimática, atuando sobre outros mecanismos bioquímicos que culminam na malignização da célula (GAIDZIK *et al.*, 2013). Apesar de preliminares e ainda pouco levados em consideração por outros autores, esses dados mostram haver uma distinção entre os dois casos. É possível que o agrupamento indevido de mutações com diferentes repercussões bioquímicas e prognósticas possa inserir viés na interpretação de alguns resultados.

2.2.2.2. Associação das mutações no DNMT3a com aspectos clinico-laboratorias e doença residual mínima

As mutações no *DNMT3a* vêm sendo associadas com alguns aspectos clínicos da LMA, tais como idade mais avançada ao diagnóstico, percentual de blastos na medula óssea, leucometria e subtipos FAB com componente monocítico e ausência de expressão do antígeno CD34 (GENOVESE *et al*, 2014; KUMAR *et al.*, 2018).

Por continuarem sendo detectadas em pacientes em remissão completa, existe uma discussão se as mutações no *DNMT3a* podem ser utilizadas como marcadores de doença residual mínima na LMA. Alguns estudos relativamente pequenos não observaram associação clara entre a detecção de mutações no *DNMT3a* pós-remissão e risco de recaída (IVEY *et al.*, 2016; JEZISKOVA *et al.*, 2015; JONGEN-LAVRENCIC *et al.*, 2018). Entretanto, por ser considerada uma mutação pré-leucêmica e dependente de uma segunda mutação para induzir LMA, o grupo de pesquisa European LeukemiaNet, recomenda que as mutações no *DNMT3a*, apenas quando combinadas a uma outra alteração clonal, podem ser utilizadas como marcador de doença residual mínima (SCHUURHUIS *et al.*, 2018). A fim de clarificar a questão, novos estudos prospectivos com análise de múltiplas amostras coletadas em tempos diferentes estão em andamento e trarão novas informações a cerca do tema (TYNER *et al.*, 2018; BRUNETTI; 2017).

2.2.3 Mutações nos genes *IDH1* e *IDH2*

A família das enzimas isocitrato desidrogenase (IDH) apresentam três classes de isoenzimas que atuam no ciclo de Krebs ao catalisar a descarboxilação oxidativa do isocitrato

em alfacetoglutarato (αKG) através da redução do NAD ou NADP à NADH ou NADPH, respectivamente. A enzima *IDH1* dependente de NADP é codificada pelo gene *IDH1*, e se localiza no citoplasma e peroxisoma. Já enzima *IDH2* atua dentro das mitocôndrias. A enzima IDH3 é codificada pelo gene IDH3 e tem sua localização também no cromossomo 15, ela catalisa a conversão dependente de NAD mitoncondrial. As IDH estão envolvidas em vários processos celulares, incluindo fosforilação oxidativa mitocondrial, metabolismo da glutamina, e lipogênese (AREF *et al.*, 2015; DE BOTTON *et al.*, 2016; GURYANOVA *et al.*, 2017).

A mutação no *IDH1* foi identificada pela primeira vez em um paciente com câncer colorretal e posteriormente as IDH mutadas foram encontradas em pacientes com gliomas de baixo grau (astrocitomas e oligodendrogliomas grau II e III) e glioblastomas secundários, onde as mutações têm uma incidência de mais de 70% (DE BOTTON *et al.*, 2016; KERNYTSKY *et al.*, 2015). Nas LMAs, elas ocorrem numa proporção de 7-15% e 8-16%, respectivamente e em conjunto, estão presentes em 20% dos pacientes (FALINI *et al.*, 2019; GROSS *et al.*, 2010; MEDEIROS *et al.*, 2017).

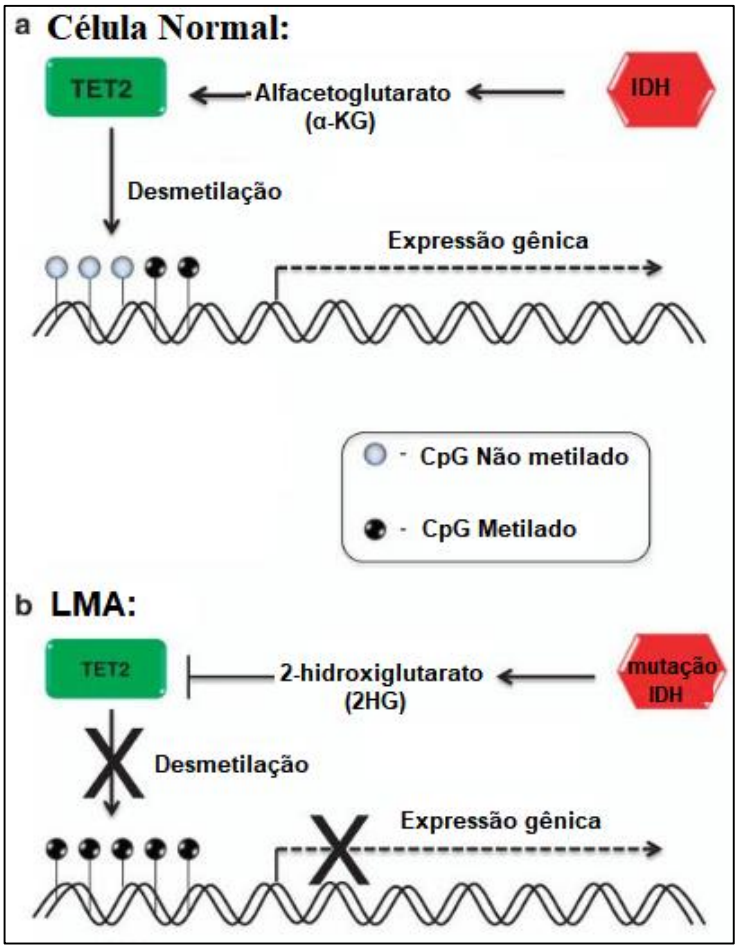
São heterozigóticas, do tipo *missense*, somáticas e parecem ser funcionalmente equivalentes entre as diferentes isoformas. Na *IDH1* ocorre a substituição do aminoácido arginina no codón 132 por outros aminoácidos, como: serina (R132S), histidina (R132H), cisteína (R132C), glicina (R132G) e leucina (R132L). No caso da mutação no *IDH2*, ocorre a mudança da arginina no códon 172 por lisina (R172K) e no codón 140, arginina por glutamina (R140Q) (AREF *et al.*, 2015; DE BOTTON *et al.*, 2016; FALINI *et al.*, 2019). Estas enzimas mutantes apresentam função neomórfica que culmina na conversão do NADPH e αKG, também produzido pela mesma enzima, em NADP+ e D-2-hidroxiglutarato (D-2HG) (GROSS *et al.*, 2010; WARD *et al.*, 2012). A produção de D-2HG em tecidos com células IDH mutantes chega a ser 50 a 100 vezes mais elevada que em tecidos em condições fisiológicas. Além de desfavorecer a formação aeróbica de adenosina trifosfato (ATP), interfere na regulação da hidroximetilação do DNA, contribuindo assim para oncogênese. O D-2HG produzido pela IDH mutante inibe as dioxigenases dependentes de α-cetoglutarato, inclusive as histonas/DNA desmetilases, resultando em uma hipermetilação do DNA nas regiões conhecidas como ilhas CpG (Figura 3). O quadro de hipermetilação do DNA, por sua vez, culmina no silenciamento de diversos genes importantes na regulação do ciclo celular e apoptose (MEDEIROS *et al.*, 2017).

Nos pacientes com LMA, as mutações nos genes *IDH1* e *IDH2* são mutualmente exclusivas entre si e também em relação ao gene *TET2* (NETWORK, 2013). Tal achado sugere que as três mutações culminam na inatividade da enzima *TET2*. Ambas inibem a

enzima *TET2*, que desmetila o DNA (RAKHEJA *et al.*, 2012). Mutações do *TET2* e *IDH1*/2 estão associadas a perfis de metilação do DNA e expressão gênica semelhantes, sugerindo redundância entre as mutações (PATEL, 2012). O perfil de expressão gênica aberrante causado por essas mutações prejudica a diferenciação mieloide e aumenta a capacidade de renovação das células progenitoras (IM *et al*., 2014).

Em doenças malignas da linhagem mieloide, a presença do IDH mutante é considerada um evento inicial em 19% dos pacientes com *IDH1* mutado e 34% dos pacientes com mutações no *IDH2*, visto que essas mutações foram encontradas não somente nos blastos leucêmicos, mas também nos percursores mais altos na hierarquia maturativa (DE BOTTON *et al.*, 2016; MOLENAAR *et al.*, 2018). Por este motivo, é provável que as mutações IDH estejam implicadas nos estágios iniciais da LMA *de novo*, assim como as mutações nos genes *DNMT3A*, *TET2* e *ASXL1* (CLARK *et al.*, 2016; XIE *et al.*, 2015).

Figura 3. Função da Isocitrato Desidrogenase. A) Função normal das enzimas *IDH1* / 2 e *TET2* na metilação do DNA e expressão gênica. B) Mecanismo de *IDH1* / 2 mutado com consequente inibição da desmetilação.



Fonte: Adaptado de AP Im et al, 2014

2.2.3.1 *Características clínicas e impacto prognóstico das mutações nos genes IDH1 E IDH2*

As características clínicas mais frequentes das mutações nos genes *IDH1*/2 são idade avançada e citogenética normal. Além disso, mutações no *IDH1*/2 e *TET2* são mutualmente exclusivas e a associação frequente com a mutação *NPM1* é sugestiva de uma interação na patogênese da LMA (AP IM, 2014).

A relação das mutações nos *IDH1* e *IDH2* com o prognóstico ainda é bastante controversa, pois diferentes estudos, mesmo analisando grandes coortes, mostraram que as mutações *IDH1*/2 podem estar associadas a um pior prognóstico (AREF *et al.*, 2015; LIU *et al.*, 2014; MARCUCCI *et al.*, 2015; PASCHKA *et al.*, 2013), um melhor prognóstico ou não ter qualquer associação (ABBAS *et al.*, 2010; DINARDO *et al.*, 2015; WAGNER *et al.*, 2010). Uma metanálise incluindo 8121 pacientes com LMA mostrou que aqueles com mutações no *IDH1* apresentaram uma menor taxa de remissão completa com tratamento convencional. Curiosamente, um estudo não observou impacto prognóstico de mutações no *IDH1*, mas encontrou um impacto adverso do polimorfismo sinônimo rs11554137, localizado no códon 105, exon 4 (WAGNER *et al.*, 2010).

Os resultados do *IDH2* também são inconsistentes, havendo relatos sem impacto prognóstico (THOL *et al.*, 2010) e relatos de prognóstico desfavorável (BOISSEL *et al.*, 2013). Estudos recentes apontaram um provável viés que possa explicar esses resultados: as mutações no *IDH2* são geralmente analisadas em conjunto, entretanto, as mutações no códon R172 parecem ter efeito adverso em comparação às demais (GREEN *et al.*, 2011; MARCUCCI *et al.*, 2015; PAPAEMMANUIL *et al.*, 2016; PATEL JP, GONEN M, 2012). Recentemente, Papaemmanuil e colaboradores sugeriram uma nova classificação genômica da LMA, incluindo de forma provisória LMA com IDH R172 mutado (excluindo assim as mutações R140) como uma entidade distinta das demais, devido ao seu prognóstico reservado (PAPAEMMANUIL *et al.*, 2016). Frente ao exposto, fazem-se necessários novos trabalhos clínicos que possam elucidar a o valor prognóstico das mutações nos *IDH1* e *IDH2*.

2.2.3.2 *Abordagens terapêuticas em pacientes com LMA com IDH1/2 mutado*

Apesar de o valor prognóstico das mutações nos genes *IDH1* e *IDH2* no contexto da LMA tratada com drogas citotóxicas ainda não estar bem definido, a detecção dessas mutações pode ser de grande valor para o uso de terapia personalizada, com uso o de inibidores das IDH mutantes. A descoberta dessas mutações resultou em uma série de novas abordagens terapêuticas, que tanto restauram a função da IDH selvagem como bloqueiam a produção ou os efeitos da D-2HG. Inibidores seletivos para *IDH1* e *IDH2* foram produzidos e

muitos se encontram atualmente em testes clínicos. Essas moléculas se ligam ao sítio catalítico das enzimas IDH mutantes e evitam a modificação conformacional necessária para a transformação de α-KG para R-2-HG (BEWERSDORF, 2019; KERNYTSKY *et al.*, 2015; WANG *et al.*, 2014).

Em estudos pré-clínicos *in vitro* e *in vivo*, a AGI-5198 e AGI-6780 que são inibidores seletivos de enzimas *IDH1*/2 mutantes, respectivamente, resultaram na normalização dos níveis de 2HG e reversão da hipermetilação do DNA e liberação de bloqueio de diferenciação celular (DE BOTTON *et al.*, 2016; WANG, 2013; KERNYTSKY *et al.*, 2015; POPOVICI-MULLER *et al.*, 2018). Os compostos AG-120, enasidenib (AG-221) e AG-881 são inibidores as IDH mutantes de uso oral. Em estudos clínicos de fase I e II, esses compostos reverteram rapidamente os níveis de R-2-HG e a hipermetilação e agora estão sendo testados em pacientes com doença hematológica avançada (BEWERSDORF, 2019; DE BOTTON *et al.*, 2016; MEDEIROS *et al.*, 2017).

Visto que a hipermetilação do DNA atua de forma central na patogenia das LMAs *IDH1*/*IDH2* mutadas, o uso de agentes hipometilantes do DNA, tais como a decitabina e azacitidina, surgem como outra possibilidade terapêutica. Essas drogas se ligam às DNA metiltransferases, inibindo o processo de metilação do DNA e já são usadas em pacientes idosos com LMA não aptos à quimioterapia convencional. Entretanto, pouco se sabe ainda sobre o seu efeito em pacientes com ou sem mutações nos genes IDH. Dois estudos pequenos que avaliaram 68 e 175 pacientes com LMA tratados com agentes desmetilantes não encontraram diferença significativa na resposta e sobrevida de pacientes com ou sem mutações nos genes IDH. Entretanto, a combinação entre agentes desmetilantes e inibidores seletivos de IDH pode trazer resultados mais consistentes e estão sendo avaliada em estudos clínicos (MEDEIROS *et al.*, 2017). Ademais, estudos pré-clínicos recentes investigaram a imunoterapia baseada em vacinação para atingir mutações *IDH1*, cuja vacinação com péptideo específico de R132H induz uma resposta antitumoral específica de MHC de classe II contra células tumorais que expressam essa mutação. Apesar de os resultados serem promissores, o uso da quimioterapia convencional ainda é necessário (DE BOTTON *et al.*, 2016).

2.3 POLIMORFISMOS NA LMA

Por muito tempo a LMA foi compreendida como uma doença originada a partir de alterações clonais. Entretanto, vários autores vêm descrevendo uma anteriormente desconhecida relação entre o *background* germinativo, representado por mutações e

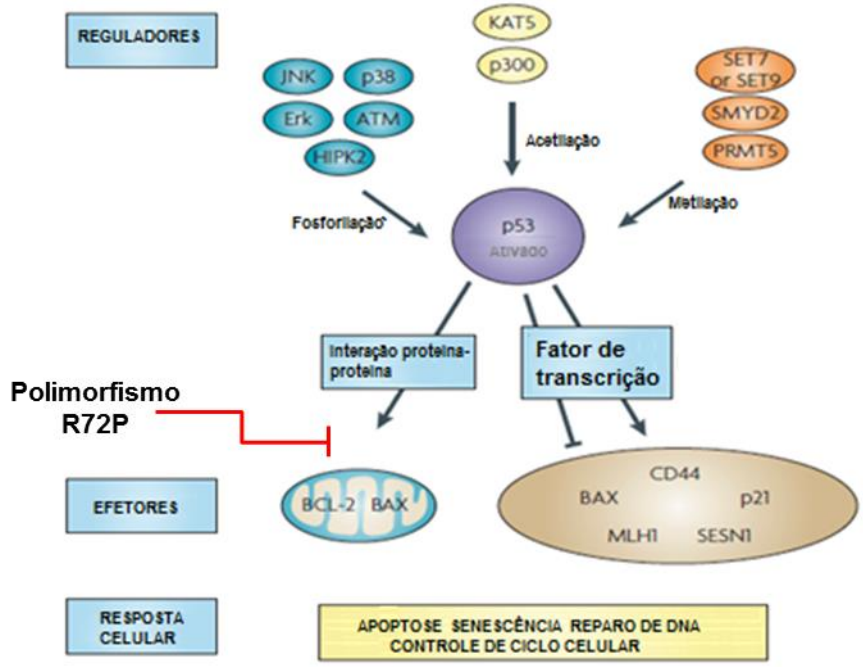
polimorfismos herdados, e o desenvolvimento da LMA. Em 2016, tal avanço resultou na criação pela OMS de novas entidades que englobam as LMAs com mutações germinativas, tais como nos genes *GATA2, RUNX1, CEBPA* e *ETV6* (ARBER *et al.*, 2016). Nesse contexto, alguns polimorfismos em genes envolvidos no ciclo celular, apoptose e metabolização de drogas, por exemplo, vêm sendo estudados não só no risco de desenvolvimento da LMA, como também na resposta clínica à quimioterapia convencional e ao transplante de medula óssea (CAO *et al.*, 2017; HJ *et al.*, 2011; KIM *et al.*, 2007; KOH *et al.*, 2012; EL-DANASOURI, 2014; ZHU *et al.*, 2018).

2.4 O GENE E A PROTEÍNA *TP53*

O gene *TP53* está localizado no braço curto do cromossomo 17 e recebe esse nome por codificar uma proteína de 53 kilodaltons, constituída por 393 aminoácidos que se distribuem em: domínio transativador N-terminal; domínio rico em prolina (PRD), domínio ligador de DNA (DBD), domínio de oligomerização(OD), sequências de localização nuclear e de exportação nuclear (BODE, 2004).

O *TP53* desempenha suas funções celulares através da indução ou repressão de genes que apresentem os chamados “elementos responsivos ao *TP53*” em sua região promotora (OLIVIER, 2010). Esses elementos já foram encontrados em mais de 100 genes humanos Dentre esses, estão incluídos genes envolvidos no ciclo celular, como o *CDKN1A* (p21), genes envolvidos na apoptose, como os *BBC3, BAX* e *PER,* genes envolvidos na inibição da angiogênese, como o THBS1, e os envolvidos na senescência celular, como o *PML* (BOND, 2005; RILEY *et al.*, 2008). Além de atuar como fator de transcrição, o *TP53* também interage diretamente com proteínas do ciclo celular, regulando-as e com a Bcl-2, aumentando a permeabilidade mitocondrial e por consequência, desencadeando os primeiros eventos da apoptose (LEU *et al.*,2004) (Figura 4).

Figura 4. Ativação e função do p53. O p53 sofre alterações pós-translacionais que o ativam e promovem sua interação com proteínas de ciclo celular, com o DNA e a membrana mitocondrial, para desempenhar suas diversas funções. O polimorfismo R72P atua na indução da apoptose na mitocôndria.



Adaptado de: OLIVIER, 2010.

Alterações no gene *TP53* ou em sua via é provavelmente o achado genético mais recorrente nos tumores malignos humanos, sendo encontradas em aproximadamente metade dos casos de alguns tipos tumorais. No câncer de ovário e colorretal, o *TP53* se encontra mutado em mais de 40% dos casos, enquanto que em tumores hematológicos e cervicais, a frequência cai para 12% e 6%, respectivamente (VOUSDEN, 2007).

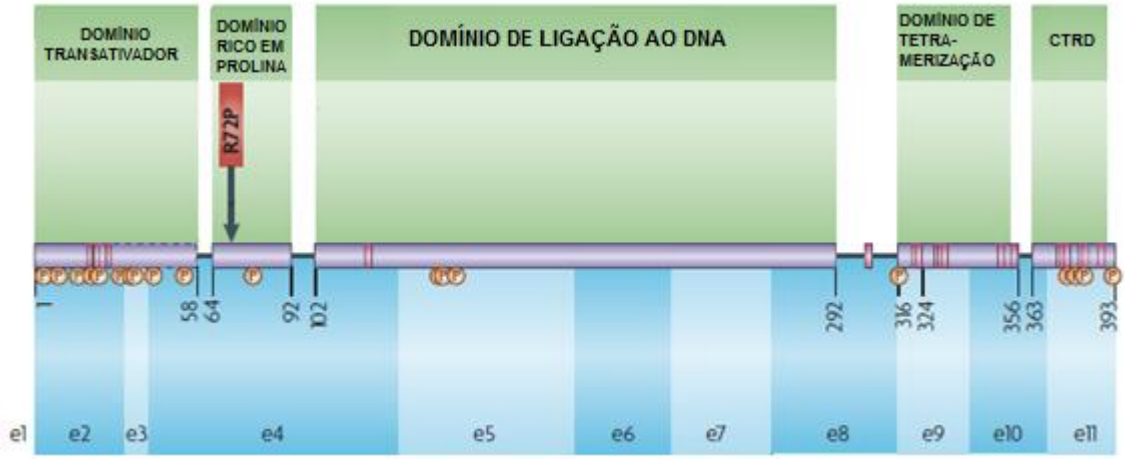
2.4.1 Polimorfismo *TP53* R72P

A grande maioria dos polimorfismos no gene *TP53* é sinônima (não altera a cadeia de aminoácidos) ou intrônica, estando situadas fora dos sítios de *splicing*, e parece não ter grande relevância médica. Entretanto, já foram identificados 11polimorfismos não-sinônimos, dos quais, o mais bem caracterizado funcionalmente e envolvido em doenças oncológicas é o R72P (*rs*1042522) (VOUSDEN, 2007).

O polimorfismo R72P consiste na troca de uma guanina na posição 2 do códon 72, éxon 4, por uma citosina, resultando na troca do resíduo de arginina por prolina. As variantes foram denominadas R72 ou ARG (arginina) e P72 ou PRO (prolina). O códon 72 está localizado no chamado domínio rico em prolina (PRD), o qual se situa entre o domínio

transativador N-terminal e o DBD (Figura 5). A função exata do PRD não está completamente elucidada. Entretanto, experimentos em linhagens celulares e em camundongos sugerem que o PRD é essencial para desencadear a apoptose frente a uma situação de estresse celular e tumorigênese (HRSTKA, 2009).

Figura 5. Localização do polimorfismo R72P no gene e na proteína *TP53.*



Fonte: adaptado de WHIBLEY *et al*., 2009.

Em 1999, Thomas e colaboradores realizaram a primeira comparação entre a atividade biológica das duas variantes para o códon 72, através de técnicas de transfecção transiente. Não foi observada diferença significativa na capacidade de ligação ao DNA, tampouco na sua função de fator de transcrição. Porém, foi visto que a forma R72 é mais eficaz em induzir a morte celular programada, chegando a induzir a apoptose cinco vezes mais rápido que a forma P72 (THOMAS *et al.*, 1999). Recentemente, Cabezas et al., ao transfectarem o polimorfismo *TP53* Arg72Pro na linhagem leucêmica Jurkat, demonstraram que a redução da capacidade apoptótica da variante P72 frente à exposição a agente citotóxico permitiu a sobrevivência de células com lesões no DNA, inclusive as anomalias cromossômicas t(15;17) e Del(5q), que são frequentemente descritas nas neoplasias mieloides (CABEZAS *et al.*, 2019).

A primeira comparação da atividade biológica em humanos das variantes P72 e R72 foi realizada em leucócitos. As células de pacientes homozigotos para R72, quando expostas a citarabina, um quimioterápico indutor de apoptose, desencadearam a apoptose em uma taxa muito maior que a da P72 (BONAFÈ *et al.*, 2002). Posteriormente, demostrou-se que a variante R72 é transportada em maior quantidade para o interior da mitocôndria, onde o *TP53* age diretamente sobre o citocromo C, causando a sua liberação no citoplasma e consequentemente, dando início à apoptose (DUMONT *et al.*, 2003). O transporte em maior

quantidade da forma R72 acontece porque o MDM2, molécula que realiza a exportação do *TP53* nuclear, apresenta maior afinidade pela R72 (MARCHENKO, 2000; WHIBLEY, 2009).

Há aproximadamente 20 anos, após uma publicação associando o polimorfismo P72 ao câncer cervical (STOREY *et al.*, 1998), deu-se início a uma busca intensiva de associação do R72P com tumores malignos, que continua até a atualidade. Foi encontrada associação com diversos tumores, entre eles, mamário, gástrico, hepático, pulmonar, entre outros (HABYARIMANA *et al.*, 2018; MULLER; VOUSDEN, 2014; OHAYON *et al.*, 2005; SAKIYAMA *et al.*, 2005; SIDDIQUE *et al.*, 2005). Apesar de alguns autores reportarem a associação do polimorfismo *TP53* R72P com o desenvolvimento d tumores hematológicos, a literatura a cerca das leucemias agudas ainda é insuficiente e contraditória, fazendo-se necessária a realização de estudos com maior número de casos e controles e em diferentes etnias, visto a diversidade na frequência do polimorfismo R72P entre diferentes populações (CABEZAS *et al.*, 2019; ELLIS *et al.*, 2008; WHIBLEY *et al.*, 2009).

3 **JUSTIFICATIVA**

Tendo em vista a considerável heterogeneidade clínica e biológica da LMA, é de grande importância a investigação de novos biomarcadores que possam refinar os algoritmos de prognóstico já existentes. Dessa forma, a proposta do presente estudo é, além de estudar isoladamente alterações moleculares com potencial prognóstico, propor um modelo que abranja a interação entre diferentes mutações. Ademais, a investigação de biomarcadores em uma coorte de pacientes brasileiros não selecionados traz resultados mais próximos da realidade de países em desenvolvimento, que diferem bastante dos ensaios clínicos realizados em países desenvolvidos.

4 MATERIAL E MÉTODOS

4.1 CASUÍSTICA

4.1.1 Pacientes com LMA

Os critérios de inclusão para pacientes com LMA foram: pacientes maiores de 18 anos, com suspeita diagnóstica de LMA, acompanhados na Fundação HEMOPE e Hospital do Câncer de Pernambuco entre 2012 e 30.05.2018. Já os critérios de exclusão foram: não confirmação do diagnóstico de LMA após análise de mielograma e imunofenotipagem, leucemia promielocítica aguda e pacientes que não foram submetidos a pelo menos um ciclo de quimioterapia de indução (o último somente para análise de sobrevida).

Amostras de medula óssea ou sangue periférico de 450 pacientes com suspeita de LMA foram coletadas durante a coleta de material para o diagnóstico na Fundação HEMOPE. Dos 450 pacientes, 100 pacientes foram posteriormente excluídos do estudo por serem diagnosticados com outras neoplasias hematológicas e 44 pacientes, com LPA. Dos 306 pacientes com diagnóstico confirmado de LMA, 79 não foram submetidos a nenhum ciclo de quimioterapia por motivos diversos, sendo portanto, excluídos do estudo (Figura 6).

O diagnóstico e classificação dos pacientes basearam-se nas características morfológicas, contagem de blastos medulares, imunofenotipagem, citoquímica e citogenética, segundo a classificação da OMS ou FAB. O tratamento consistiu em um ou dois ciclos de terapia de indução com 100 mg/m 2 /dia de arabinosídeo citosina (Ara-C) administrados por 7 dias com uma perfusão contínua e mais 60 mg/m 2 /dia de daunorrubicina durante 3 dias de tratamento. Para os pacientes que alcançaram remissão completa (RC) após a indução, a terapia pós-remissão consistiu de dois a quatro ciclos de consolidação com citarabina em altas doses, de 1 a 5 dias.

4.1.2 Grupo controle

Para o grupo de controles saudáveis, foram coletadas amostras de sangue periférico de 224 indivíduos saudáveis, sem antecedentes de doença oncológica ou hematológica, residentes na região metropolitana do Recife (média de idade de 51 anos, variando de 21-83 anos, sendo 55% do sexo feminino). Não foram incluídas nesse grupo, pessoas com parentesco próximo declarado.

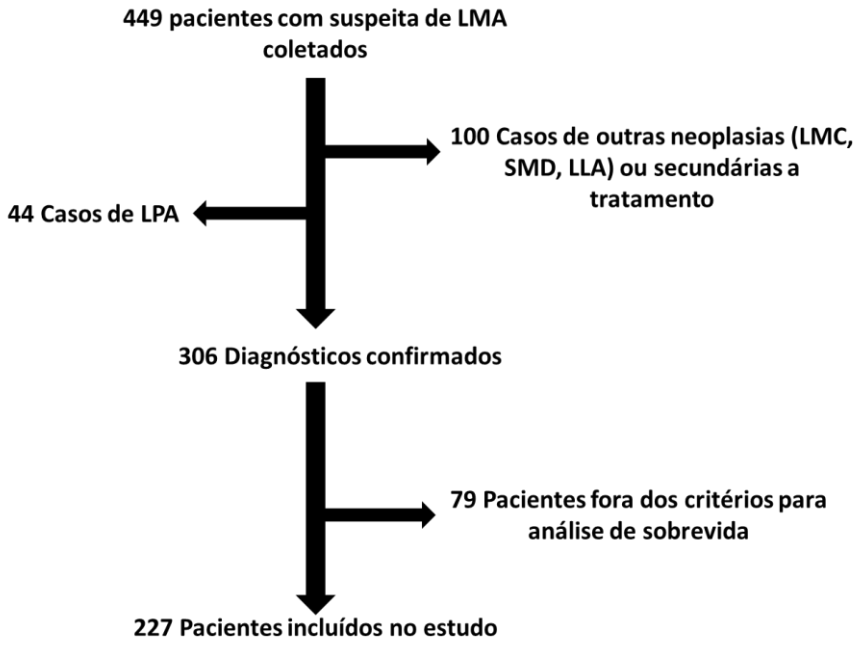


Figura 6. Critérios de inclusão e exclusão de amostras utilizados pelo estudo.

4.2 OPERACIONALIZAÇÃO DA PESQUISA E TÉCNICAS

4.2.1. Procedimentos de coleta de dados

A coleta de informações clínicas foi realizada através de consulta aos prontuários da Fundação HEMOPE e Hospital do Câncer de Pernambuco.

4.2.2. Extração de DNA

As amostras de sangue periférico e/ou medula óssea foram obtidas do material enviado para o serviço de diagnóstico do hospital, não tendo sido necessária coleta específica para a pesquisa. Para extração de DNA, foi realizada através do método de fenol-clofórmio descrito po Isola *et al*., (1994). Resumidamente, realiza-se a lise de hemácias pela adição de uma solução de cloreto de amônio e bicarbonato de amônio. Após centrifugação e descarte de sobrenadante, adiciona-se o tampão TKM1 (TRIS-HCl, KCl, EDTA, $MgCl_2$), TKM2 (TKM1 acrescido de NaCl 5M) e o agente surfactante dodecil sulfato sódico (SDS) para lise dos leucócitos. Após centrifugação, o sobrenadante é transferido para novo tubo onde se adiciona um preparo de clorofórmio/álcool isoamílico fenol (desnaturação de proteínas e isolamento do DNA). Após nova centrifugação e transferência do sobrenadante para novo tubo, adiciona-se acetato de sódio 3M e isopropanol gelado para precipitação do DNA. Após nova centrifugação, lava-se o precipitado de DNA com etanol 70% e centrifuga novamente. Após

descarte do etanol por inversão do tubo, deixa-se o precipitado de DNA secar por pelo menos 20 minutos e reidrata-se o DNA com deionizada.

Duas horas após a hidratação, as amostras foram quantificadas em NanoDrop 2000c (ThermoFischer Scientific).

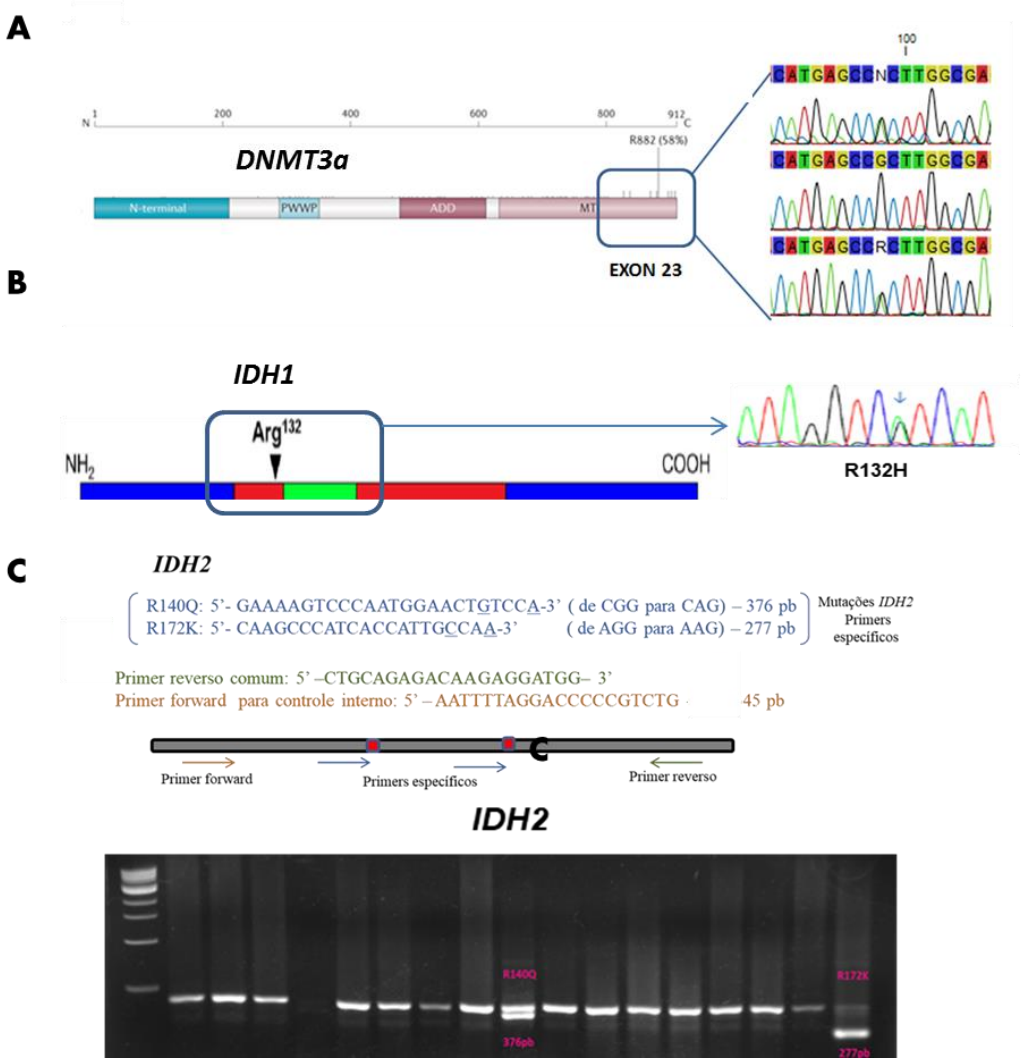
4.2.3 Pesquisa de mutações nos genes *IDH1, IDH2, DNMT3a, FLT3 e NPM1*

Para pesquisa de mutações no gene *DNMT3a* exon 23 e *IDH1* foi utilizado o sequenciamento bidirecional de Sanger e para o gene *IDH2,* PCR alelo-específica, estabelecido por ASHRAF *et al.*, 2013, onde se utiliza quatro primers: um *forward* e um *reverse* externos e dois primers alelo-específicos para as mutações R140Q e R172K (Figura 7).

Os primers utilizados para o sequenciamento do *DNMT3a* foram: *DNMT3A*-EXON23 FW 5'-GTGTGGTTAGACGGCTTCC-3' e *DNMT3A*-EXON23 RV 5'- CTCTCCCACCT TTCCTCTG–3', que flanqueiam a região de interesse, incluindo o códon R882. Já para o *IDH1*, foram utilizados os seguintes primers: *IDH1* FW 5'- TGAGAAGAGGGTTG AGGAGTTCAAGT–3' e *IDH1* RV 5'- AATGTGTTGAGA TGGACGCCTATTTGT–3'. Os produtos de PCR foram purificados com ExoSAP-IT™ PCR Product Cleanup Reagent (ThermoFischer Scientific) e as reações de sequenciamento foram preparadas com o BigDye® Terminator v3.1 Sequencing Standard Kit e aplicadas no ABI 3500 (Applied Biosystems). Para todos os procedimentos, foram seguidas as instruções do fabricante.

Seguindo protocolo previamente descrito (LIMA *et al*, , 2015), a pesquisa de mutações nos genes *FLT3* e *NPM1* foi realizada por PCR convencional, com primers flanqueando as regiões onde ocorrem as mutações do tipo inserção, com posterior confirmação dos casos positivos através de análise de fragmento.

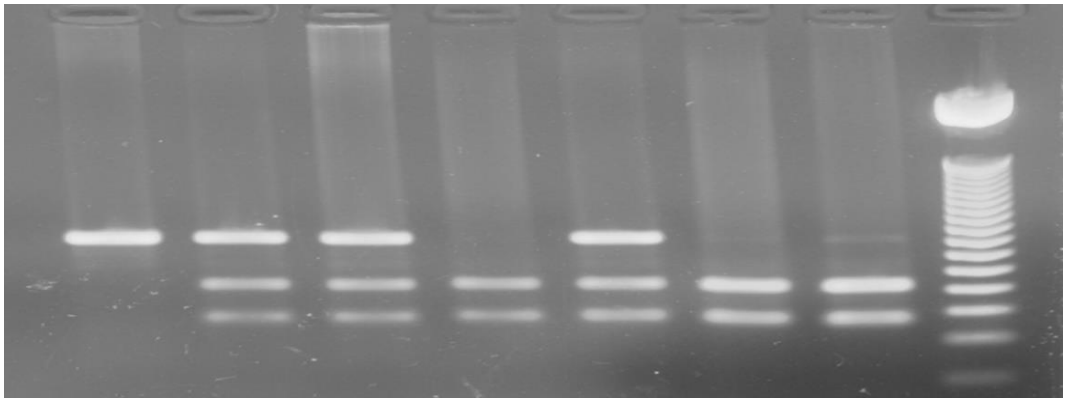
Figura 7. Pesquisa de mutações nos genes *DNMT3a*, *IDH1* e *IDH2*. Estratégias adotadas para a pesquisa de mutações no *DNMT3a* e *IDH1* por sequenciamento de Sanger (**A**-**B**). Sequência de primers e representação esquemática da estratégia de PCR multiplex alelo-específica, seguida de eletroforese em gel de agarose **(C)**.



4.2.4. Genotipagem do polimorfismo TP53 R72P

Para detecção do polimorfismo TP53 R72P, foi primeiramente realizada uma PCR com primers flanqueando a região do exon 4 (TP53 Exon 4 Fw: 5'-TGCTCTTTTCACCCATCTAC-3' e TP53 Exon 4 RV: 5'- ATACGGCCAG GCATTGAAGT-3') onde se localiza o polimorfismo. Em seguida, o produto de 353 pares de base da PCR foi digerido com a enzima BstuI (New England Biolabs), clivando exclusivamente o alelo R72 (ARG) em dois fragmentos menores de 214 e 139 pares de base. Os fragmentos de PCR foram então visualizados em gel de agarose 1,5% corado com brometo de etídio (Figura 8).

Figura 8. PCR-RFLP realizada com a enzima BstuI para detecção do TP53 R72P. Genótipo Pro/Pro: poço 1; genótipo Arg/Pro: poços 2, 3 e 5; Arg/Arg: poços 4, 6 e 7. Último poço: ladder: 50pb



4.3. VARIÁVEIS ANALISADAS E ANÁLISE ESTATÍSTICA

Além das mutações nos genes *DNMT3a*, *IDH1*, *IDH2* e do polimorfismo *TP53* R72P, foram coletados dados clínicos e laboratoriais, como : idade ao diagnóstico, sobrevida global, sobrevida livre de doença, taxa de remissão citológica (constatada por mielograma pós-indução com menos de 5% de blastos), subtipo FAB, leucometria, percentual de blastos na medula óssea, imunofenotipagem, citogenética e mutações nos genes *FLT3* e *NPM1*. A sobrevida global foi definida como intervalo de tempo em meses entre a data do diagnóstico e data do óbito ou censura. Já a sobrevida livre de doença foi definida como o tempo em meses entre a data de remissão completa e a data de recaída, óbito ou censura.

Para processamento dos testes estatísticos e confecção dos gráficos, foram utilizados os softwares SPSS Statistics versão 19, GraphPad Prism 5, R e as ferramentas Oncoprinter e MutationViwer disponibilizadas no www.cbioportal.org (acesso realizado em Abril/2018). Para as variáveis categóricas, aplicou-se o teste qui-quadrado bilateral ou teste de Fischer; para as variáveis contínuas o Teste de Mann-Whitney; para análise de sobrevida a Curva de Kaplan-Meier acoplado ao teste log-rank. Todos os testes foram considerados significativos quando $p<0,05$. As razões de risco (hazard ratios) uni e multivariadas foram determinadas pelos modelos de regressão proporcional de Cox e reportados com intervalos de confiança de 95%. No estudo de associação caso-controle, foram avaliados cinco modelos genéticos: frequência alélica, codominante, dominante, sobredominante e recessivo.

4.4 BANCO DE DADOS THE CANCER GENOME ATLAS

O banco de dados do TCGA inclui 200 pacientes com LMA, disponibilizando os dados clínicos, RNAseq, exoma, metilação do DNA, entre outros. Das 200 amostras, 20 casos de leucemia promielocítica aguda foram excluídos das análises, restando dessa forma, 180 pacientes do consórcio. Os dados foram obtidos através do website www.cbioportal.org (acesso realizado em Abril/2018). Esses dados foram utilizados para composição de coorte independente e análises de bioinformática (TCGA NETWORK, 2013). Tratamento dos pacientes foi baseado nas diretrizes propostas pelo National Comprehensive Cancer Network (NCCN) (TCGA NETWORK, 2013, O'Donnel, 2017).

4.5 QUESTÕES ÉTICAS

A coleta de amostras e dados clínicos foi iniciada após a aprovação do Comitê de Ética em Pesquisa em Seres Humanos do Centro de Ciências da Saúde da Universidade Federal de Pernambuco (CAAE 50987015.3.0000.5208 e 30229114.1.0000.5208). Os pacientes assinaram Termo de Consentimento Livre e Esclarecimento, autorizando a coleta de dados e a utilização de suas amostras biológicas na pesquisa.

5 RESULTADOS

5.1 Artigo 1

Co-occurrence of *DNMT3A*-R882 and *FLT3*/*NPM1* mutations identifies a subset of AML patients with particularly adverse prognosis

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Abstract

The use of new generation sequencing methods in AML research revealed new recurrent mutations, of which, those occurring in DNA-methylation modifiers, such as *DNMT3a*, *IDH1* and *IDH2* are found in 60% of AML patients. Despite been widely investigated as prognostic markers, the clinical relevance of these genes remains unclear and available data are somewhat conflicting. Here, we aimed to **(i)** evaluate the effect of *DNMT3a*, *IDH1* e *IDH2* mutations in two independent cohorts from Brazil (n=227) and The Cancer Genome Atlas (TCGA, n=180) and **(ii)** investigate the clinical repercussions of the cooperation between *DNMT3a* and *FLT3*/*NPM1* mutations in AML. Although *IDH1* and *IDH2* mutations were associated with specific features such as advanced age, *NPM1* mutations (*IDH2*), FAB M1 subtype, normal karyotype (*IDH1*), there was no impact of these mutations on patient's outcome. *DNMT3a* R882 (hotspot) mutations, when compared to non-R882 and wildtype *DNMT3a*, were associated with monocytic subtypes of AML, higher leukocyte counts, normal karyotype and more importantly, *FLT3* and *NPM1* mutations. RNAseq data revealed that *DNMT3a*-mutated patients form different clusters according to the presence of these partner mutations. Based on these results, patients harboring *DNMT3a* R882 mutations with at least one partner mutation in *FLT3* or *NPM1* were grouped into a composite variable (R882/*FLT3*/*NPM1)* for survival analysis. Patients assigned to R882/*FLT3*/*NPM1* had shorter survival rates in both cohorts. Results were also reproduced when limited to normal-karyotype patients. The composite variable had stronger survival prediction power than individual analysis of *DNMT3a*, *FLT3* or *NPM1* and was independent of age, leukocyte count or cytogenetic risk in the multivariate analysis. In summary, by transposing this biological concept into a model for outcome prediction, we show that the well-recognized cooperation of *DNMT3a* with *FLT3* and *NPM1* identifies a subset of AML with adverse prognosis more efficiently than the classical analysis of these mutations individually.

Keywords: *IDH1*, *IDH2*, *DNMT3a* mutations, AML prognosis

1 Introduction

The last two decades of research in acute myeloid leukemia (AML) were characterized by outstanding advancements in the comprehension of its molecular landscape, enabling a better understanding of AML physiopathology and the refinement of molecular-based prognostication algorithms (DÖHNER *et al.*, 2017; MEDINGER, 2016). In this context, new generation sequencing methods revealed recurrent mutations in AML patients, of which, those occurring in DNA-methylation modifiers, such as DNA methyltransferase 3a (*DNMT3a),* isocitrate dehydrogenase 1 (*IDH1) and 2 (IDH2)* are found in 60% of AML patients (TCGA NETWORK, 2013).

DNMT3A mutations occur in 22% of adult AML patients and are associated with a higher age, monocytic subtypes and hyperleukocytosis (LEY, 2010). They are more commonly found in the hotspot at codon R882, however, less common mutations of unknown biochemical effects were identified in other sites of the enzyme (IM, 2014). R882 mutations had been shown to be an early event in leukemogenesis, causing impaired DNA methylation and consequently, aberrant methylation and gene expression profiles (FERRANDO, 2017; JEONG, 2018; SHLUSH *et al.*, 2014; YAN *et al.*, 2011). results from population-based and functional studies suggest that, although mutated *DNMT3a* cause clonal hematopoiesis, a second mutation in genes such as *FLT3* or *NPM1* are necessary to develop AML (GURYANOVA *et al.*, 2017; BUSCARLET, 2017; MEYER *et al.*, 2016; VAN DEN AKKER *et al.*, 2019; YANG, 2016).

IDH1 and *IDH2* are important enzymes from the Krebs circle that catalyze isocitrate in alpha-ketoglutarate. Approximately 20% of AML patients carry gain-of-function mutations in these genes cause a neomorphic function: metabolizing alpha-ketoglutarate into D-2-hydroxiglutarate (D-2HG). High levels of D-2HG inhibits DNA demethylases, such as TET2, culminating in a hypermethylated DNA with abnormal gene transcription (LIN *et al.*, 2018).

Despite widely investigated, *DNMT3a*, *IDH1* and *IDH2* role on AML prognosis remains unclear and current data is somewhat conflicting (GAIDZIK *et al.*, 2013; YUAN *et al.*, 2016). Here, we evaluated the impact of these mutations on survival in two independent cohorts. Led by the concept that mutated *DNMT3a* interplays with cooperative mutations, such as *FLT3* or *NPM1* to develop a fully leukemic phenotype, we propose that the interaction between *DNMT3a* and *FLT3*/*NPM1* is potentially a more powerful prognosis predictor than single mutational status of each gene alone.

2 Methods

2.1 Patients

Two hundred twenty seven adult patients diagnosed with *de novo* AML followed from 2012 to 2018 in a single reference center in Recife, northeast Brazil were included. Additionally, 180 patients from the The Cancer Genome Atlas (TCGA) databank were included as an independent cohort. Acute promyelocytic leukemia (AML-M3) patients were not included in the study. Upon clinical evidence of acute leukemia, diagnosis was performed by combining bone marrow cytology, immunophenotyping, cytochemistry and karyotyping.

Patients from the Brazilian cohort were treated with daunorubicin (45-90 mg/m^2/d for 3 days) and cytarabine (100-200 mg/m^2/d for 7 days) induction, followed by three or four cycles of consolidation therapy with cytarabine in high doses (above 1 g/m^2/d). For patients who did not achieve complete remission (CR) after one course of chemotherapy, a second course was administered between days 28 and 35 after the end of the first course. CR was assessed by bone marrow examination on day 28 after each course of chemotherapy. For patients older than 60 years, the treatment protocol was adapted according to performance status and the presence of comorbidities. Patients from the TCGA cohort were treated according to the National Comprehensive Cancer Network (NCCN) recommendations (TCGA NETWORK, 2013). Genomic, transcriptomic and clinical information from the TCGA cohort were obtained at the website www.cbioportal.org.

In accordance with the Declaration of Helsinki, informed consent for the use of genomic and clinical information was obtained from all patients. Ethical approval was obtained from the local research ethics board (CAAE 50987015.3.3001.5205).

2.2 Investigation of *DNMT3a, IDH1, IDH2, NPM1* and *FLT3* mutations

For molecular analysis, diagnostic samples from bone marrow or peripheral blood (only when blasts in peripheral blood were > 20%) from 227 adult patients were collected. Following DNA extraction, *DNMT3a* exon 23 (flanking R882 region) and *IDH1* mutations were detect by direct Sanger sequencing (ABI 3500 sequencer and BigDye Terminator v3.1, Applied Biosystems), whereas *IDH2* mutations were identified by allele-specific PCR, as described by Ashaf et al., 2013.

Primer set for detecting *DNMT3a* mutations were: *DNMT3A*-EXON23 FW 5'-GTGTGGTTAGACGGCTTCC-3' and *DNMT3A*-EXON23 RV 5'- CTCTCCCACC TTTCCTCTG–3', and for *IDH1*: *IDH1* FW 5'- TGAGAAGA

GGGTTGAGGAGTTCAAGT–3’ and *IDH1* RV 5’- AATGTGTTGAGA TGGACGCCTATTTGT–3’. *FLT3* and *NPM1* mutations were also performed with a previously described method (LIMA *et al*, , 2015).

2.3 Variables and statistical analysis

The following variables were evaluated: *DNMT3a*, *IDH1*, *IDH2*, *FLT3* and *NPM1* mutations, overall survival (OS) and disease-free survival (DFS), age at diagnosis, gender, FAB subtype, leukocytes count, karyotype and immunophenotyping. OS was defined as the amount of time from diagnosis to death and DFS, the time from cytological remission (bone marrow blasts < 5%) to relapse or death.

Categorical variables were analyzed by Pearson’s chi-square or Fischer’s exact test, while Mann-Whitney test was used for continuous variables. OS and DFS were estimated using Kaplan-Meier and comparisons were evaluated by log-rank test. Hazard ratios were determined by Cox’s proportional regression. All statistical analysis were reported with a 95% confidence interval ($p<0,05$). Data analysis was performed with GraphPad Prism 5 and R program for Windows, version 3.2.2 (2015 R Foundation for Statistical Computing). For illustrating patient’s mutational profile, Oncoprinter and MutationViewer tool (available at www.cbioportal.org) were used.

3 Results

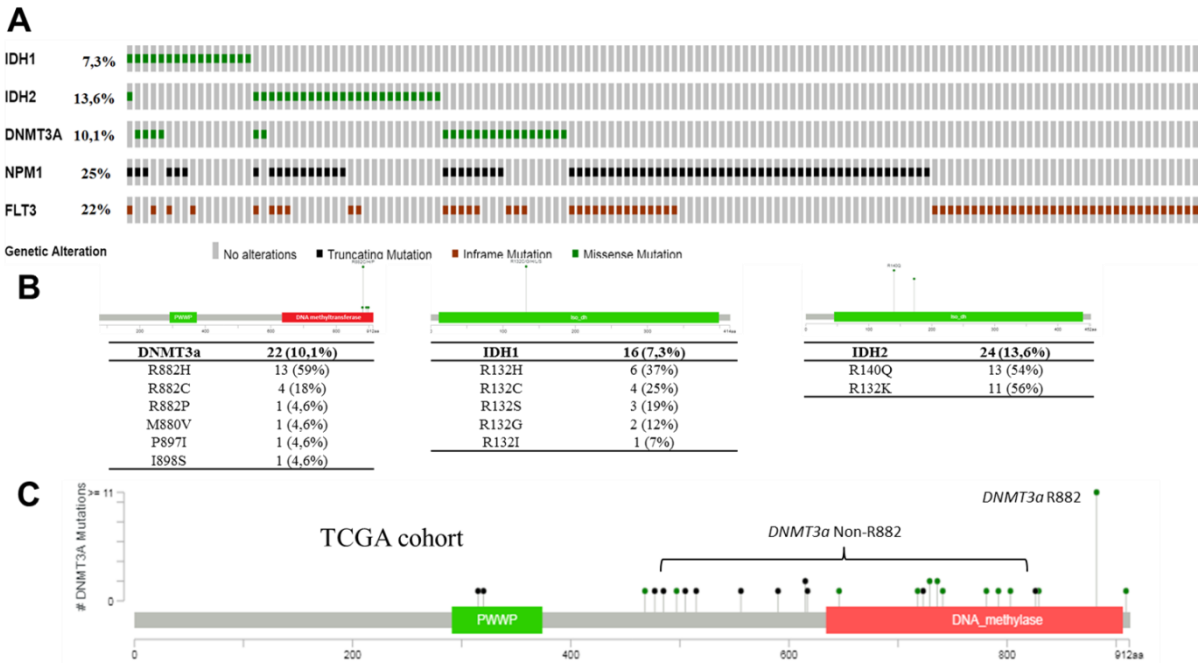
3.1 Cohort features

From the 227 patients from the Recife cohort, 118 (52%) were females. Average age at diagnosis was 49,5 years, ranging from 18 to 93 years. Average hemoglobin concentration at diagnosis was 7,89g/dL (3,1-16,0g/dL), average WBC was 68.768400 cells/µL (490 – 435.400 cells/µL) and platelets count was 75.000 (2.000 – 465.000 cells/µL). Average bone marrow blast count was 60%, ranging from 21% to 97%. Lactic dehydrogenase average serum levels were 923 U/L (116 - 4.192 U/L).

From the 227 patients, 172 received complete 3+7 protocol treatment and checked for cytological remission 28 days after chemotherapy, whereas the remaining 55 died during treatment or before remission assessment (usually due to opportunist infections during aplasia). Cytologic remission was achieved in 107 (62%) of those who survived chemotherapy and following aplasia. Estimated OS in three and five years were, respectively, 19% and 14%, while estimated DFS in three and five were 30% and 24%.

3.2 *DNMT3A*, *IDH1* and *IDH2* mutations

In patients from the Recife cohort, *DNMT3a* R882 mutations were identified in 22/218 (10,2%) patients, *IDH1* mutations in 16/220 (7,3%) e and *IDH2* in 24/177 (13,6%). All mutations were previously reported (PAPAEMMANUIL *et al.*, 2016) and *IDH1*/2 were mutually exclusive among each other, while *DNMT3a* co-occurred with *NPM1* and *FLT3* (Figure 1). When compared to the Recife cohort, TCGA cohort had a slightly higher proportion of *DNMT3a* R882 (28/180 = 15%) and *IDH1* mutations (19/180 = 10%).



DNMT3a	22 (10,1%)
R882H	13 (59%)
R882C	4 (18%)
R882P	1 (4,6%)
M880V	1 (4,6%)
P897I	1 (4,6%)
I898S	1 (4,6%)

IDH1	16 (7,3%)
R132H	6 (37%)
R132C	4 (25%)
R132S	3 (19%)
R132G	2 (12%)
R132I	1 (7%)

IDH2	24 (13,6%)
R140Q	13 (54%)
R132K	11 (56%)

Figure 1. Mutational profile of AML patients. Distribution (A), frequency and type (B) of *DNMT3a*, *IDH1* and *IDH2* mutations in cohort Recife. (C) DNMT3a mutations in 180 AML patients from the TCGA cohort.

3.3 Association of *IDH1* and *IDH2* with clinical-laboratorial features and survival

In both cohorts, *IDH1* mutations were significantly more prevalent in FAB M1 AML subtype. However, only in Recife cohort *IDH1* mutations were associated with normal karyotype (NK). We found no associations of *IDH1* mutations with immunophenotypic features or WBC (Table 1). On the other hand, *IDH2* mutations were associated with a higher age at diagnosis in both cohorts, but associated with *NPM1* mutations only in Recife cohort. Additionally, an association with loss of the HLA-DR antigen was also observed (Table 2).

No significant differences were observed for *IDH1* mutations in survival analysis for both cohorts. In the Recife cohort, estimated 3-year OS rate for mutated and wildtype *IDH1* were, respectively 8% and 15% (p=0,172), whereas DFS rates were 20% and 21%, respectively (p=0,559). In a similar trend, estimated 3-year OS rate for mutated and wildtype *IDH1* in the TCGA cohort were, respectively 38% and 30% (p=0,310) and estimated 3-year DFS rate were 32% and 31%, respectively (p=0,542).

Likewise, there was no significant impact regarding *IDH2* status in both Recife cohort (estimated 3-year OS rate for mutated *IDH2*: 22%, wildtype *IDH2*: 18%, p=0,213; DFS rate: mutated *IDH2*: 41%, wildtype *IDH2*: 18%, p=0,918) and TCGA cohort (estimated 3-year OS rate for mutated *IDH2*: 23%, wildtype *IDH2*: 32%, p=0,923; DFS rate: mutated *IDH2*: 41%, wildtype *IDH2*: 18%, p=0,587)(Figure 2). Further analysis restricted to normal karyotype patients was also performed, but results were similar to those observed in the full cohorts (data not shown).

Table 1. Association of IDH1 mutations with clinical and laboratorial features

IDH1	**Cohort Recife**			**Cohort TCGA**		
	Mut (%)	**Wt (%)**	***p*-value**	**Mut (%)**	**Wt (%)**	***p*-value**
Age			1,000			0,342
>60	5 (7)	72 (93)		7 (8)	79 (92)	
<60	10 (7)	127 (93)		12 (13)	82 (87)	
Gender			0,801			0,628
Male	7 (7)	97 (93)		9 (9)	89 (91)	
Female	9 (8)	107 (92)		10 (12)	72 (88)	
FAB subtype			0,002			0,020
M0	0 (0)	13 (100)		2 (11)	17 (89)	
M1	5 (18)	23 (82)		12 (26)	34 (74)	
M2	5 (7)	64 (93)		2 (5)	42 (95)	
M4	5 (8)	58 (92)		2 (5)	39 (95)	
M5	0 (0)	17 (100)		1 (4)	21 (96)	
M6	0 (0)	5 (100)		0 (0)	3 (100)	
M7	1 (100)	0 (0)		0 (0)	3 (100)	
Cytogenetic risk			0,112			0,248
Favourable	0 (0)	21 (100)		0 (0)	19 (100)	
Intermediate	9 (16)	46 (84)		13 (12)	100 (88)	
Adverse	1 (7)	13 (93)		6 (14)	37 (86)	
NK-AML			0,046			0,810
Yes	8 (18)	36 (82)		10 (11)	77 (89)	
No	2 (4)	45 (96)		9 (10)	82 (90)	
NPM1			0,556			0,110
Mutated	5 (9)	50 (91)		9 (17)	45 (83)	
Non-mutated	11 (7)	153 (93)		10 (8)	116 (92)	
FLT3-ITD			0,753			0,596
Mutated	4 (9)	41 (91)		4 (8)	46 (92)	
Non-mutated	12 (7)	152 (93)		15 (12)	115 (88)	
WBC			0,669			0,698
>100.000	2 (13)	13 (87)		1 (5)	18 (95)	
<100.000	6 (11)	51 (89)		18 (11)	143 (89)	
CD34			0,361			
Positive	12 (10)	120 (90)		-	-	-
Negative	0 (0)	17 (100)		-	-	-
HLA-DR			0,199			
Positive	9 (7)	122 (93)		-	-	-
Negative	3 (6)	17 (94)		-	-	-

Table 2. Association of *IDH2* mutations with clinical and laboratorial features. Mut: mutated; Wt: wildtype ;NK-AML: normal karyotype AML.

IDH2	**Cohort Recife**			**Cohort TCGA**		
	Mut (%)	**Wt (%)**	***p*-value**	**Mut (%)**	**Wt (%)**	***p*-value**
Age			0,009			0,003
>60	14 (23)	46 (77)		16 (19)	70 (81)	
<60	9 (8)	101 (92)		4 (4)	90 (96)	
Gender			0,520			0,813
Male	10 (12)	75 (88)		10 (10)	88 (90)	
Female	14 (15)	78 (85)		10 (12)	72 (88)	
FAB subtype			0,329			0,325
M0	2 (14)	12 (86)		4 (21)	15 (79)	
M1	3 (11)	25 (89)		8 (17)	38 (83)	
M2	12 (22)	43 (78)		5 (11)	39 (89)	
M4	5 (11)	42 (89)		3 (7)	38 (93)	
M5	0 (0)	17 (100)		0 (0)	22(100)	
M6	0 (0)	3 (100)		0 (0)	3 (100)	
M7	-	-		0 (0)	3 (100)	
Cytogenetic risk			0,278			0,064
Favourable	1 (5)	19 (95)		0 (0)	19(100)	
Intermediate	4 (9)	40 (91)		16 (14)	97 (86)	
Adverse	3 (21)	11 (79)		2 (5)	41 (95)	
NK-AML			0,710			0,471
Yes	4 (13)	28 (86)		11 (13)	76 (87)	
No	4 (9)	42 (91)		8 (9)	83 (91)	
NPM1			0,005			0,438
Mutated	11 (29)	27 (71)		4 (7)	50 (93)	
Non-mutated	13 (9)	126 (91)		16 (13)	110(87)	
***FLT3*-ITD**			0,143			0,067
Mutated	7 (23)	23 (77)		2 (4)	48 (96)	
Non-mutated	17 (12)	125 (88)		18 (14)	112(86)	
WBC			0,139			0,700
>100.000	5 (36)	9 (64)		1 (5)	18 (95)	
<100.000	9 (16)	46 (84)		19 (12)	142(88)	
CD34			0,371	-	-	-
Positive	16 (14)	98 (86)				
Negative	3 (27)	8 (73)				
HLA-DR			0,004	-	-	-
Positive	12 (11)	97 (89)				
Negative	7 (41)	10 (59)				

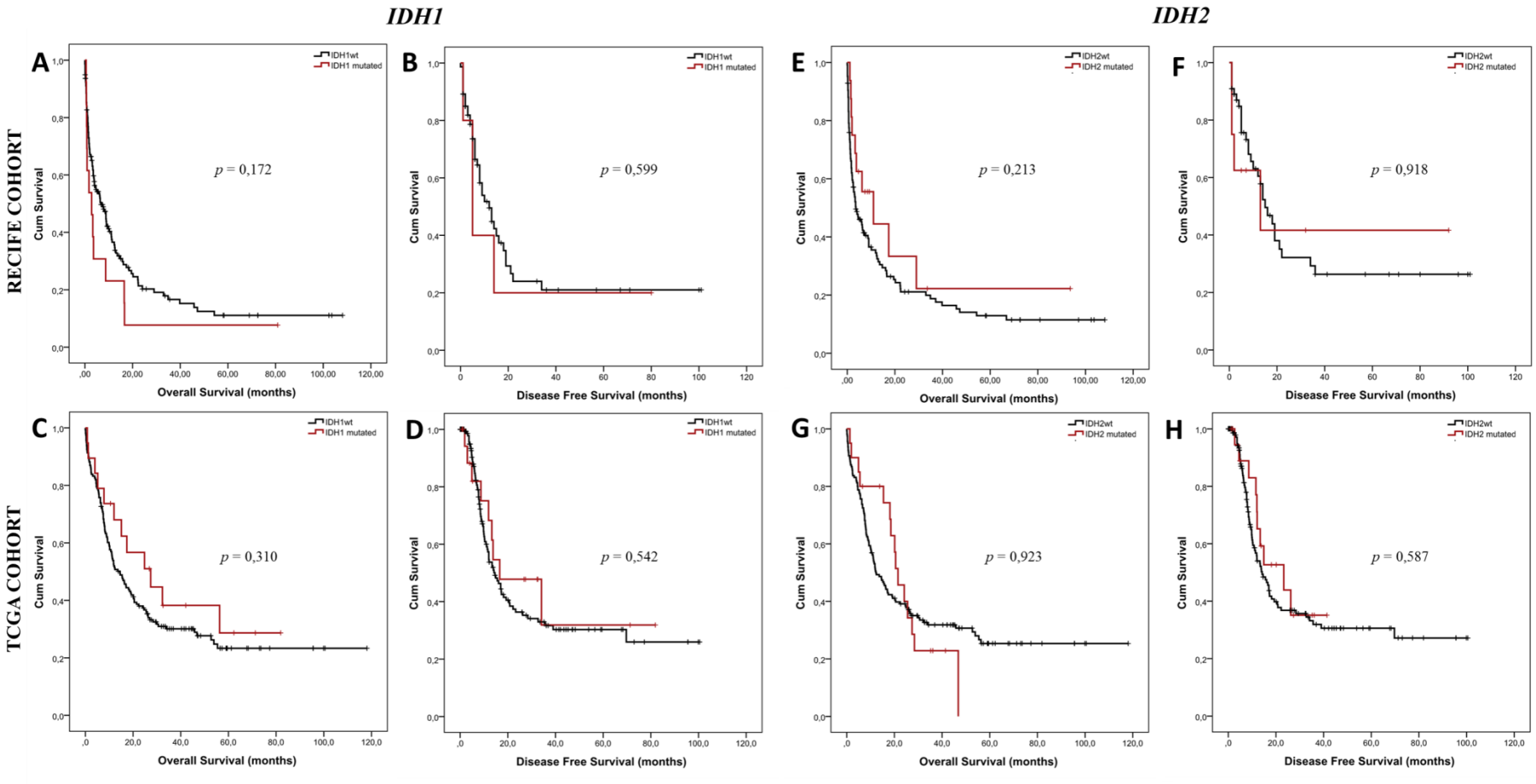


Figure 2. Survival analysis for *IDH1/2* mutations. OS and DFS from the Recife cohort (A, B, E, F) and TCGA cohort (C, D, G, H) according to IDH1/2 mutations.

3.4 Association of *DNMT3a* R882 mutations with clinical-laboratorial features and survival

DNMT3a R882 mutations were significantly more prevalent in normal karyotype AML and consequently, in the intermediate group for karyotypic risk. Additionally, these mutations were associated with the presence *FLT3* and *NPM1* mutations and hyperleukocytosis (WBC>100.000 cells/µL). R882 mutated patients had also an increased frequency of AML monocytic subtypes (FAB M4 and M5) (Table 3).

Survival analysis indicates that *DNMT3a* R882 mutated patients tended to have shorter survival, but it did not reached statistical significance (Figure 3). Estimated 3-year OS rates for mutated and wildtype *DNMT3a* R882 in the Recife cohort were, respectively 6% and 19% (p=0,180) while estimated 3-year DFS rates were 13% and 34% (p=0,199). Similar results were observed in the TCGA cohort as estimated 3-year OS rates for mutated and wildtype *DNMT3a* R882 were, respectively 21% and 32% (p=0,105) and estimated 3-years DFS rates were 21% and 34%, respectively (p=0,225).

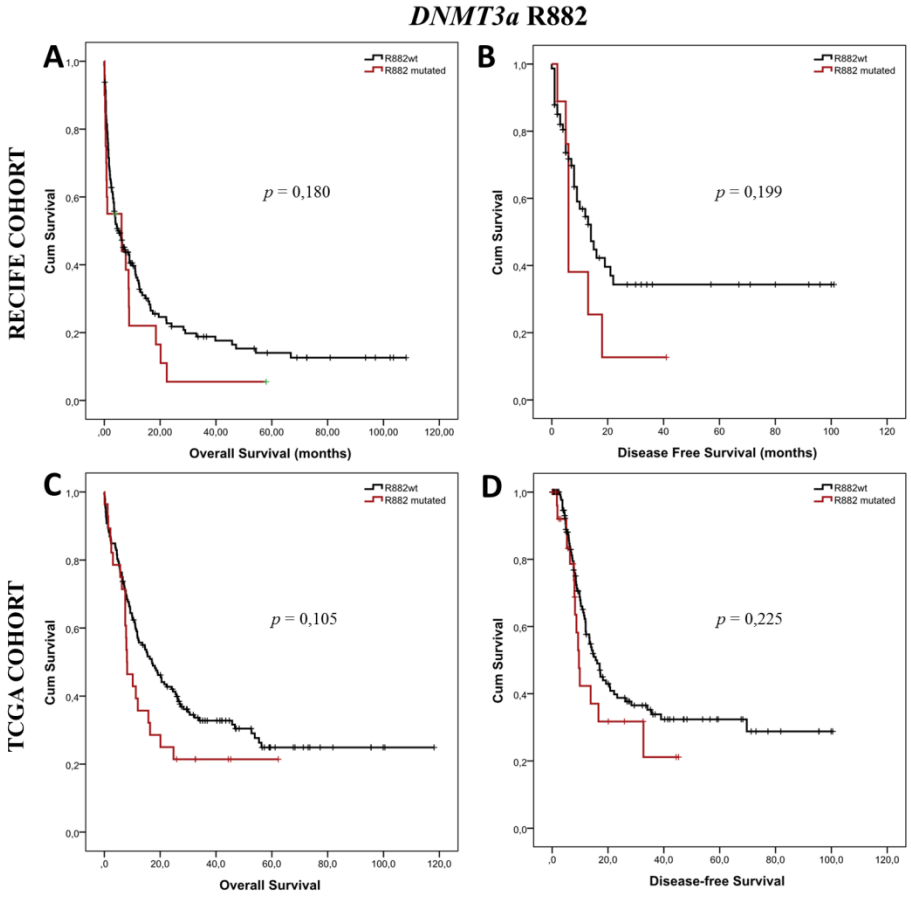


Figure 3. Survival analysis for *DNMT3a* R882 mutations. OS and DFS from the Recife cohort (A and B,) and TCGA cohort (C and D) according to *DNMT3a* R882 mutations.

Table 3. Association of *DNMT3a* R882 mutations with clinical and laboratorial features. Mut: mutated; Wt: wildtype ;NK-AML: normal karyotype AML.

***DNMT3a* R882**	**Cohort Recife**			**Cohort TCGA**		
	Mut (%)	**Wt (%)**	***p*-value**	**Mut (%)**	**Wt (%)**	***p*-value**
Age			0,180			0,411
>60	8 (11)	64 (89)		17 (18)	77 (82)	
<60	8 (6)	128 (94)		11 (13)	69 (87)	
Gender			0,328			0,411
Male	6 (6)	94 (94)		13 (13)	85 (87)	
Female	12 (10)	106 (90)		15 (18)	67 (82)	
FAB subtype			0,406			0,075
M0	1 (6)	14 (94)		1 (5)	18 (95)	
M1	1 (4)	26 (96)		5 (11)	41 (89)	
M2	5 (7)	65 (93)		5 (11)	39 (89)	
M4	9 (15)	52 (85)		9 (22)	32 (78)	
M5	1 (6)	16 (94)		8 (36)	14 (64)	
M6	1 (25)	3 (75)		0 (0)	3 (100)	
M7	-	-		0 (0)	3 (100)	
Cytogenetic risk			0,12			0,033
Favourable	0 (0)	23 (100)		0 (0)	19 (100)	
Intermediate	9 (15)	53 (85)		23 (20)	90 (80)	
Adverse	1 (6)	15 94)		4 (9)	25 (91)	
NK-AML			0,007			0,021
Yes	9 (18)	40 (82)		19 (22)	68 (78)	
No	1 (2)	51 (98)		8 (9)	74 (91)	
NPM1			0,018			<0,0001
Mutated	9 (17)	44 (83)		19 (35)	35 (65)	
Non-mutated	9 (5)	156 (95)		9 (7)	105 (93)	
***FLT3*-ITD**			0,011			0,01
Mutated	8 (18)	37 (82)		14 (28)	29 (72)	
Non-mutated	9 (5)	160 (95)		14 (11)	116 (89)	
WBC			0,006			0,085
>100.000	4 (25)	12 (75)		6 (32)	13 (68)	
<100.000	1 (2)	59 (98)		22 (14)	139 (86)	
CD34			0,193			
Positive	14 (11)	114 (89)		-	-	-
Negative	3 (23)	10 (77)		-	-	-
HLA-DR			0,702			
Positive	16 (13)	109 (87)		-	-	-
Negative	3 (16)	16 (84)		-	-	-

3.5 *DNMT3A* R882 has distinct molecular and clinical features from *DNMT3a* non-R882 mutations

In the TCGA cohort, 28 (15%) patients had the hotspot *DNMT3a* R882 mutations (R882H, R882C and R882P) while 21 (11%) had non-R882 mutations, which are dispersed in other *DNMT3a* domains. In order to address whether these classes of mutations have different effects from each other, we evaluated its mutational landscape and clinical features that are usually associated with *DNMT3a* mutations.

Striking differences were found regarding the molecular profile of each class of *DNMT3a* mutation: while mutated *NPM1* and *FLT3* (and the co-occurrence *NPM1*/*FLT3*) were significantly more frequent in the R882 group, *IDH2* and NRAS mutations were more frequent in the non-R882 group. The fact that half of R882 patients are mutually positive for *FLT3* and *NPM1* (observed in both cohorts), points towards a strong synergism between these three mutations.

Additionally, the R882 group had a higher occurrence of NK-AML than non-R882, although both were higher than *DNMT3a* wildtype. With regard to the clinical features, R882 mutations were associated with monocytic subtypes (M4 and M5) and higher WBC, whereas non-R882 individuals were similar to those with wildtype *DNMT3a* (Figure 4 A-B).Both R882 and non-R882 mutations were mutually exclusive to chromosomal rearrangements such as *CBFB-MYH11*, *RUNX1-RUN1T1*.

Based on the evident co-occurrence of *DNMT3a* and *FLT3/NPM1* mutations, we compared RNAseq data from patients with only *DNMT3a* mutation to those carrying *DNMT3a* plus *FLT3* and/or *NPM1*. Principal component analysis (PCA) indicates distinct clusters for each group, suggesting that the gain of the second mutation interacts with mutated DNMT3a, transforming its gene expression signature (Figure 4C).

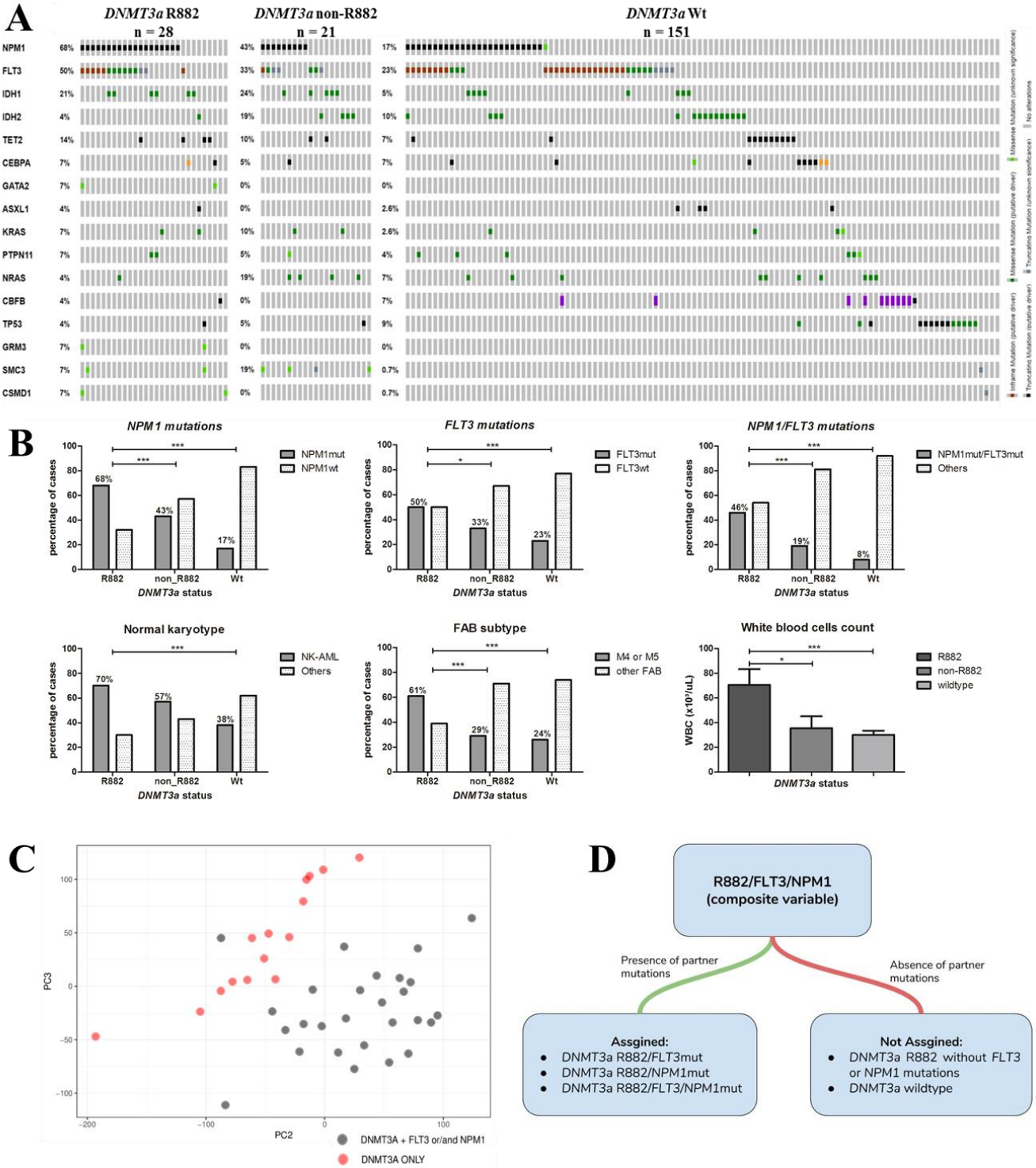


Figure 4. Clinical and biological aspects of *DNMT3a* mutations. Mutational landscape of TCGA AML patients according to *DNMT3a* status reveals that *FLT3* and *NPM1* are enriched in the *DNMT3a* R882 group, while IDH2 and NRAS are enriched in the non-R882 group (**A** and **B**). Normal karyotype, as well as monocytic FAB subtypes and higher WBC are more common in *DNMT3a* R882 patients than non-R882 or *DNMT3a* wildtype (**B**). Principal Component Analysis (PCA) from RNAseq data shows that *DNMT3a* forms distinct clusters depending on the presence of the partner mutations in *FLT3* or *NPM1* (**C**). Criteria for patients inclusion in the R882/*FLT3*/*NPM1* composite variable (**D**).

3.6 Prognostic impact of *DNMT3a* R882, *FLT3* and *NPM1* cooperation

Together with current results, solid data from literature indicate the existence of a specific leukemic entity that is defined not by harboring a *DNMT3a* R882 mutation alone, but by its combination with the partner mutations *FLT3* and *NPM1*, with unique mutational, gene expression and DNA methylation signatures (GURYANOVA *et al.*, 2017; MEYER *et al.*, 2016; TCGA NETWORK, 2013; RIBEIRO *et al.*, 2012; YANG *et al.*, 2016). To address whether the cooperation among these three genes also have prognostic relevance, we created a composite variable, here named "R882/*FLT3*/*NPM1*" that includes R882-positive patients with at least one partner mutation (R882mut/*FLT3*mut or R882mut/*NPM1*mut or R882mut/*FLT3*mut/*NPM1*mut) (Figure 4D).

In the Recife cohort, patients assigned to the R882/*FLT3*/*NPM1* group (n=14) had a notably poorer outcome, with an estimated 3-year OS rate of 1% (all deceased), while patients not included in the composite variable (n=190) had estimated 3-year OS rate of 20% (p=0,002; HR: 2,44 (CI95%: 1,35-4,44, p=0,003)). In DFS analysis, patients included in R882/*FLT3*/*NPM1* (n=5) had an estimated 3-year OS rate of 1% (all relapsed/deceased), while patients not included in the composite variable (n=90) had estimated 3-year OS rate of 31% (p=0,086, HR: 2,1 (CI95%: 0,74-5,92, p=0,163)). These finding were validated in the TCGA cohort, as individuals assigned in the R882/*FLT3*/*NPM1* group (n=20) had an estimated 3-year OS rate of 15%, while patients not included in the composite variable (n=160) had estimated 3-year OS rate of 33% (p=0,021, HR: 1,83 (CI95%: 1,09-3,06, p=0,022)). Estimated 3-year DFS rate for the TCGA R882/*FLT3*/*NPM1* group was 16%, while for those not assigned in R882/*FLT3*/*NPM1*, was 34% (p= 0,018, HR: 2,06 (CI95%: 1,11-3,81, p=0,021) (Figure 5 A-D).

Remarkably, the negative effect of R882/*FLT3*/*NPM1* remained significant after adjustment for age, cytogenetic risk stratification, and WBC as cofounders in multivariate analysis (Table 4). Due to the well-known clinical heterogeneity of normal karyotype patients, we also restricted survival analysis to this group (Figure 5 E-F). Furthermore, the lack of impact of *NPM1*, *FLT3* and *DNMT3a* R882 mutations on survival in Cox's regression for univariate analysis suggest that the negative effect of R882/*FLT3*/*NPM1* is not caused by individual effects from any of the studied genes. (Table 4).

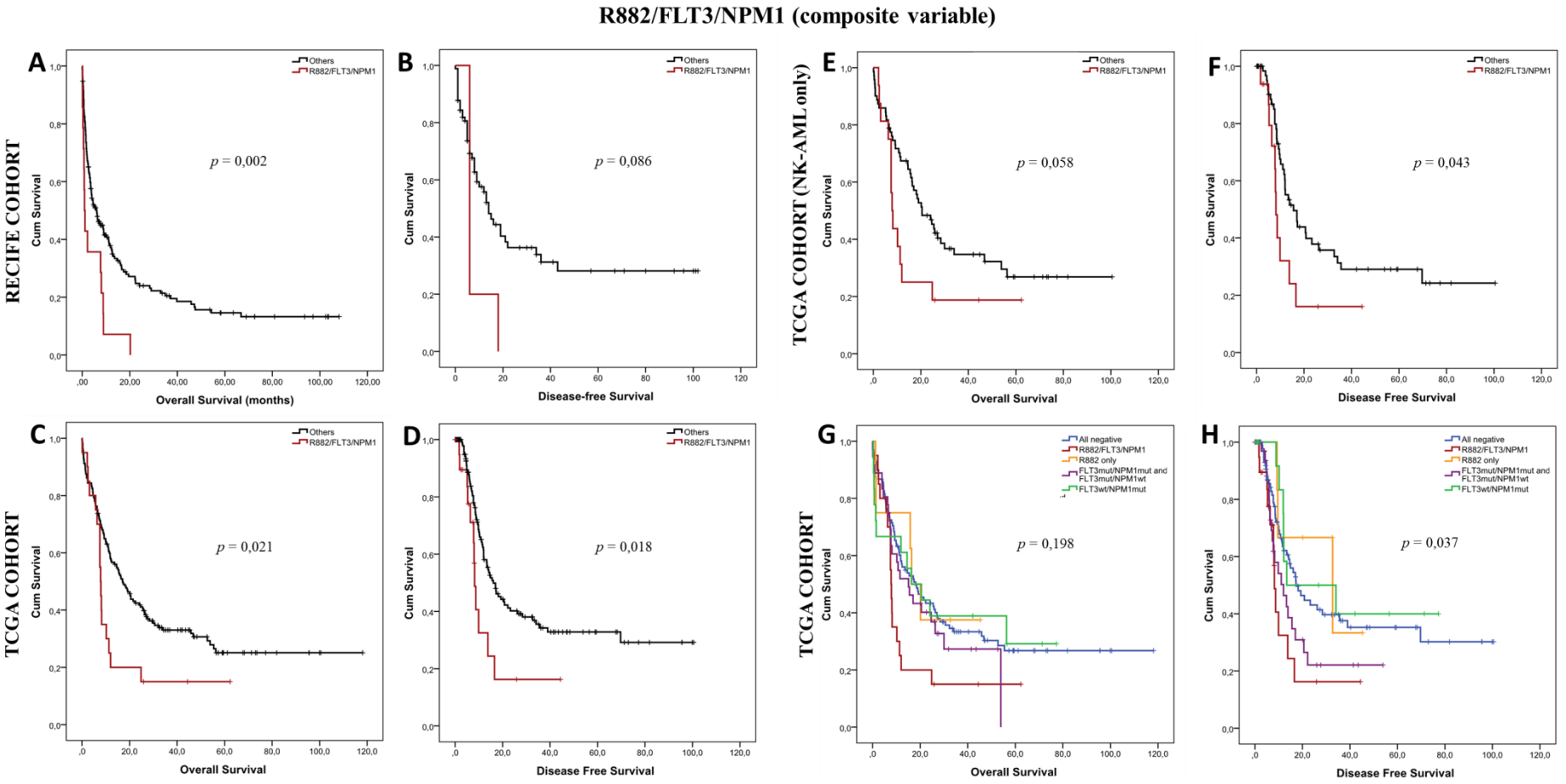


Figure 5. Survival analysis for the R882/*FLT3*/*NPM1* composite variable. OS and DFS in cohort Recife (**A** and **B**) and TCGA (**C** and **D**) according to the R882/FLT3/NPM1 composite variable. **E** and **F**: survival analysis restricted to normal karyotype patients. **G** and **H**: survival analysis regarding distinct molecular groups in TCGA cohort (red: R882/*FLT3*/*NPM1*, blue: triple negative, orange: only R882 mutated, purple: FLT3mut/*NPM1*wt and FLTmut/*NPM1*mut, green: *NPM1*mut/*FLT3*wt.

Table 4. Cox's regression analysis for factors associated with outcome in AML. HR: hazard ratio, 95%CI: 95% confidence interval, Cox proportional regression. Cytogenetic risk according to Medical

End point	Variable	Univariate analysis		Multivariate analysis	
		HR (95% CI)	P-value	HR (95% CI)	P-value
Cohort Recife					
Overall survival	R882/FLT3/NPM1	2,44 (1,35-4,44)	**0,003**	1,97 (1,06-3,84)	**0,048**
	Age > 60	1,96 (1,41-2,74)	**6,9 10^{-5}**	1,78 (1,19-2,67)	**0,005**
	Cytogenetic risk	1,14 (0,8 – 1,61)	0,47	1,17 (0,77-1,77)	0,464
	WBC > 10^5	1,45 (0,97-2,17)	0,072	2,03 (0,96-2,72)	0,076
	R882	1,32 (0,77-2,26)	0,309	-	-
	FLT3	1,02 (0,69-1,5)	0,93	-	-
	NPM1	0,9 (0,63-1,29)	0,573	-	-
Disease-free survival	R882/FLT3/NPM1	2,1 (0,74-5,92)	0,163	1,64 (0.45-5,99)	0,450
	Age > 60	1,79 (0,87-3,7)	0,116	0,74 (0,28-1,94)	0,543
	Cytogenetic risk	1,18 (0,65-2,13)	0,582	1,62 (0,73-3,59)	0,24
	WBC > 10^5	0,9 (0,43-1,89)	0,788	1,4 (0,59-3,35)	0,54
	R882	1,77 (0,7-8-4,0)	0,168	-	-
	FLT3	0,9 (0,44-1,86)	0,784	-	-
	NPM1	0,81 (0,45-1,48)	0,508	-	-
Cohort TCGA					
Overall survival	R882/FLT3/NPM1	1,83 (1,09-3,06)	**0,022**	2,14 (1,24-3,68)	**0,006**
	Age > 60	2,61 (1,82-3,73)	**1,5 10^{-7}**	2,66 (1,83-3,87)	**2,8 10^{-7}**
	Cytogenetic risk	1,80 (1,33-2,44)	**1,3 10^{-4}**	1,84 (1,32-2,56)	**3,6 10^{-4}**
	WBC > 10^5	1,25 (0,72-2,18)	0,43	1,68 (0,92-3,05)	0,089
	R882	1,46 (0,92-2,32)	0,11	-	-
	FLT3	1,28 (0,86-1,88)	0,22	-	-
	NPM1	1,05 (0,72-1,53)	0,8	-	-
Disease-free Survival	R882/FLT3/NPM1	2,06 (1,11-3,81)	**0,021**	2,27 (1,21-4,25)	**0,01**
	Age > 60	1,24 (0,81-1,90)	0,318	1,31 (0,84-2,04)	0,23
	Cytogenetic risk	1,23 (0,86-1,74)	0,257	1,22 (0,84-1,78)	0,3
	WBC > 10^5	0,97 (0,47-2,01)	0,938	0,98 (0,48-2,15)	0,957
	R882	1,40 (0,81-2,46)	0,23	-	-
	FLT3	1,63 (1,04-2,55)	**0,035**	-	-
	NPM1	1,27 (0,81-1,97)	0,298	-	-

Research Council (MRC).

4 Discussion

4.1 *IDH1* and *IDH2* mutations

Recent studies have demonstrated that abnormal function of epigenetic modifiers, such as *DNMT3a*, *IDH1* and *IDH2* are found in the majority of AML cases and play a central role in leukemic transformation of hematopoietic precursors (LIN *et al.*, 2018; JEONG, 2018). However, the prognostic significance of mutations in these genes is still under investigation and there is still inconsistence among different reports, probably due to diverging population features and treatment courses (AP IM, 2014).

As several selective IDH inhibitors are being tested in clinical trials, it is important to study *IDH1* and *IDH2* mutational frequency and clinical features across several populations (BEWERSDORF *et al.*, 2019; POPOVICI-MULLER *et al.*, 2018). Here, we report results from a Brazilian cohort of non-selected AML patients, with further validation in an independent cohort from the TCGA consortium. Although *IDH1* and *IDH2* mutations were associated with specific disease features that had been previously described, such as advanced age, *NPM1* mutations (*IDH2*), FAB M1 subtype, normal karyotype (*IDH1*) and mutual exclusivity among them (ABBAS *et al.*, 2010; DINARDO *et al.*, 2015), there was no impact of these mutations on patient's outcome. In that regard, there are both reports of a negative impact on prognosis (AREF *et al.*, 2015; LIU *et al.*, 2014; MARCUCCI *et al.*, 2015; PASCHKA *et al.*, 2013) and no significant impact (CRUZ, 2016; DINARDO *et al.*, 2015; WAGNER *et al.*, 2010). Interestingly, Wagner *et al.*, described an adverse outcome for AML patients carrying the synonymous polymorphism on *IDH1*, rs11554137, while no impact for the mutation was found.

It is important to highlight that distinct mutations in the same gene might have different repercussions on disease features and survival (FALINI *et al.*, 2019; MARCUCCI *et al.*, 2012; PAPAEMMANUIL *et al.*, 2016). Papaemmanuil and colleagues recently reported that *IDH2* R172, but not R140 has prognostic importance and proposed the creation of a provisional AML entity for patients harboring this mutation (PAPAEMMANUIL *et al.*, 2016). Due to our sample size, we could not test R172 individually.

4.2 *DNMT3a* R882 mutations

DNMT3a mutations had been shown to be one of the earliest events in leukemogenesis, skewing hematopoietic stem cells into division and self-renewal (JEONG *et al.*, 2018; POTTER *et al.*, 2018; SHLUSH *et al.*, 2014). Despite been first described as an adverse prognosis marker, its effect on survival is contradictory and many studies report

significant results only in particular subsets of patients, regarding age, karyotype and type of mutation (KUMAR *et al.*, 2018; MARCUCCI *et al.*, 2012; ROLLER *et al.*, 2013; SCHMALBROCK *et al.*, 2018; YANG *et al.*, 2016; YUAN *et al.*, 2016).

Here, we observed clear differences between the clinical/molecular aspects from patients carrying R882 (hotspot) or non-R882 mutations. In corroboration with Ley et al., and Gaidzik et al., R882 mutations were associated with monocytic subtypes of AML (M4 and M5), higher leukocyte counts and normal karyotype. While patients assigned to the non-R882 had a higher prevalence of *IDH2* and NRAS mutations, those assigned to the R882 group were enriched for *FLT3* and *NPM1* mutations. Patients with R882 also had a significant higher rate of *FLT3*/*NPM1* double-positivity when compared to non-R882 mutations or wildtype *DNMT3a*. Of interest, although Buscarlet *et al.*, and Genovese *et al.*, described a significant prevalence of *DNMT3a* mutations in healthy older individuals, only 9% to 12% of those mutations were in codon R882, while in AML patients, they correspond to 58% of all mutations in this gene. The difference between R882 frequency in healthy and AML suggest that R882 has a major role in leukemogenesis when compared to non-R882. However, further functional investigations to elucidate the biological differences between these mutations are necessary.

As previously reported elsewhere, half of *DNMT3a* R882 patients also carry *FLT3* and *NPM1* mutations, suggesting that the interplay between these genes is necessary to drive leukemia. (LOGHAVI *et al.*, 2014; PAPAEMMANUIL *et al.*, 2016; RENNEVILLE *et al.*, 2012; YUAN *et al.*, 2016). In support, recent population studies identified *DNMT3a* mutations in healthy (non-leukemic) individuals and the mutation was associated to age-related clonal hematopoiesis, but not AML (BUSCARLET, 2017; GENOVESE *et al.*, 2014). Studies conducted in murine models had shown that *DNMT3a* mutation alone causes some level of dysplasia but not fully-developed AML, whereas when combined to *NPM1* or *FLT3*, the animal develops an aggressive, low-latency and transplantable AML. These data support a model in which an hematopoietic stem-cell acquires a *DNMT3A* mutation that undergoes clonal expansion until an additional genetic lesion is acquired, leading to leukemic transformation (GURYANOVA *et al.*, 2017; LOBERG *et al.*, 2019; MEYER *et al.*, 2016; YANG *et al.*, 2016).

Moreover, analysis from transcriptome, miRNA (TCGA NETWORK, 2013) and methylome (YANG *et al.*, 2016) revealed that when combined, these mutations forms distinct clusters, not derived from single-mutation effects, and therefore, pointing towards the existence of a distinct and well-defined subtype of AML. Corroborating these findings,

Ribeiro et al, while analyzing DNA methylation from AML patients, describes a cluster that harbor concomitant *DNMT3a*/*NPM1*/*FLT3* mutations, are M4/M5 AMLs and has an unique gene expression signature, marked by overexpression of HOX genes (RIBEIRO *et al.*, 2012). Interestingly, when analyzed alone, *DNMT3a* mutations had rather unspecific methylation signatures. Beyond its biological features, a recent study demonstrated that AML cells derived from patients harboring *DNMT3a*/*FLT3*/*NPM1* mutations have distinct sensitivity to specific treatments, when compared to cells harboring individual mutations (TYNER *et al.*, 2018). Altogether, there are solid evidences that points towards the existence of an unique AML entity originated from the cooperative effect of *DNMT3a* R882, *FLT3* and *NPM1*.

Despite being well-accepted, this concept is rather limited to the biological comprehension of AML and the data concerning its clinical relevance is scarce, as most studies evaluate single effects of each mutation. To address this question, we transposed the *DNMT3a* + partner mutation model into a composite variable (here called R882/*FLT3*/*NPM1*). At transcriptome level, patients assigned to the composite variable R882/*FLT3*/*NPM1* had distinct mRNA signatures from those carrying R882 but no partner mutations. In survival analysis, patients assigned to the R882/*FLT3*/*NPM1* group had shorter survival rates in both cohorts. The composite variable had stronger survival prediction power than individual analysis of *DNMT3a*, *FLT3* or *NPM1* and was independent of age, leukocyte count or cytogenetic risk on multivariate analysis. Because *FLT3* and *NPM1* are well-established prognostic markers, we then stratified survival analysis according to distinct mutational status. Those assigned to the composite variable had a worse outcome than the other groups, including *FLT3*mut/*NPM1*wt and *FLT3*/*NPM1*mut patients, which is a well-described for its unfavorable outcome in AML.

Supporting current results, Papaemmanuil, by analyzing a cohort of 1540 AML patients, showed that gene-gene interaction can largely modify prognostic effect of single mutations and the three-way interaction of *DNMT3a/FLT3/NPM1* confered a significantly more deleterious prognosis than individual mutations in those genes. Similarly, Metzeler et al., reported significantly shorter survival rates when *DNMT3A* mutations were studied in conjunction with the combined *NPM1*/*FLT3*-ITD genotype.

5 Conclusion

The frequency and types of *DNMT3a*, *IDH1* and *IDH2* mutations were described are similar to those reported elsewhere. Although *IDH1*/*IDH2* mutations were associated with

specific clinical and molecular features of AML, we observed no significant impact on patient's outcome.

The traditional concept that leukemia is caused by single mutations has shifted to a complex network of mutations, epigenetic changes and gene expression data. Thus, by transposing this biological concept into a model for outcome prediction in two independent cohorts, we show that the well-recognized cooperation of *DNMT3a* with *FLT3* and *NPM1* identifies a subset of AML with adverse prognosis more efficiently than the classical analysis of these mutations individually. We expect that current observations can be further tested in larger AML cohorts.

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5.2 Artigo 2

Association between the *TP53* Arg72Pro polymorphism and clinical outcomes in acute myeloid leukemia

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In acute myeloid leukemia (AML), alterations involving the tumor suppressor gene *TP53*, including mutations, deletions, or both, are frequently associated with older age and very poor prognosis.[1] In addition, they are closely associate with complex aberrant karyotype and, outside this group, *TP53* alterations are rarely observed.[2] On the other hand, several polymorphisms in the *TP53* gene have been described,[3] of which the nonsynonymous *TP53* Arg72Pro (G215C) polymorphism appears to be a promising genetic modifier in human tumors, particularly because of its role as a modulator of the apoptotic activities of the encoded p53 protein.[4] In terms of the biological significance of the *TP53* Arg72Pro polymorphism, there are discernible functional differences between variants at this site. While the *TP53* variant that encodes proline (Pro72) results in 3- to 5-fold decreased apoptotic activity[5] and an increased risk of cancer,[6] the *TP53* arginine (Arg72) variant has an increased ability to induce apoptosis and to repress cellular transformation.[5]

To date, little is known about the frequency and prognostic impact of the *TP53* Arg72Pro polymorphism in hematological malignancies, particularly in AML. The few available data suggest that the interaction between the *TP53* Arg72Pro polymorphism and the *MDM2* SNP309 variant could modulate responses to genotoxic therapy and increase the risk of therapy-related AML.[7] Here we assessed the frequency of the *TP53* Arg72Pro polymorphism in healthy volunteers and patients with AML and evaluated its clinical impact on outcomes of non-selected patients with AML (excluding acute promyelocytic leukemia) at two university hospitals who were followed from June 2003 to January 2016.

Overall, 429 subjects were included. Two-hundred five adult patients with *de novo* AML were retrospectively analyzed. One hundred fifty-one (74%) patients were treated in Recife (northeast Brazil), while 54 (26%) patients were treated in Ribeirao Preto (southeast Brazil). The baseline features were similar between centers. The treatment protocol was described previously.[8] Briefly, patients up to 60 years of age, the treatment protocol was adapted according to performance status and the presence of comorbidities (in particular, cardiac disorders). Conventional chemotherapy consisted of daunorubicin (60-90 mg/m^2/d for 3 days) and cytarabine (100-200 mg/m^2/d for 7 days) or TAD-9 as induction, followed by three or four cycles of consolidation therapy with high doses of cytarabine (above 1 g/m^2/d). For patients who did not achieve complete remission (CR) after one course of chemotherapy, a second course was administered between days 28 and 35 after the end of the first course. CR

was assessed by bone marrow examination on day 28 after each course of chemotherapy. For those who needed it, a post-remission therapy based on autologous or allogeneic transplantation was performed.

For the healthy control group, peripheral blood samples from 224 age- and sex-matched healthy volunteers (median age: 51 years, range: 21–83 years; 55% female) with no history of hematological disease were obtained from the University Hospital, Federal University of Pernambuco (Recife, Brazil). Patients with therapy-related AML or with a previous history of myelodysplastic syndrome were not included. In accordance with the Declaration of Helsinki, informed consent was obtained from all patients and healthy volunteers. Ethical approval was obtained from the local research ethics board. The *TP53* Arg72Pro polymorphism was evaluated by polymerase chain reaction-restriction fragment length polymorphism.[9] Details of the statistical analysis and clinical endpoints were described previously.[10]

The *TP53* Arg72Pro polymorphism was successfully genotyped in 413 (96%) subjects, including 189 of 205 patients with AML (92%) and 224/224 of healthy volunteers (100%). No deviation from Hardy-Weinberg equilibrium was detected in the patients ($P>0.05$) or controls groups ($P>0.05$). In addition, there were no differences in the baseline characteristics or outcomes of the patients included in this study versus patients who not included because of poor quality genomic DNA or unavailable DNA samples (data not shown). To determine whether the *TP53* Arg72Pro polymorphism is associated with risk of AML, we compared the frequency of this polymorphism in patients with AML and healthy subjects. The allelic ($P=0.004$) and genotypic ($P=0.008$) frequencies of the *TP53* Pro72 variant were higher in patients with AML (Table 1). Next we restricted our analysis to patients with AML and analyzed the association of the frequency of the *TP53* Arg72Pro polymorphism with clinical and laboratory features. We also evaluated its clinical impact on induction and post-induction outcomes. The recessive model (i.e., Pro/Pro *versus* Arg/Arg+Arg/Pro, hereafter called non-Pro/Pro) was used because it had the best fit to our data (Table 1). There were no significant differences between patients with the Pro/Pro genotype (43 patients, 23%) *versus* the non-Pro/Pro genotype (146 patients, 77%) with respect to clinical and laboratory features (Table 2).

Table 1. Distribution of allele and genotype frequencies of the *TP53* Arg72Pro polymorphism.

***TP53* Arg72Pro**	**Acute myeloid leukemia, n (%)**	**Healthy volunteers, n (%)**	**Odds ratio (95% CI)**	***P*-value**
Allele				
Arg (G allele)	203 (54)	285 (64)	Reference	
Pro (C allele)	175 (46)	163 (36)	1,51 (1.14 to 1.99)	0.004
Codominant				
Arg/Arg	57 (30)	89 (40)	Reference	0.011
Arg/Pro	89 (47)	107 (48)	1.30 (0.84 to 2.01)	0.286
Pro/Pro	43 (23)	28 (12)	2.40 (1.34 to 4.29)	0.004
Dominant				
Arg/Arg	57 (30)	89 (40)	Reference	
Arg/Pro + Pro/Pro	132 (60)	135 (60)	1.53 (1.01 to 2.30)	0.054
Recessive				
Arg/Arg + Arg/Pro	146 (77)	196 (88)	Reference	
Pro/Pro	43 (23)	28 (12)	2.06 (1.22 to 3.48)	0.008
Over dominant				
Arg/Arg + Pro/Pro	100 (53)	117 (52)	Reference	
Arg/Pro	89 (47)	107 (48)	1,03 (0.70 to 1.51)	0.969

Table 2. Baseline characteristics of AML patients.

	All		*TP53* Arg72Pro polymorphism (recessive model) Pro/Pro		non-Pro/Pro		*P*-value[4]
	No.	%	No.	%	No.	%	
Age (years)							0.62
18-40	66	34.9	17	39.5	49	33.6	
40-60	59	31.2	14	32.6	45	30.8	
>60	64	33.9	12	27.9	52	35.6	
Age, median (range)	47 (18, 93)		41 (18, 80)		49 (18, 93)		0.203
Gender							0.862
Female	102	54	24	55.8	78	53.4	
Male	87	46	19	44.2	68	46.6	
FAB subtype							0.364
M0	16	9.1	6	16.7	10	7.2	
M1	27	15.4	7	19.4	20	14.4	
M2	59	22.7	8	22.2	51	36.7	
M4	52	29.7	11	30.6	41	29.5	
M5	16	9.1	4	11.1	12	8.6	
M6	3	1.7	-	-	3	2.2	
M7	2	1.1	-	-	2	1.4	
Missing data	14	-	7	-	7	-	
Cytogenetic abnormalities							0.215
Normal karyotype	47	42	10	40	37	42.5	
t(8;21)(q22;q22)	14	12.5	-	-	14	16.1	
inv(16)(p13;q22)/t(16;16)(p13;q22)	10	5.3	4	16	6	6.9	
Monosomy 7	4	3.6	1	4	3	3.4	
Trisomy 8	2	1.8	1	4	1	1.1	
Complex karyotype	7	6.3	2	8	5	5.7	

11q23 abnormalities	1	0.9	1	4	-	-	
Others	12	10.7	2	8	10	11.5	
Missing data[1]	77	-	18	-	59	-	
Cytogenetic risk stratification[2]							0.778
Favorable	24	24.7	4	19	20	26.3	
Intermediate	57	58.8	13	62	44	57.9	
Adverse	16	16.5	4	19	12	15.8	
Missing data	92	-	22	-	70	-	
FLT3-ITD							0.832
Mutated	38	20.3	8	18.6	30	20.8	
Non-mutated	149	79.7	35	81.4	114	79.2	
Missing data	2	-	-	-	2	-	
NPM1 mutations							0.687
Mutated	46	24.6	9	20.9	37	25.7	
Non-mutated	141	75.4	34	79.1	107	74.3	
Missing data	2	-	-	-	2	-	
Molecular risk group[3]							0.815
Low-risk group	31	16.6	6	14	25	17.4	
High-risk group	156	83.4	37	86	119	82.6	
Missing data	2	-	-	-	2	-	
WBC ($\times 10^9$/L), median	46.5		45.5		47.5		0.695
Range	0.5, 312		2, 285		0.5, 312		
Hemoglobin (g/dL), median	7.8		8.3		7.7		0.393
Range	3, 14		5.5, 11		3, 14		
PLT ($\times 10^9$/L), median	47		48		47		0.614
Range	6, 652		6, 143		8, 625		

Abbreviations: AML, acute myeloid leukemia; FAB, French-American-British; WBC, white blood cells; PLT, platelets; *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; *NPM1*, nucleophosmin.

NOTES:

1: Material not available or no metaphases detected.

2: The cytogenetic risk groups were defined according to Medical Research Council (MRC) criteria.[16]
3: The high-risk molecular group was defined as *NPM1*$^{\text{non-mutated}}$/*FLT3*-ITD$^{\text{negative}}$, *NPM1*$^{\text{non-mutated}}$/*FLT3*-ITD$^{\text{positive}}$, or *NPM1*$^{\text{mutated}}$/*FLT3*-ITD$^{\text{positive}}$. The low-risk molecular group was defined by the presence of any *NPM1* mutations and the absence of *FLT3*-ITD mutations.
4: Missing values were excluded for calculation of P-values.

Of the 189 enrolled patients, two (1%) patients who started the induction treatment were lost to follow-up without being assessed for CR. In addition, 68 (36%) patients did not receive conventional chemotherapy (main reasons for treatment failure were previously described[8]) and were considered ineligible for the induction and post-induction therapy analyses. CR was achieved in 68/119 (57%) patients, of which 18/27 (67%) and 50/92 (54%) patients were assigned to the Pro/Pro and non-Pro/Pro groups, respectively. The *TP53* Arg72Pro polymorphism had no impact on CR (*P*=0.278). Of the 51 patients who failed to reach CR, 16 (33%) experienced early mortality, mainly due to bacterial and fungal infections. Although the early mortality rate was proportionally higher in patients with the non-Pro/Pro genotype (44% *versus* 30%), this did not differ between groups (*P*=0.449).

The median follow-up for the entire cohort was 135 days (95% confidence interval (CI): 69–200 days) with an estimated 5-year OS rate of 22% (95% CI: 17%–29%). Patients with the Pro/Pro genotype had significantly longer survival (median: 264 days, 95% CI: 201–657 days) than patients with the non-Pro/Pro genotype (median: 114 days, 95% CI: 70–158 days). Univariate analysis showed that patients with the Pro/Pro genotype had a higher 5-year OS rate (42%) than patients with non-Pro/Pro genotype (12%) (*P*=0.031) (Figure 1A), although this difference was no longer significant after adjustment for age, cytogenetic risk stratification, and leukocyte counts at diagnosis as cofounders (hazard ratio (HR): 0.66, 95% CI: 0.37–1.17; *P*=0.163). We also analyzed the clinical impact of the *TP53* Arg72Pro polymorphism in each cytogenetic risk group. The *TP53* Arg72Pro polymorphism had no impact on the clinical outcome of patients assigned to the favorable (24 patients; *P*=0.6) and adverse groups (16 patients; *P*=0.561). In contrast, the *TP53* Pro/Pro genotype was significantly associated with longer survival (*P*=0.035) for patients assigned to the intermediate cytogenetic risk group (57 patients) (Figure 1B), albeit these results were not consistent with the multivariate analysis (HR: 0.44, 95% CI: 0.14–1.37; *P*=0.164). The *TP53* Arg72Pro polymorphism had no impact on disease-free survival (*P*=0.77).

Considering the prognostic relevance of age in AML, we opted to evaluate the prognostic impact of the *TP53* Arg72Pro polymorphism separately in younger (up to 60 years of age; 125 patients) and older patients (older than 60 years; 64 patients). For patients up to 60 years of age, the *TP53* Arg72Pro polymorphism had no impact on CR (*P*=0.648), and DFS (*P*=0.856), but there was a trend towards a higher OS rate for patients with Pro/Pro genotype (17% *versus* 46%; *P*=0.059). *TP53* Arg72Pro polymorphism was not associated with

treatment outcomes in patients older than 60 years old (CR: *P*=0.154; DFS; *P*=0.201; OS; *P*=0.643).

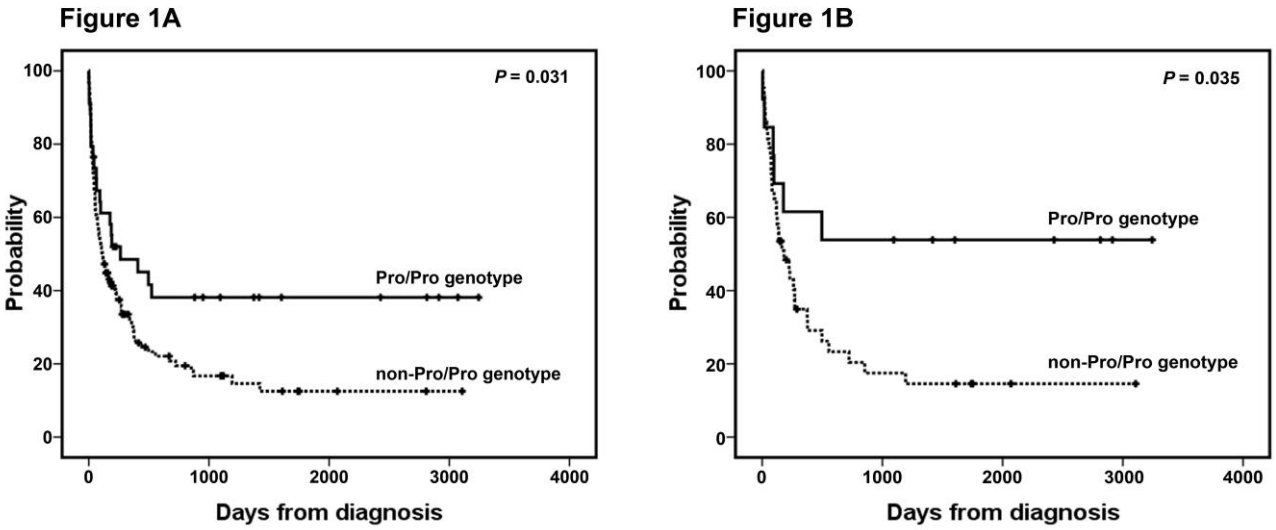

Figure 1. **Patients survival**. The probability of overall survival (A) in patients with AML according to the presence of the *TP53* Arg72Pro polymorphism (entire cohort). Overall survival (B) in patients with intermediate-risk karyotype according to the presence of the *TP53* Arg72Pro polymorphism. Survival curves were estimated using the Kaplan-Meier method and the log-rank test was used for comparison.

Although most studies have described the *TP53* Pro72 allele as a poor prognostic factor in several type of cancer,[6] there is some evidence that the Pro72 variant is more efficient in both activating DNA-repair target genes[11] and inducing cell-cycle arrest.[12] Particularly in myeloid neoplasms, the *TP53* Pro72 variant exerts a protective effect against therapy-related AML in individuals with lower levels of the MDM2 protein.[7] Finally, Pro72 allele carriers show significantly lower frequency of *TP53* mutations in specific types of human non-hematological tumors.[13] Here, we demonstrated that the *TP53* Pro/Pro genotype was associated with higher risk of leukemia and favorable outcome in AML patients treated with conventional therapy, particularly those assigned to the intermediate cytogenetic risk group. Therefore, it seems that the *TP53* Arg72Pro polymorphism may have different tissue- and context-specific functions, and the prognostic importance of each allele may depend on the type of cancer and on the particular treatment. Importantly, screening for the *TP53* mutations was not performed in the present study. Nevertheless, we should point out that in

our cohort only 6% of patients had complex karyotype and abnormalities involving chromosome 17 (17p-, monosomy 17, or i(17q)) was not observed. One may argue that *TP53* alterations could be the responsible for inferior outcomes in our cohort, but considering the rarity of these mutations in non-complex karyotype AML this possibility seems unlikely.

Several published data have investigated the relationship between the *TP53* Arg72Pro polymorphism and risk of leukemia, but the impact of this polymorphism on predisposition to leukemia remains controversial. Most recently, Tian et al. in a meta-analysis involving seven AML cohorts with 1,054 patients and 4,337 healthy subjects, reported an absence of association between the *TP53* Arg72Pro polymorphism and increased risk of AML.[14] Nevertheless, it is important to note that the authors conducted theirs analyses based on studies from Asian population (eight studies) and Caucasian population (five studies), which may not reflect the Brazilian genetic background, a well-known mixed population.

Although we have demonstrated that the *TP53* Arg72Pro polymorphism could be prognostically relevant in AML, such results must be taken with caution. First, a sizeable number of patients were excluded from induction and post-induction analyses because did not receive conventional chemotherapy (36%) or experienced early mortality (33%), which could bias our analyses. In addition, because of the relatively small sample size and the current lack of validation in independent cohorts, it is probably premature to use *TP53* Arg72Pro genotype information to make treatment decisions. It would be reasonable that our findings are confirmed by others groups with larger sample sizes and well-designed studies. Despite limitations, the germline genetic profile should not be overlooked, taking into account the genomic landscape of AML and its role in the clonal evolution of the disease.[15]

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Supplement

Supplemental Table 1. Univariate and multivariate analysis for overall survival.

End point	Model Variables	Model 1				Model 2				Model 3				Model 4			
		HR	**95% CI**		***P*-value**	**HR**	**95% CI**		***P*-value**	**HR**	**95% CI**		***P*-value**	**HR**	**95% CI**		***P*-value**
Overall survival	***TP53* Arg72Pro polymorphism**: Pro/Pro *vs* non-Pro/Pro	0.59	0.37	0.95	0.032	0.63	0.39	1.02	0.065	0.61	0.33	1.33	0.12	0.66	0.37	1.17	0.163
	Age (years): continuous variable					1.02	1.01	1.03	< 0.001	1.01	0.99	1.02	0.119	1.01	0.99	1.49	0.213
	Cytogenetic risk stratification: adverse *vs* intermediate *vs* favourable									1.24	0.85	1.77	0.24	1.02	0.63	1.65	0.915
	Leukocyte counts (x10^9/L): continuous variable													1.09	1.05	1.14	< 0.001

NOTE: Hazard ratios (HR) > 1 or < 1 indicate an increased or decreased risk, respectively, of an event for the first category listed.

6 CONCLUSÕES

A frequência, distribuição e tipo das mutações nos genes *IDH1*, *IDH2* na coorte de LMA brasileira está de acordo com o descrito em outras populações. Apesar de associadas a características clinico-laboratoriais específicas, essas mutações demonstraram não exercer impacto significativo no prognóstico dos pacientes.

Foi observada uma forte associação entre mutações no *DNMT3a*, especialmente do tipo R882, e mutações nos genes *FLT3*/*NPM1*. A interação entre essas mutações resultam em padrões de expressão gênica distintos dos observados quando as mesmas ocorrem isoladamente, o que sugere a existência de entidade leucêmica específica, formada a partir dessa combinação. Ao transpor esse conceito para a formulação de uma variável composta, observou-se um prognóstico marcadamente adverso em pacientes que possuíam mutações *DNMT3a* combinadas a *FLT3* ou *NPM1*. Dessa forma, o presente trabalho demonstra que o efeito cooperativo entre as mutações estudadas é mais representativo do aspecto biológico da doença e apresenta maior valor prognóstico que a análise isolada de cada mutação. Esses achados precisam, no entanto, ser posteriormente validados em coortes maiores.

Já em relação ao polimorfismo *TP53* R72P, os resultados do presente trabalho sugerem que o alelo 72Pro (prolina) está associado de forma recessiva a um maior risco de desenvolvimento de LMA. Ainda no modelo recessivo, o alelo 72Pro foi associado a um desfecho clínico favorável. Esse mesmo efeito foi observado também em análise restrita ao pacientes com cariótipo normal. Apesar de o mecanismo em que atua o polimorfismo R72P na sobrevida LMA ser desconhecido, os presentes resultados apontam para seu potencial uso como marcador de prognóstico, principalmente no grupo com cariótipo normal.

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Fut3 role in breast invasive ductal carcinoma: Investigating its gene promoter and protein expression

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ABSTRACT

Fucosylated glycans synthesized by α1,3/4-fucosyltransferase (FUT3) enzyme play an important role in breast cancer prognosis and metastasis, being involved in the binding of circulating tumor cells to the endothelium and being related to tumor stage, metastatic potential and chemoresistance. Despite the pro-tumor action of this enzyme, studies have demonstrated its role in natural killer-induced cytotoxicity through the recognition of sialyl Lewis X by C-type lectin receptors and through extrinsic apoptosis pathway triggered by Apo2L-TRAIL. This study aimed to investigate the expression pattern of FUT3 in invasive breast carcinoma (IDC) from patients of Pernambuco state, Northeast of Brazil, and genotype *FUT3* promoter region to identify possible SNPs that could be associated with variations in FUT3 expression. Immunohistochemistry assay was used to access the FUT3 expression in normal (n = 11) and tumor tissues (n = 85). DNA sequencing was performed to genotype the *FUT3* promoter region in patients with IDC (n = 109) and healthy controls (n = 110). Our results demonstrated that the absence of FUT3 enzyme is related to breast's IDC. The non-expression of FUT3 was more frequent in larger lesions and also in HER2 negative IDC tumors. Genomic analysis showed that two variations localized in *FUT3* promoter region are possibly associated with IDC. Our results suggest that minor allele T of SNP rs73920070 (−6933 C > T) confers protection whereas minor allele T of SNP rs2306969 (−6951 C > T) triggers to susceptibility to IDC in the population of Pernambuco state, Northeast of Brazil.

1. Introduction

Breast cancer is the most incident tumor among women worldwide, having affected 1.67 million women in 2012 (Ferlay et al., 2014). Although early diagnosis associated with new therapies had been giving the chance of cure for many breast cancer patients, oncology field is still far away to comprehend all factors that trigger and rule the disease development.

Fucosylated glycans play an important role in breast cancer prognosis and metastasis, being involved in the binding of circulating tumor cells to the endothelium (Yuan et al., 2008; Zen et al., 2008) and also related to tumor stage, metastatic potential (Jeschke et al., 2005) and chemoresistance (Ma et al., 2013).

Fucosyltransferases are a family of enzymes that catalyzes the L-fucose transfer from GDP-L-fucose to a nascent glycan (Miyoshi et al., 2008). There are 13 genes encoding fucosyltransferases in human genome which are called *FUT1* to *FUT11*, *POFUT1* and *O-FUT2* (Becker and Lowe, 2003). *FUT3* gene encodes a fucosyltransferase with α1,3 and α1,4 L-fucose bonding activity. This gene is localized in the short arm of chromosome 19 and is formed by three exons, A, B and C (Kukowska-Latallo et al., 1990). The expression of *FUT3* is dependent on its hypomethylation (Serpa et al., 2006) and is regulated by transcription factors, such as HNF1α (Lauc et al., 2010), p53 (Katkoori et al., 2012) and c-Myc (Sakuma et al., 2012), and interleukins IL-8, TNF-α (Groux-Degroote et al., 2008), IL-1β and IL-10 (Padró et al., 2011b).

The mainly products of FUT3 enzyme are the Lewis antigens. Sialyl Lewis X (sLe^x) and its isomer sialyl Lewis A (sLe^a) are glycans, recognized by the E-selectins, involved in adhesion of tumor cells to the endothelium and consequent escape of these cells from blood stream, a crucial step in hematogenous metastasis (Burdick et al., 2012). Although the mechanism mediated by Lewis antigens and E-selectin represents an important aspect in tumor biology, FUT3 is also related to other pathways in neoplastic cells. In colorectal cancer, the fucosylation by FUT3 of TβR I and TβR II receptors is essential for epithelial–mesenchymal transition (EMT) induced by TGF-β (Hirakawa et al., 2014). However, tumor death pathways are also dependent on fucosylation. Overexpression of sLe^x in melanoma and erythroleukemia cell lines by *FUT3* gene transfection leads to cytotoxicity mediated by natural killer cells through ligation to C type lectin receptors, such as CD94 (Higai et al., 2006;

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In spite of studies that had found a relationship between the expression of Lewis antigens and the metastatic spread of human breast cancers, indicating a pro-tumor action of FUT3 processing (Listinsky et al., 2011), the importance of NK-induced cytotoxicity for antitumor efficacy against breast cancer cells suggests an involvement of this enzyme in antitumor pathways (Kajitani et al., 2012). In order to a better understanding of FUT3 role in breast tumors this study aimed to investigate the expression pattern of FUT3 enzyme in invasive breast carcinoma (IDC) samples from Pernambuco State, Northeast of Brazil. Genotype of *FUT3* promoter region was also performed to identify possible single nucleotide polymorphisms (SNPs) that could be associated with variations in FUT3 tumors expression.

2. Materials and methods

2.1. Samples

Formalin-fixed, paraffin-embedded (FFPE) tumor samples of 127 patients diagnosed with breast invasive ductal carcinoma (IDC) from 2009 to 2014, were collected from the tissue bank of the Hospital das Clínicas da Universidade Federal de Pernambuco (HC-UFPE) and from the Instituto de Medicina Integral Professor Fernando Figueira (IMIP). The diagnosis of breast cancer was based on hematoxylin and eosin histopathology analyzed by two independent pathologists. Patients' clinical data were obtained from medical files and included age, tumor size, metastasis to regional lymph nodes and tumor staging. In the breast cancer patients' samples (127), 65 samples had DNA extraction and immunohistochemistry assay developed. Forty-four out of 127 samples had only DNA extracted while 20 out of 127 samples (not belonging to previous 44 sample group) were only immunohistochemistry assayed. DNA from 15 blood samples was obtained from the 44 sample's group for genotyping evaluation of germline or somatic origin of *FUT3* gene variations. Control samples included 11 normal breast tissues obtained from cosmetic surgery and 110 blood samples of cancer-free patients. This study was approved by the local Research Ethics Committee (CAAE 06586612.9.0000.5208 — No. 140.876) and all participants signed the written informed consent.

2.2. Immunohistochemistry assay

FFPE tissue samples from 85 IDC patients and 11 healthy controls were sliced in sections of 4 μm and immunohistochemistry assay was performed according to Vasconcelos et al. (2013). Sections were deparaffinized in xylene and hydrated with decreasing ethanol concentration. Tissues were antigen retrieved with 10 mM citrate buffer (pH 6.0), followed by endogenous peroxidase blocking with methanolic H_2O_2 solution and finally incubated with 1% bovine serum albumin dissolved in phosphate buffered saline (PBS-BSA). Sections were incubated, separately, with primary antibodies: goat polyclonal antibody anti-estrogen receptor L-20, mouse monoclonal antibody anti-progesterone receptor AB-52, mouse monoclonal antibody anti-Ki-67 (all at 1:50 dilution, Santa Cruz Biotechnology, Santa Cruz, California), rabbit polyclonal antibody anti-c-erbB-2 (HER-2) oncoprotein (1:100, DAKO, Carpinteria, California) and rabbit polyclonal antibody anti-FUT3 (1:200, SigmaAldrich, St. Louis, Missouri). Immunocomplex detection were done by the polymer-based detection system ADVANCE™ HRP kit (DAKO, Carpinteria, California) and the reaction was reveled with 3,3′-Diaminobenzidine (DAKO, Carpinteria, California) and counterstaining with hematoxylin (QEEL, São Paulo, São Paulo). Positive breast tissues for the corresponding antibody were used as internal staining controls. Negative staining controls were prepared replacing the primary antibody for 1% PBS-BSA.

2.3. Immunohistochemistry analyzes

Tissues were analyzed using an image system coupled to an Eclipse 50i microscope (Nikon, Melville, New York). The estrogen receptor (ER) and progesterone receptor (PR) expression analyzes were conducted following the recommendations of the ASCO/CAP ER/PR guidelines (2010) (Hammond et al., 2010) and HER-2 expression was evaluated according to the ASCO/CAP HER2 guideline (2013) (Wolff et al., 2013). Ki67 expression was evaluated according to Fountzilas and collegues (2012) (Fountzilas et al., 2012) and cases were considered positive when more than 14% of neoplastic cells nuclei expressed this protein. Tissue staining for FUT3 was classified based on the percentage of stained cells and dichotomized in positive and negative groups, considering positive samples when more than 50% of neoplastic cells were reactive to FUT3 antibody in at least 3 fields in a 100× magnification (Uhlen et al., 2010).

2.4. DNA extraction

DNA was extracted from 109 FFPE tumor samples following an optimized protocol adapted from Ramalho et al. (2014). The FFPE tissues used on the DNA extraction were previously characterized by the pathologist as containing tumor cells in their majority. Ten FFPE tissue slices (2 μm) from each sample were dried under vacuum and deparaffinized with 1 mL of xylene for 30 min at 55 °C under gentle stirring. After discard of previous xylene, deparaffinization was repeated. Ethanol (1 mL) was added to the sample, mixed by inversion and incubated at 55 °C for 5 min, followed by centrifugation at 14,000 *g* for 3 min. Ethanol was removed and ethanol/centrifuge process was repeated using 70% ethanol. Samples were dried under vacuum. After drying, deparaffinized tissue was digested with 400 μL of cell lysis buffer (0.5 M EDTA, 5 M NaCl, 1 M Tris), 36 μL of 20% SDS solution, 24 μL of proteinase K (20 mg/mL) and 480 μL of deionized water at 65 °C for 18 h. Proteins were precipitated with 420 μL of 5 M NaCl followed by centrifugation at 14,000 *g* for 20 min. Supernatant was transferred to a microtube containing 800 μL of cold isopropyl alcohol. After centrifugation at 14,000 *g* for 30 min, supernatant was discarded and 500 μL of ethanol was added and briefly mixed. Samples were centrifuged at 14,000 *g* for 15 min and the supernatant was carefully discarded. DNA was dried under vacuum, resuspended in 50 μL of TE buffer pH 8.0 (10 mM Tris.Cl,1 mM EDTA) and stored at −20 °C until use. DNA quantification was performed by Nanodrop 2000 Spectrophotometer (ThermoScientific, Waltham, Massachusetts). DNA was also extracted from blood samples of 109 health controls and 15 IDC patients. Extraction was performed with Wizard® Genomic DNA Purification Kit (Promega, Madison, Wisconsin) according to manufacturer's protocol.

2.5. FUT3 promoter genotype

Regulatory region of *FUT3* gene was amplified by PCR and sequenced using the primers ProFUT3_fw (5′-CTGGGTCTGGTATGCAAT-3′) and ProFUT3_rv (5′-GGTAGAATGGGATAACTT-3′). All PCR reactions were performed using Platinum®Taq DNA Polymerase (Invitrogen, Grand Island, New York) according to manufacturer's protocol. Each reaction contained 2 μM of each primer, 0.2 μM of each dNTP and 200 ng of DNA template for a final reaction volume of 25 μL. Amplification conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 62 °C for 1 min and 72 °C for 45 s and a final step at 72 °C for 5 min. Amplification was confirmed through visualization of a 507 bp fragment after electrophoresis on 1% agarose gel at 100 V for 90 min, stained with ethidium bromide (0.5 μg/mL). Amplicons' purification was performed using Exonuclease 1 and Shrimp alkaline phosphatase (USB, Cleveland,

Ohio). Fragments were sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) according to manufacturer's protocol adapted for MegaBACE 500 system (General Electric, Fairfield, Connecticut). After precipitation with ammonia acetate and ethanol, DNA was resuspended in 10 μL of MegaBACE Loading Buffer (General Electric, Fairfield, Connecticut) and samples were injected for electrophoresis running and sequencing in MegaBACE 500 (General Electric, Fairfield, Connecticut). Nucleotide sequences were analyzed using the CLC Main Workbench v.6 software (Quiagen, Venlo, Netherlands).

2.6. Prediction of transcription factor binding sites

The in silico prediction of transcription factors whose binding sites could be formed or blocked by different *FUT3* promoter region genotypes was performed using Alggen Promo software, V 3.0.2.

2.7. Statistical analysis

Statistical association was analyzed using the nonparametric Chi-square test, for genotype data, and Fisher's Exact test, for expression data. p value (p), odds ratio (OR) and its 95% confidence interval (CI) were calculated. $p < 0.05$ was considered statistically significant. Student's t-test was used for analysis of variances' homogeneity. All Statistical analysis was performed using the software GraphPad Prism version 5.

3. Results

A total of 127 breast IDC patients and 121 health controls were included in this study. Clinical and histopathological data were collected from medical files and molecular expression pattern of classic breast tumor markers was assessed by immunohistochemistry assay for IDC samples. Mean age of IDC patients was 53.6 ± 14.1 (range 31–90) and health controls were 53.6 ± 13.3 (range 21–85), showing no statistically significant difference between the two groups ($p = 0.8650$, D'Agostino & Pearson omnibus normality test). IDC population had a predominance of tumors classified as G2 or G3 (94% of all cases) according to Modified Bloom–Richardson histological grade system (Lakhani et al., 2012). All other histopathologic parameters, such as tumor size, limph node metastasis and molecular expression pattern, showed a homogeneous distribution.

From the 127 IDC samples, 85 were analyzed by immunohistochemistry assay. Normal ductal breast tissues obtained from 11 donors submitted to cosmetic surgery were used as immunohistochemistry assay controls. FUT3 staining in positive tissues showed a granular cytoplasmic pattern (Fig. 1). Positive FUT3 staining was more frequent in health breast tissues than in IDC tissues ($p = 0.0034$, OR = 7.55, 95% CI 1.97–28.93), suggesting an association of absent FUT3 expression and IDC development (Table 1). Among IDC samples, no statistic significant differences were found between patients with different histological grades or lymph node metastasis status. However, tumor size analyzes demonstrated that non-expression of FUT3 was more frequent in larger IDC tumors ($p = 0.0116$, OR = 10.22, 95% CI 1.23–84.74) and in HER-2 negative samples ($p = 0.006$, OR = 18.2, 95% CI 2.23–148.4) (Table 1).

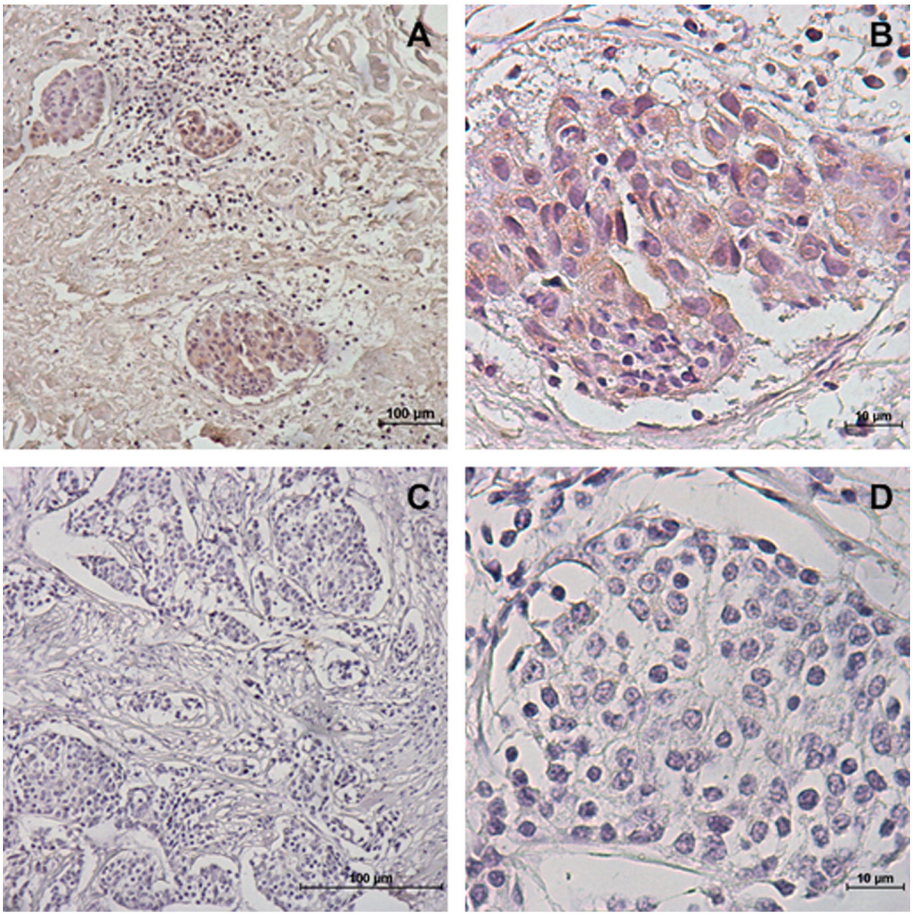

Fig. 1. Immune staining pattern of FUT3 in breast invasive ductal carcinoma (IDC): positive staining - magnification of 100× (A) and 400× (B); negative staining - magnification of 100× (C) and 400× (D).

Table 1
FUT3 expression pattern in patients with breast invasive ductal carcinoma (IDC: case) and normal breast tissue (control).

FUT3 expression	Positive	Negative	*p* value	Odds ratio
Control (n = 11)	7 (0.64)	4 (0.36)	Ref.	
Case (n = 85)	16 (0.19)	69 (0.81)	0.0034**	7.55 (1.97–28.93)
Histological grade				
I	0 (0)	7 (1)	Ref.	
II	8 (0.25)	24 (0.75)	0.3077	
III	7 (0.19)	30 (0.81)	0.5753	
Lymph Node metastasis				
Absent	2 (0.09)	21 (0.91)	Ref.	
Present	11 (0.23)	36 (0.77)	0.1959	
Tumor diameter (cm)				
≤5 cm	12 (0.31)	27 (0.69)	Ref.	
>5 cm	1 (0.04)	23 (0.96)	0.0116*	10.22 (1.23–84.74)
Estrogen receptor				
Positive	7 (0.2)	28 (0.8)	Ref.	
Negative	6 (0.22)	21 (0.78)	1.00	
Progesterone receptor				
Positive	7 (0.2)	27 (0.8)	Ref.	
Negative	5 (0.19)	21 (0.81)	1.00	
HER-2				
Positive	13 (0.34)	25 (0.66)	Ref.	
Negative	1 (0.03)	35 (0.97)	0.006*	18.2 (2.23–148.4)
Ki-67				
Positive	5 (0.29)	12 (0.71)	Ref.	
Negative	8 (0.22)	29 (0.78)	0.7328	

Statistical significance and odds ratio measured by Fisher exact test using the software GraphPad Prism v. 5.
* $p < 0.05$.
** $p < 0.005$.

Table 2
Genotype distribution of rs73920070 and rs2306969 *FUT3* promoter SNPs in samples with breast invasive ductal carcinoma (IDC) and normal breast.

rs73920070	Case (n = 109)	Control (n = 110)	OR (CI 95%)	*p* value
Allele				
C	216 (0.99)	211 (0.96)	Ref.	
T	2 (0.01)	9 (0.04)	0.22 (0.05–1.02)	0.0338*
Codominant				
C/C	107 (0.98)	101 (0.92)	Ref.	
C/T	2 (0.02)	9 (0.08)	0.21 (0.04–0.99)	0.0315*
T/T	0 (0)	0 (0)		
Dominant				
C/C	107 (0.98)	101 (0.92)	Ref.	
C/T + T/T	2 (0.02)	9 (0.08)	0.21 (0.04–0.99)	0.0315*
Recessive				
C/C + C/T	109	110		
T/T	0	0		
Overdominant				
C/C + T/T	107 (0.98)	101 (0.92)	Ref.	
C/T	2 (0.02)	9 (0.08)	0.21 (0.04–0.99)	0.0315*
rs2306969†	(n = 109)	(n = 109†)		
Allele				
C	115 (0.53)	151 (0.69)	Ref.	
T	103 (0.47)	67 (0.31)	2.02 (1.36–2.99)	0.0004**
Codominant				
C/C	11 (0.10)	57 (0.52)	Ref.	<0.0001***
C/T	93 (0.85)	37 (0.34)	13.02 (6.15–27.56)	<0.0001***
T/T	5 (0.05)	15 (0.14)	1.73 (0.52–5.74)	0.3685
Dominant				
C/C	11 (0.10)	57 (0.52)	Ref.	
C/T + T/T	98 (0.90)	52 (0.48)	9.77 (4.72–20.22)	<0.0001***
Recessive				
C/C + C/T	104 (0.95)	94 (0.86)	Ref.	
T/T	5 (0.05)	15 (0.14)	0.30 (0.11–0.86)	0.019*
Overdominant				
C/C + T/T	16 (0.15)	72 (0.66)	Ref.	
C/T	93 (0.85)	37 (0.34)	11.31 (5.83–21.94)	<0.0001***

Statistical significance and odds ratio mensured by two-tailed Chi-square test using the software GraphPad Prism v.5.
* $p < 0.05$.
** $p < 0.005$.
*** $p < 0.001$.
† Genotyping data ware not possible to be acquired in one control sample.

In attempt to elucidate the reason of the absence of FUT3 expression in IDC patients, we extracted DNA from 109 FFPE tissue samples, out of the 127 IDC group, and sequenced the *FUT3* promoter region. As control, DNA was also extracted and sequenced from 110 blood samples from cancer-free donors. A 507 bp fragment was amplified to genotype 10 single nucleotide polymorphisms (SNPs) previously reported in dbSNP database, NCBI (Sherry et al., 2001). Among all SNPs verified in this study, four of them were biallelic in the study population (Supplementary Table 1). Variations rs73920070 (−6933 C > T) and rs2306969 (−6951 C > T) showed statistically significant differences in their genotype frequency between IDC patients and healthy controls. At position −6933 (rs73920070) the heterozygous genotype C/T was more frequent in healthy subjects than IDC patients (for the co-dominant model, $p = 0.0315$, OR = 0.21, 95% CI 0.04–0.99), as well as the minor allele T ($p = 0.0338$, OR = 0.22, 95% CI 0.05–1.02). On the other hand, the heterozygous genotype C/T at the position −6951 (rs2306969) was more frequent in IDC patients than in health subjects (for the co-dominant model, $p < 0.0001$, OR = 13.02, 95% CI 6.154–27.56), as well as the minor allele T ($p = 0.0004$, OR = 2.02, 95% CI 1.36–2.99). However, the homozygous genotype for the minor allele T showed a similar frequency between the two groups ($p = 0.51$). The genotype distribution of rs2306969 and rs73920070 *FUT3* SNPs in different models is showed in Table 2. Blood samples from 15 IDC patients were genotyped to confirm whether the variations observed in this study were really germline polymorphisms. The blood genotype for all SNPs from all samples analyzed matched with those obtained from the respectively tumor tissue samples, confirming the germinal origin of the polymorphisms.

The in silico prediction of transcription factors whose binding sites could be formed or blocked by the SNPs rs73920070 and rs2306969 in *FUT3* promoter region was performed using Alggen Promo software, V 3.0.2 (Farré et al., 2003). The presence of allele T instead C in SNP rs73920070 leads to the formation of 2 new transcription factor binding sites, to the TFIID and GR-α. In turn, the presence of allele T instead C in SNP rs2306969 leads to loss of 6 transcription factor binding sites, TFII-I, STAT-4, c-Ets-1, Ebk-1, MAZ and GR-α.

4. Discussion

Alpha1,3-fucosyltransferases are a family of enzymes related to the synthesis of Lewis antigens and constituted by FUT3-FUT7 and FUT 8[9]. Involved in the leukocyte diapedesis, sialyl Lewis X antigen (sLe^x, Neu5Acα2-3Galα1-4(Fucα1-3)GlcNAc) and its isomer sialyl Lewis A antigen (sLe^a, Neu5Acα2-3Galα1-3(Fucα1-4)GlcNAc) are fucosylated glycans, recognized by E-selectins, in cell-cell adhesion molecules localized in vascular endothelium. Interactions between sLe^x/sLe^a and E-selectins mediate the leukocyte rolling on the endothelium, an essential step for the escape of these cells to inflammatory sites (Dube and Bertozzi, 2005). It is well known that this mechanism is also used by tumor cells to mediate their extravasation to secondary sites during hematogenous metastasis (Burdick et al., 2012). SLe^x and sLe^a antigens are overexpressed in solid tumors such as head and neck (Czerwinski et al., 2013), renal (Borzym-kluczyk and Radziejewska, 2013), lung (Komatsu et al., 2013), colon (Mare et al., 2013), gastric (Kim et al., 2011),

pancreatic (Ballehaninna and Chamberlain, 2012), bile duct (Kikkawa et al., 2012), prostate (Dimitroff et al., 2004) and breast (Jeschke et al., 2005) carcinomas.

The FUT3 enzyme has an important role in Lewis antigens synthesis. Reduced expression of FUT3 in colon, prostate, gastric and cholangiocarcinoma tumor cell lines leads to suppression in Lewis antigens levels (Padró et al., 2011a; Silsirivanit et al., 2013; Weston et al., 1999; Xin et al., 2012; Yin et al., 2010) and overexpression of this enzyme results in raised levels of sLe^a and sLe^x in pancreatic and prostatic tumor cell lines, respectively (Aubert et al., 2000; Barthel et al., 2009). In colon cancer cell lines, FUT3 is also involved in ephitelial-mesenchymal transition (EMT) stimulated by TGF-β. TβR I and TβR II receptor's fucosylation mediated by this enzyme is fundamental for TGF-β signaling of EMT process (Hirakawa et al., 2014). Acquisition of a mesenchymal phenotype is a mechanism used by tumor cells to allow them to disseminate from primary sites and self-renew in a secondary site, leading to motility and metastasis (Wang and Zhou, 2011). Thus, due the role of FUT3 in two important steps of metastatic process, we previously hypothesized that an overexpression of this enzyme would be involved in IDC, leading to a more malignant and metastatic tumor.

In contrast, we did not find any correlation between the FUT3 expression and tumor malignancy or metastatic properties of IDC. However, we observed that lack of FUT3 enzyme expression is more frequently found in breast invasive ductal carcinoma patients from the State of Pernambuco, Northeast Brazil. Furthermore, tumor size analyzes demonstrated the correlation between FUT3 non-expression with larger lesions and HER-2 negative IDC tumors. Our results corroborate with those obtained by Teresa et al. (2010) that showed a high incidence of negative *FUT3* genotypes, which leads to the synthesis of inactive truncated enzyme, in Southeastern Brazilian patients with IDC when compared to health controls. However, this previously study did not predict any correlation between negative *FUT3* genotype and tumor size or molecular pattern (Teresa et al., 2010). Although these are unexpected results since high levels of Lewis antigens have been seen in breast invasive carcinomas, sharing of α1,3-fucosyltransferase activity with other fucosyltransferases could, at least partially, explain this contradiction. Fucosylation of sLe^x antigen could also be performed by FUT4, FUT6 and FUT7 (Kannagi, 2004). In fact, breast carcinomas showed high levels of FUT4 when compared with normal tissues and this enzyme is overexpressed in high metastatic breast cancer cell lines (Yan et al., 2015). Therefore, the increase in sLe^x levels observed in breast carcinoma could be a result of FUT4 elevated activity in these lesions. Nevertheless, sLe^a fucosylation is mediated only by FUT3 but enzymatic activity and mRNA levels of this enzyme were not associated with sLe^a overexpression in gastrointestinal and ovarian tumors, respectively (Dohi et al., 1994; Escrevente et al., 2006). So, more studies are necessary to understand the mechanisms involved in the regulation of sLe^a expression.

Few investigations of FUT3 expression in tumor tissue samples have been made. *In vitro* studies with tumor cells lines demonstrated a major metastatic potential in prostatic and pancreatic tumor cells which overexpressed FUT3 (Aubert et al., 2000; Barthel et al., 2009) but this methodology do not completely reproduce the tumor heterogeneity and microenvironment, and could bring bias to the analysis (Vargo-Gogola and Rosen, 2007). Unlike the observed in metastatic prostate tumor cell lines, the analysis of FUT3 in Pernambuco State patient's tissue samples identified lower levels of this enzyme in prostatic adenocarcinoma than in benign prostatic hyperplasia (Vasconcelos et al., 2013). In patients with colorectal carcinoma, low expression of *FUT3* mRNA was related to tumor infiltration and distant metastasis (Petretti et al., 2000).

The differences between the results obtained by in vitro and *in vivo* analysis could be related to the role of immunologic system against the tumor cells *in vivo* and the involvement of FUT3 in this mechanism. Transfection of melanoma and erythroleukemia cells with *FUT3* gene led to a high cytotoxicity mediated by NK cells and this process was inhibited by anti-sLe^x antibody addition. The recognition of tumor cells via sLe^x by NK cells and triggering of cytotoxicity is mediated by C-type lectin receptors, such as CD-94 and NKG2D (Higai et al., 2006; Ohyama et al., 2002). Another death pathway against tumor cells stimulated by NK cells and influenced by the fucosylation in the Apo2L/TRAIL. The TNF-related apoptosis-inducing ligand (also known as Apo2L/TRAIL) mediates an extrinsic apoptosis pathway shooted by its ligation to TRAIL receptors DR4 and DR5. This ligation leads to receptor oligomerization on the cell membrane and initiation of apoptosis of the target cell. TRAIL can also interact with decoy receptors DCR1 and DCR2 resulting in possible inhibition of the pathway. TRAIL pathway is related to NK cell induced cytotoxicity against tumor cells (Johnstone et al., 2008). In colorectal cancer, overexpression of *FUT3* mRNA was found in Apo2L/TRAIL sensitive cell lines. Reducing the O-glycosilation in these cell lines results in reduced sensitivity to this pathway (Wagner et al., 2007). Colon cancer cell line HCT116 that did not express fucosylated antigens due to expression of a truncated GMDS enzyme resulted in larger tumors *in vivo* than the same cell line that express fucosylated antigens after GMDS transfection (rescue). HCT116 rescued cells were more sensitive to TRAIL stimulation than not rescued ones (Moriwaki et al., 2009). These finds showed a possible relationship between the expression of FUT3 enzyme and NK-induced cytotoxicity through C-type lectin receptors and Apo2L/TRAIL pathway. Due the importance of NK cell in immune system antitumor action against breast cancer cells (Kajitani et al., 2012), we propose that no expression of FUT3 enzyme in breast IDC samples observed in this study would be result of an adaptive advantage that led to an immunoresistance of the tumor cells (Fig. 2).

Genomic analysis of *FUT3* promoter region showed that two variations localized in this region might be associated with IDC in Brazilian patients. Minor allele T of SNP rs73920070 (−6933 C > T) were more frequently observed in the cancer-free control group whereas minor allele T of SNP rs2306969 (−6951 C > T) were more frequently observed in IDC Brazilian patients from Pernambuco state. Few studies have reported these polymorphisms in other populations. In a Caucasian population, rs2306969 T allele was associated with ovarian cancer risk (Sellers et al., 2008). The same SNP in a European population showed that the minor allele T have a weak relationship with intestinal gastric cancer and its heritage with mutant variations of other two *FUT3* SNPs (rs778986 and rs11673407) is associated with diffuse gastric cancer (Duell et al., 2014). Minor allele T of rs2306969 in patients with colorectal cancer from southern China was associated with high levels of serum carcinoembryonic antigen (CEA) a colorectal cancer biomarker (He et al., 2014). However, until the present moment studies were not found correlating rs73920070 with health disorders.

The Allgen PROMO *in silico* analysis of transcription factor binding sites using different genotypes for rs73920070 and rs2306969 gave us insight how those SNPs could influence the *FUT3* mRNA transcription. The T allele of SNP rs73920070, more frequent in our control samples, leads to the gain of two potential sites for the transcription factors TFII-D and GR-α, respectively. Those transcription factors could positively modulate the transcription of *FUT3* gene, possibly increasing the expression of FUT3 enzyme, which was also associated with absence of IDC. The opposite was seen for T allele of SNP rs2306969. The minor allele for this variation, more frequent in IDC patients, alters six potential sites for the transcription factors TFII-I, STAT-4, c-Ets-1, Ebk-1, MAZ and GR-α, respectively, impairing their ligation. Transcription of *FUT3* gene and enzyme expression would be reduced, which was also associated with presence of IDC according to our immunohistochemistry results. However, in vitro assays have to be done to evaluate the real impact of SNPs rs73920070 and rs2306969 in FUT3 expression.

The dual role of the FUT3 in tumor cells could indicate that these cells modulate its expression to acquire beneficial characteristics during their establishment in the primary site and later during metastasis. Our results demonstrated that the non-expression of FUT3 enzyme is related to invasive ductal carcinoma in Brazilian patients and this pattern is

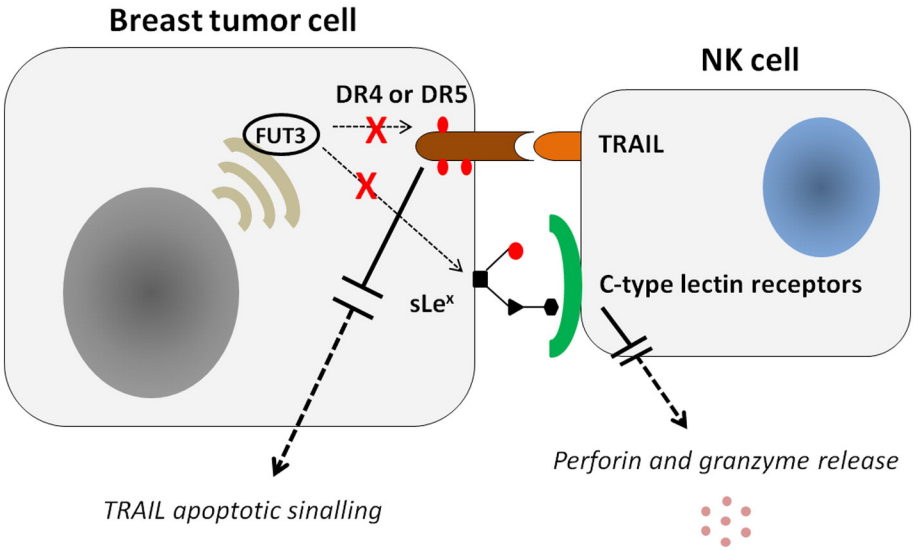

Fig. 2. Schematic representation α1,3/4-fucosyltransferase (FUT3) enzyme in Natural killer-induced cytotoxicity against breast tumor cells. The lack of FUT3 enzyme by breast cancer cells could result in no fucosylation of DR4 and DR5 receptors, blocking the TRAIL apoptotic signaling. FUT3 absence would also reduce fucosylated antigens in cell surface leading to non-recognition of Lewis antigens in tumor cell surface by C-type lectin receptor, preventing release of perforin and granzyme by NK-cells. Both mechanisms could promote the immunoresistance of breast tumor cell. NK: Natural killer; DR4: Death receptors 4; DR5: Death receptor 5; TRAIL: TNF-related apoptosis-inducing ligand; sLe^x: Sialyl Lewis X antigen.

more frequent in larger and HER-2 negative tumors. Moreover, two SNPs localized in *FUT3* promoter region were found in this population, suggesting that rs73920070 (−6933 C > T) confers protection whereas rs2306969 (−6951 C > T) triggers to susceptibility to IDC. Analyzing previous studies, we hypothesized that this FUT3 down regulation is an adaptive advantage developed by breast tumor cells to acquire an immune-resistant phenotype while the up-regulating of others α1,3-fucosyltransferases, such as FUT4, contributes to the metastatic power. Studies are under development to better understand the role of the FUT3 expression in breast tumor cells regarding the immune response and the functional role of the SNPs rs73920070 and rs2306969 in FUT3 expression and IDC development.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yexmp.2015.08.015.

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Prognostic importance of CD56 expression in intermediate risk acute myeloid leukaemia

Recently, we showed that clinical outcomes of Brazilian acute myeloid leukaemia (AML) patients were significantly inferior to those in the USA and Europe. Excluding well-recognized differences between developed and developing countries, heterogeneity in outcome still exists in patients with the same cytogenetic risk (Lima *et al*, 2015). Therefore, it would be interesting to identify supplementary biological markers that yield non-redundant biological information for further prognostic refinement of AML patients. In this context, aberrant expression of CD56 antigen (also termed NCAM1) in leukaemic blasts has been frequently associated with inferior outcome in patients with AML, particularly those with t(8;21) (Iriyama *et al*, 2013) and t(15;17) abnormalities (Montesinos *et al*, 2011). Nevertheless, knowledge regarding its prognostic value in other subtypes of AML remains limited. Here, we evaluated clinical outcomes of non-selected patients with AML (non-acute promyelocytic leukaemia) according to CD56 expression. Our data suggest that aberrant expression of this antigen may predict inferior outcome in patients with intermediate outcome.

One hundred and forty-one newly diagnosed AML patients were included between August 2008 and April 2015, with last follow-up in May 2015. Patients were classified according to morphological, immunophenotypic and cytogenetic findings. Treatment protocol was previously described (Lima *et al*, 2015). The local Research Ethics Board approved the study. To determine CD56 antigen expression, immunophenotypic analysis was accomplished using diagnostic bone-marrow samples and standard flow-cytometry methods. Patients were designated 'CD56-positive' when ≥20% of leukaemic cells expressed CD56 antigen (Bene *et al*, 1995). Fisher's exact test or Chi-square test, as appropriate, was used to compare categorical variables. Wilcoxon rank-sum test was used to compare continuous variables. Overall survival (OS) and disease-free survival (DFS) were calculated using the Kaplan–Meier method. OS was defined as the time from the initiation of induction therapy to death from any cause; those alive or lost to follow-up were censored at the date last known alive. DFS was defined as the time from complete remission to disease relapse, development of secondary malignancy, or death from any cause, whichever occurred first. Patients who were alive without disease relapse or secondary malignancy were censored at the time last seen alive and disease-free. The log-rank test was used for comparisons of Kaplan–Meier curves.

Univariate and multivariate proportional hazards regression analysis was performed for potential prognostic factors for OS and DFS (Table I). Potential prognostic factors examined in multivariate regression analysis were CD56 expression, cytogenetic risk stratification, age at diagnosis and leucocyte counts. Proportional hazards assumption for each variable of interest was tested. Linearity assumption for all continuous variables was examined using restricted cubic spline estimates of the relationship between the continuous variable and log relative hazard/risk. All *P*-values were two sided with a significance level of 0·05. All calculations were performed using Stata Statistic/Data Analysis version 12 (Stata Corporation, College Station, TX, USA) and R 2.10.1 (The CRAN project, www.r-project.org) software.

Table I. Univariate and multivariate analysis for overall survival and disease-free survival.

		Univariate analysis				Multivariate analysis			
End point	Model Variables	HR	95% CI		*P*-value	HR	95% CI		*P*-value
Overall survival	CD56 expression: positive *versus* negative	1·79	1·28	2·49	0·001	1·94	1·09	3·45	0·022
	Age (years): continuous variable	1·1	1·06	1·2	0·001	1·1	1·01	1·3	0·041
	Cytogenetic risk stratification: adverse *versus* intermediate *vs* favourable	1·47	1·27	2·33	0·003	1·19	0·73	1·93	0·48
	Leucocyte counts (x 10^9/l): continuous variable	1·03	1·01	1·05	0·014	1·02	0·99	1·07	0·297
Disease-free survival	CD56 expression: positive *versus* negative	4·49	1·93	10·5	<0·001	6·35	1·85	21·7	0·003
	Age (years): continuous variable	0·99	0·97	1·02	0·797	0·99	0·97	1·02	0·96
	Cytogenetic risk stratification: adverse *versus* intermediate *versus* favourable	1·64	0·57	4·69	0·354	3·52	1·12	11·1	0·031
	Leucocyte counts (x 10^9/l): continuous variable	1·08	1·03	1·14	0·001	0·99	0·98	1·09	0·581

Hazard ratios (HR) >1 or <1 indicate an increased or decreased risk, respectively, of an event for the first category listed.

Patient baseline characteristics were reported descriptively. The median age was 49 years (range: 18–84 years) with 64 males (45%). Thirty-nine patients (28%) were ≥ 60 years. Pre-treatment bone marrow samples were analysed by G-banding cytogenetics, of which 105 (75%) were successful. Patients were stratified according to Grimwade *et al* (2010) as follows: favourable (28/105; 27%), intermediate (64/105; 61%) and adverse (13/105; 12%); 50 patients (48%) were cytogenetically normal. CD56 analysis was accomplished in 127/141 patients (90%). The remaining 14 patients (10%) had no CD56 data or samples available for further characterization, and were excluded from analysis. To test whether the samples without CD56 results were missing at random, the OS was evaluated for patients with and without CD56 data. Five-year OS rate did not differ between patients with (35%) and without (25%) available CD56 data ($P = 0{\cdot}96$). Aberrant CD56 expression was identified in 33 patients (26%). There were no significant differences among CD56-negative and CD56-positive patients with respect to baseline characteristics. The frequencies of CD56 expression in favourable, intermediate and adverse groups were 31%, 22%, and 55%, respectively ($P = 0{\cdot}102$).

Three of the 141 included patients were lost to follow-up prior to the assessment of remission status and were excluded from subsequent analyses. Overall, 72/138 patients (52%) achieved complete remission (CR). CD56 expression

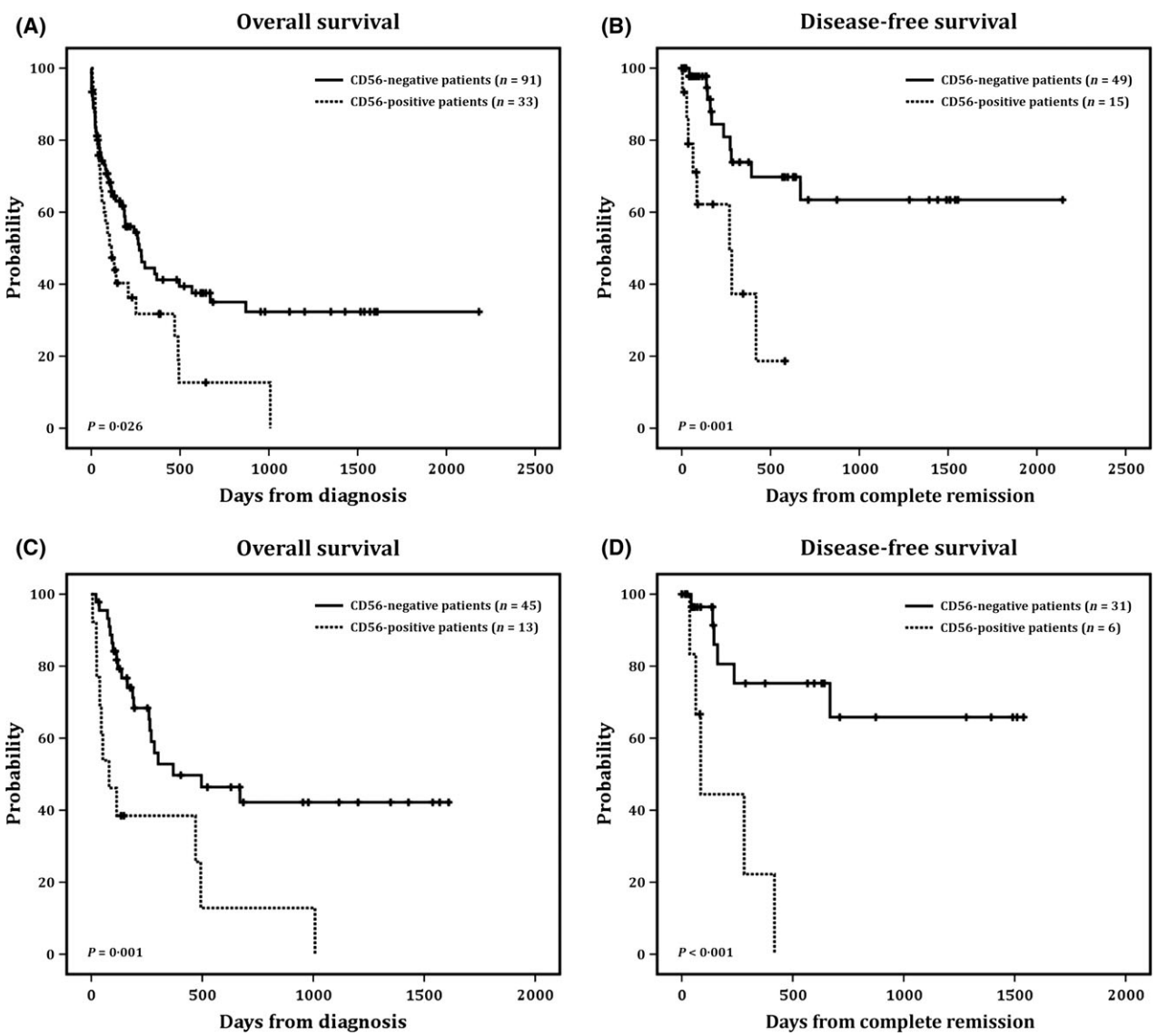

Fig 1. Patient survival. Probability of overall survival (**A**) and disease-free survival (**B**) in acute myeloid leukaemia patients according to CD56 expression (entire cohort). Overall survival (**C**) and disease-free survival (**D**) in patients with intermediate outcome according to CD56 expression. Overall survival and disease-free survival were estimated using the Kaplan-Meier method and the log-rank test was used for comparison of the survival curves. n, the number of patients included in each analysis.

was not associated with CR achievement ($P = 0{\cdot}424$). The median follow-up was 208 d (95% confidence interval [CI]: 106–309 d). CD56-positive patients exhibited significantly shorter survival (114 d, 95%CI: 48–179 d) than CD56-negative patients (270 d, 95%CI: 161–378 d) ($P = 0{\cdot}026$; Fig 1A). The median DFS for CD56-positive and CD56-negative groups was 269 d (95%CI: 28–509 d), and 1467 d (95%CI: 1120–1815 d), respectively ($P = 0{\cdot}001$; Fig 1B). In multivariate proportional hazards regression analysis, CD56 expression was independently associated with shorter OS (hazard ratio [HR]: 1·94, 95%CI: 1·09–3·45; $P = 0{\cdot}022$) and shorter DFS (HR: 6·35, 95%CI: 1·85–21·7; $P = 0{\cdot}003$), considering cytogenetic risk stratification, leucocyte counts and age at diagnosis as confounders. Next, we analysed the impact of CD56 expression in each cytogenetic risk group. For patients assigned to the favourable group, CD56 expression had no impact on CR ($P = 0{\cdot}202$), OS ($P = 0{\cdot}161$) or DFS ($P = 0{\cdot}642$). Similar results were observed in the adverse group (CR, $P = 0{\cdot}635$; OS, $P = 0{\cdot}14$; DFS, $P = 0{\cdot}317$). In contrast, aberrant expression of CD56 identified patients assigned to the intermediate group with shorter OS ($P = 0{\cdot}001$; Fig 1C) and DFS ($P < 0{\cdot}001$; Fig 1D), but had no impact on CR ($P = 0{\cdot}191$). These results were consistent with the multivariate analysis (OS, HR: 3·19, 95%CI: 1·49–6·84; $P = 0{\cdot}003$; DFS, HR: 7·79, 95%CI: 1·8–33·6; $P = 0{\cdot}006$).

We and others (Raspadori *et al*, 2001; Alegretti *et al*, 2011; Djunic *et al*, 2012) have demonstrated that aberrant expression of CD56 antigen is associated with inferior outcome in AML. Of interest, some evidence suggests that CD56-positive blasts may emerge from less-differentiated leukaemic stem cells (Montesinos *et al*, 2011) and are less sensitive to standard chemotherapy schemes (Chapiro *et al*, 2006). Furthermore, co-expression with multidrug resistance (Raspadori *et al*, 2001) and extramedullary infiltrates (Chang *et al*, 2004) are frequent findings in CD56-positive patientsand may underlie the adverse outcomes predicted by CD56 antigen. To the best of our knowledge, our study represents the first to evaluate the prognostic impact of CD56 expression in a real-life setting and address these findings to a large and heterogeneous subset of AML, such as intermediate cytogenetic risk group. However, limitations of our study include limited number of patients and a relative short follow-up. These results need to be confirmed in an independent cohort. If confirmed, CD56 measurement may constitute a cheap and effective alternative for the management of AML patients in less privileged countries, where the global majority of AML patients will probably be treated and where access to molecular tests are still limited.

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Conflicts of Interest

The authors have no competing financial interests to declare.

Authorship Contributions

J.L.C-S. collected, analysed and interpreted data and drafted the manuscript. A.R.L-A performed the statistical analyses, interpreted data and drafted the manuscript. L.E.C., M.F.B., M.M.O., P.L.F-N., M.A.N., M.C.B-C., C.G.M. and D.M.M. obtained the samples, updated the clinical data and reviewed the manuscript. M.A.B., D.M.M. and A.R.L-A reviewed the paper and gave final approval for the submitted version.

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To the editor:

Clinical outcomes of patients with acute myeloid leukemia: evaluation of genetic and molecular findings in a real-life setting

Despite its undeniable importance, standard cytogenetic analysis is still not a routine practice for most reference centers in developing countries, and its accomplishment is practically limited to university research centers. Particularly in Brazil, little is known about the cytogenetic and molecular characterization of patients with acute myeloid leukemia (AML), and results about clinical outcomes remain scarce. In an effort to minimize the lack of results and contribute new insights in our setting, we reported clinical features and outcomes of 241 nonselected Brazilian patients with AML (nonacute promyelocytic leukemia) from 2 university hospitals, followed from November 2005 to April 2015.

Patients were classified according morphologic, immunophenotypic, and cytogenetic findings. Treatment protocols were similar between centers. For patients up to 60 years of age, the treatment protocol was adapted according to performance status and the presence of comorbidities (in particular, cardiac disorders). Briefly, the conventional chemotherapy consisted of daunorubicin (45-90 mg/m^2 per day for 3 days) and cytarabine (100-200 mg/m^2 per day for 7 days) or thioguanine, cytosine arabinoside, and daunorubicin as induction,[1] followed by 3 or 4 cycles of consolidation therapy with high doses of cytarabine (>1 g/m^2 per day). For patients who did not achieve complete remission (CR) after 1 course of chemotherapy, a second course was administered between days 28 and 35 after the end of the first course. CR was assessed by bone marrow examination on day 28 after each course of chemotherapy. For those who needed it, a postremission therapy based on autologous or allogeneic transplantation was performed. Patients older than 60 years were treated with low-dose cytarabine; a combination of etoposide, thioguanine, and idarubicin; or best supportive care. The local research ethics board of each participating center approved the study. Research was conducted in accordance with the Declaration of Helsinki.

The baseline characteristics are summarized in supplemental Table 1. One hundred thirty patients were enrolled in Recife (northeast Brazil, 54%), and 111 patients were enrolled in Campinas (southeast Brazil, 46%). Baseline features were similar between centers. The median age was 47 years (range, 18-97 years) with 114 males (47%). Sixty-two patients (26%) were older than 60 years. Pretreatment bone marrow samples were analyzed by G-banding cytogenetics, of which 187 (78%) were successful. According to Medical Research Council trials,[2] patients were stratified as follows: favorable (30/187, 16%), intermediate (119/187, 64%), and adverse (38/187, 20%). Overall, 101 patients (42%) were cytogenetically normal. To test whether the samples without cytogenetics results were missing at random, the overall survival (OS) was evaluated for patients with and without cytogenetics data. The 5-year OS rate did not differ between patients with (18%) and without (23%) available cytogenetics data (P = .372). Additionally, the entire cohort was fully characterized for *NPM1* and *FLT3*-ITD mutations. Details can be found in the supplemental Data, available on the *Blood* Web site.

Out of 241 enrolled patients, 39 patients (16%) who started the induction treatment were lost to follow-up without assessment for CR. Of 202 evaluable patients, 115 (57%) achieved complete hematologic remission. CR rates according to the cytogenetic risk stratification were 33%, 64%, and 77% for adverse, intermediate, and favorable groups, respectively (P = .001). The logistic regression analysis revealed that age (odds ratio [OR], 0.95; 95% confidence interval [CI], 0.93-0.98; P = .002) and cytogenetic risk stratification (OR, 0.41; 95% CI, 0.19-0.89; P = .024) were independently

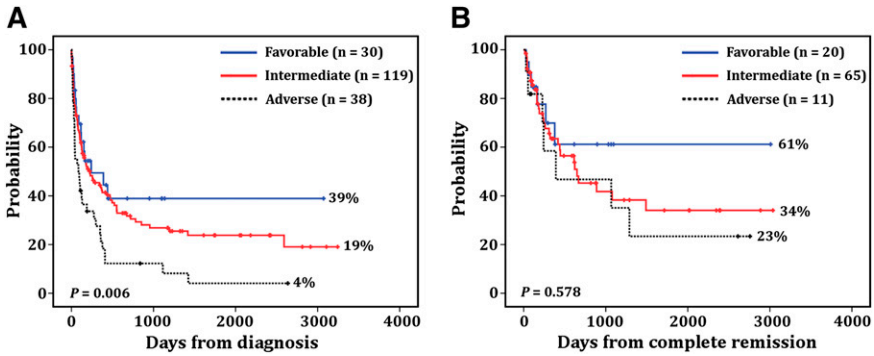

Figure 1. Patient survival. Probability of OS (A) and DFS (B) in patients with AML according to cytogenetic risk stratification.[1] The percentages of each group are presented on the graph. The "n" indicates the number of patients included in each analysis.

associated with CR. The median follow-up among survivors was 177 days (95% CI, 85-268 days). According to Medical Research Council criteria, patients assigned to the adverse group exhibited significantly shorter survival (85 days; 95% CI, 43-169 days) than patients assigned to the intermediate (224 days; 95% CI, 72-375 days) and favorable groups (241 days; 95% CI, 168-572 days) ($P = .006$; Figure 1A). Age (hazard ratio [HR], 1.1; 95% CI, 1.01-1.2; $P = .029$), cytogenetic risk stratification (HR, 1.45; 95% CI, 1.02-2.06; $P = .036$), and white blood cell counts (HR, 1.03; 95% CI, 1-1.06; $P = .021$) were independently associated with poor OS. The median disease-free survival (DFS) for adverse, intermediate, and favorable groups was 392 days (95% CI, 37-1544 days), 650 days (95% CI, 208-1091 days), and 668 days (95% CI, 57-1278 days), respectively. In contrast to OS, cytogenetic risk stratification had no impact on DFS ($P = .578$; Figure 1B).

In summary, we identified patients with favorable, intermediate, and adverse outcomes with frequencies very similar to those reported by other groups.[2,3] However, our patients' clinical outcomes were significantly inferior to those reported by developed countries. The most significant difference was observed in the favorable group, whose survival rate was significantly lower (39%) than rates reported by studies conducted in the United States and Europe.[2,4] Furthermore, the early mortality rate was especially higher in our cohort (42%), which is probably a major cause of lower OS rate. Among the main reasons for high early mortality in Brazil, we highlight the lack of adequate hospital infrastructure, especially during induction therapy.[5,6] As a consequence, high incidence of bacterial and fungal infections are frequently reported.[7] It is important to note that our results are representative of a real-life setting, which strongly differs from the well-controlled clinical trials conducted in developing countries.

Altogether, our results and others[1,5,6,8] draw attention to an urgent need for improved clinical support and treatment of patients with AML in order to obtain results comparable to those reported in developed countries. Such improvements may be achieved through international collaborative efforts, which have already proved their effectiveness in economically less privileged countries. A great example is the International Consortium on Acute Promyelocytic Leukemia study.[9] This initiative considerably reduced the differences in treatment outcome of patients with acute promyelocytic leukemia between developed and developing countries through the dissemination of knowledge and exchange of experience from well-established cooperative groups in the United States and Europe. We hope that with better hospital infrastructure and such initiatives, the clinical outcomes of our patients will improve in the near future.

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To the editor:

GM-CSF stimulates granulopoiesis in a congenital neutropenia patient with loss-of-function biallelic heterozygous *CSF3R* mutations

Severe congenital neutropenia (CN) is a heterogeneous disease characterized by an absolute neutrophil count (ANC) below 500 cells per microliter and recurrent, life-threatening bacterial infections. Treatment with recombinant human granulocyte colony-stimulating factor (rhG-CSF) was shown to increase ANCs in the majority of patients and dramatically improves the quality of life.[1] In a previous study, we demonstrated major differences in responses to G-CSF and granulocyte macrophage CSF (GM-CSF) in patients with CN.[2] In the majority of these patients, GM-CSF failed to induce neutrophil granulocyte counts but did induce monocytosis and eosinophilia.[2] Many underlying genetic defects have been identified. Among them are mutations in the *ELANE*, *HAX1*, and *G6PC3* genes and many others.[3] A recent study identified patients with biallelic loss-of-function *CSF3R* mutations who were considered to have a novel subtype of CN.[4] These patients did not respond to G-CSF therapy, and no treatment options were available for them. Heterozygous acquired mutations in the *CSF3R* gene were also reported in CN patients.[5]

In this study, we identified a CN patient who did not respond to G-CSF treatment. Three days after birth, the female patient was diagnosed with CN with blood ANC below 250 cells per microliter. White blood cell differential counts were as follows: 6% neutrophils, 7% monocytes, 4% eosinophils, and 83% lymphocytes. A bone marrow (BM) evaluation at 3 weeks of age revealed granulopoietic hypoplasia with reduction of all stages but no maturation arrest or increase in blasts. Erythropoiesis, lymphopoiesis, and megakaryopoiesis were normal. No antineutrophil antibodies were detected. Mutational screening revealed no mutations in *ELANE*, *HAX1*, or *G6PC3*. Cytogenetic evaluation revealed a normal karyotype.

Sequencing of the *CSF3R* gene showed 2 heterozygous mutations in this patient that revealed a compound heterozygous mode of inheritance of *CSF3R* mutations. In one allele intronic mutation, c.998-2A>T leads to the skipping of exon 9 and introduces an aberrant sequence downstream of exon 8 and a shift in the reading frame. In the second allele, we detected a stop-codon (p.W547*) mutation in the extracellular part of the G-CSF receptor (G-CSFR) (Figure 1A). The p.W547* mutation was inherited from the father and the c.998-2A>T mutation was inherited from the mother.

No expression of G-CSFR was detected on the patient's neutrophils or monocytes in contrast to blood cells from the healthy donors (Figure 1B). Granulocyte macrophage CSFR (GM-CSFR) expression on $CD33^+$ cells from the patient's BM was similar to that observed for BM cells from healthy donors. In addition, we plated the patient's BM mononuclear cells in a semisolid medium supplemented with 10 ng/mL G-CSF, 10 ng/mL GM-CSF, or a cytokine cocktail containing G-CSF, GM-CSF, interleukin-3, stem cell factor, and erythropoietin, and cultured them for 14 days. No granulocyte colony-forming unit (CFU-G), granulocyte macrophage CFU, or macrophage CFU colonies were found in plates supplemented with 10 ng/mL rhG-CSF. In sharp contrast, all types of colonies grew on plates containing either rhGM-CSF or the cytokine cocktail.

G-CSF treatment of this patient was initiated at the age of 3 weeks, but ANCs failed to increase with doses up to 110 μg/kg per day. At the age of 7 months, treatment with GM-CSF (6 μg/kg per day) was initiated. In the first year of GM-CSF treatment, peripheral blood ANCs ranged from 860 to 3744 cells per microliter, enabling the GM-CSF dose to be reduced to 3 μg/kg per day twice a week (Figure 1C). To evaluate whether GM-CSF was still required to maintain sufficient ANCs, GM-CSF administration was stopped with prophylactic administration of oral antibiotics. Because of the development of otitis media, GM-CSF treatment was restarted at a dose of 3 μg/kg twice a week. This dose was sufficient to maintain the patient's ANC above 1000 cells per microliter and kept her free from infections. The patient has remained on GM-CSF treatment for the last 12 years without any adverse events.

In summary, we provide the first demonstration of the successful treatment of a CN patient harboring biallelic loss-of-function *CSF3R* mutations who did not respond to G-CSF by administering GM-CSF. These findings suggest that all CN patients who do not respond to G-CSF should be screened for germ-line *CSF3R*

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Clinical outcomes of patients with acute myeloid leukemia: evaluation of genetic and molecular findings in a real-life setting

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Brief communication

Schistosomal liver fibrosis and hepatocellular carcinoma – case series of patients submitted to liver transplantation

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ABSTRACT

Schistosomiasis affects approximately 207 million people in 76 countries. The association between hepatocellular carcinoma and *Schistosoma mansoni* infection has been investigated. Studies using animal models suggest that the parasite may accelerate the oncogenic process when combined with other factors, such as hepatitis C virus infection or exposure to a carcinogen. Herein, we report a case series of six hepatocellular carcinoma patients from Northeast Brazil, with negative serology for both hepatitis B and C virus, submitted to liver transplantation, whose explant showed evidence of schistosomal liver fibrosis. Since all patients enrolled in this study were submitted to liver transplantation, we were able to access the whole explanted liver and perform histopathological analysis, which is often not possible in other situations. Although 50% of them showed signs of liver failure, no cirrhosis or any liver disease other than schistosomal fibrosis had been detected. These uncommon findings suggest that *Schistosoma mansoni* infection might predispose to hepatocellular carcinoma development, regardless of the absence of other risk factors.

The World Health Organization estimated that 207 million people from 76 countries were infected by the various species of *Schistosoma* in 2016.[1] The Brazilian Ministry of Health estimates that up to 6 million people are infected with *Schistosoma mansoni* in Brazil, of whom 5–10% will develop the hepatosplenic form. Most cases occur in the Northeast region and the state of Pernambuco accounts for 35% of national deaths due to this condition.[2]

The hepatosplenic form of *S. mansoni* infection (SMI) is due to egg-induced inflammation and fibrosis in liver presinusoidal portal areas, leading to portal hypertension, esophageal varices formation and digestive bleeding. A typical feature of

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Table 1 – Demographic, clinical, laboratory and histopathological data of six patients with HCC and SLF submitted to liver transplantation in Recife – Brazil.

	Patients					
	1	2	3	4	5	6
Sex	Male	Male	Male	Female	Male	Male
Age (years)	53	45	46	34	70	57
UGB	Yes	No	No	Yes	Yes	No
Splenectomy	No	No	No	Yes	Yes	No
LFS	Ascites	No	No	Ascites	Ascites + jaundice	No
MELD	12	7	7	10	15	8
Comorbidities	None	DM, AC	None	None	DM, AC	DM
Liver/body weight (%)	1.61	4.50	2.22	2.14	1.34	1.60
Liver parenchyma	SLF + SG	SLF + SG	SLF	SLF + SG	SLF	SLF + SG
Milan criteria	In	Out	Out	In	In	Out

HCC, hepatocellular carcinoma; SLF, schistosomal liver fibrosis; UGB, upper gastrointestinal bleeding; LFS, liver failure signs; MELD, model for end-stage liver disease; DM, diabetes mellitus; AC, alcohol consumption; SG, schistosomal granuloma.

this condition is the maintenance of liver perfusion due to an increase in hepatic arterial flow, allowing the preservation of hepatocyte function.[3]

The association between SMI and hepatocellular carcinoma (HCC) has been evaluated over the last 40 years in both experimental and clinical studies, of which many were performed in Egypt. However, due to the use of non-sterile shared syringes for SMI treatment in the past, that country has a high prevalence of hepatitis C and schistosomal coinfection.[4] Therefore, the co-occurrence of HCV and SMI in the Egyptian population makes it difficult to comprehend the real contribution of SMI in the oncogenesis of HCC. Importantly, the previous studies lack histopathological material that analyzes the whole liver. Such resource is essential to rule out other conditions that may contribute to carcinogenesis.

The aim of this study is to identify the frequency of schistosomal liver fibrosis (SLF) in patients submitted to liver transplantation (LT) due to HCC in a reference center in Northeastern Brazil, describing the clinical, laboratory and histological characteristics of the patients whose explant showed only SLF. A case series was drawn up from data collected from the medical records of 170 patients submitted to LT for HCC treatment at the Liver Transplantation Unit of the Oswaldo Cruz Hospital in Recife, Pernambuco, Northeast Brazil, from 1999 to December 2016. The study included cases for which the histopathological examination of the explant demonstrated the presence of SLF. Patients with current or previous evidence of hepatitis B or C and with histopathological evidence of cirrhosis or other liver diseases were excluded.

Of the 170 patients submitted to LT for HCC treatment between 1999 and 2016, six (3.5%) were identified with an isolate finding of SLF when the explant was analyzed. A summary of these six included cases is shown in Table 1. Five of them were male and all were from endemic areas of Northeast Brazil. The age at transplantation ranged from 34 to 70 years, with a median of 53 years. Three patients reported a previous episode of upper gastrointestinal bleeding (UGB) due to rupture of esophageal varices, and two of them had undergone splenectomy. In three patients, the indication of LT as HCC therapy was due to bilobar tumor involvement and in the remaining three due to signs of hepatic failure, such as ascites and/or jaundice. All patients with clinical signs of liver failure had previous UGB.

All explants showed stellate portal fibrosis with vascular proliferation, typical of Symmers' fibrosis and in four cases there were schistosomal granulomas (Fig. 1). As to the weight of the explant, three cases had a reduction in the ratio explant/total body weight, two of which had signs of liver failure. Although three patients had other risk factors for HCC,

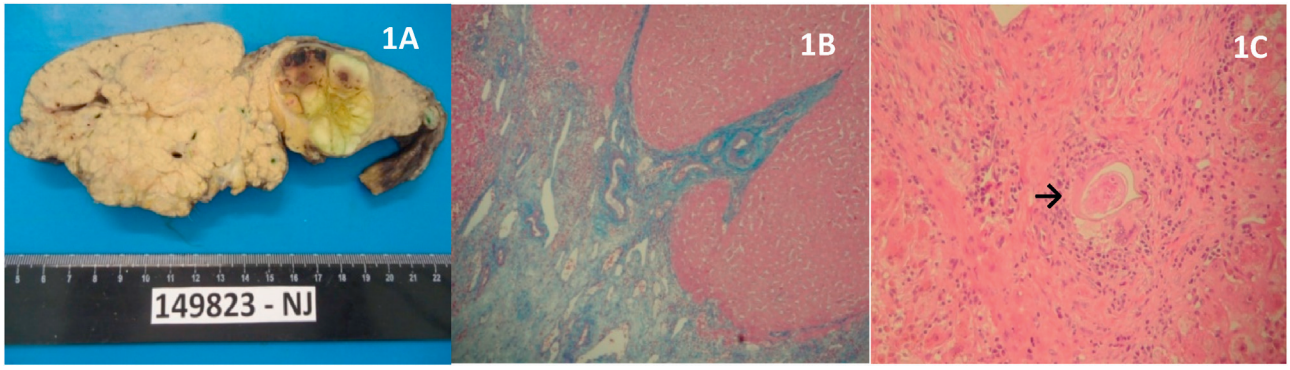

Fig. 1 – Explant showing a hepatocellular carcinoma in the left lobe of a liver with schistosomal fibrosis and absence of nodular transformation (A). Microscopic slides showing stellate portal fibrosis with vascular proliferation (B – Masson's trichrome, 40×) and a schistosomal granuloma (C, arrow, hematoxylin–eosine, 100×).

such as diabetes and alcohol consumption, the histopathological analysis of their explants did not show any cirrhosis, steatosis, or other liver diseases. No complete portal vein thrombosis was found in any case; only one patient had partial occlusion of this vessel.

It is speculated that parasitic infections may play a role in carcinogenesis due to changes of the host inflammatory response. The association between *Schistosoma haematobium* and squamous cell carcinoma of the bladder is already well established.[5] The association between SMI and HCC has been studied since the 1970s. Studies with experimental animals showed a higher frequency of liver dysplasia and HCC in the group exposed concomitantly to carcinogens and SMI.[6,7]

In a case–control study with 75 patients with HCC and HCV divided according to the presence of SMI, El-Tonsy et al. observed that the co-infected patients were younger and had a higher proportion of larger and multifocal tumors.[8] Sabry et al. found serologic evidence of SMI in 20 of 60 HCC cases in Egypt, of whom only four were co-infected with HCV.[9] Another Brazilian cases series described seven HCC patients with schistosomiasis; however, some of these patients had portal thrombosis or previous hepatitis B.[10] It is important to highlight that none of these studies performed histopathological analysis of the liver to exclude other predisposing diseases or some degree of liver cirrhosis.

It is important to note that, in this study, half of the patients had clinical signs of liver failure, with no histological evidence of cirrhosis, and they were precisely those who had a history of UGB. Due to portal obstruction, schistosomiasis patients present hepatic sinusoidal arterialization. Consequently, they are more vulnerable to sudden falls in blood pressure due to UGB episodes, developing focal areas of hepatic necrosis.[11]

In summary, by studying HCC patients from a schistosomiasis endemic region who had undergone liver transplantation, we were able to perform histopathologic analyses of the explanted liver, which is not commonly available in non-transplanted HCC patients due to biopsing-related risks. Therefore, the present case series is the first to describe an association between HCC and SLF based on the whole analysis of the liver, which permits the exclusion of other associated hepatic diseases. Although this case series brings clinical evidence about the possible association between SMI and HCC, it does not establish a causal relation, thus, further investigations are required to better understand the current observations.

Conflicts of interest

The authors declare no conflicts of interest.

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Research Article

Glycophenotype Evaluation in Cutaneous Tumors Using Lectins Labeled with Acridinium Ester

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Background. Tumor cells show alterations in their glycosylation patterns when compared to normal cells. Lectins can be used to evaluate these glycocode changes. Chemiluminescence assay is an effective technique for quantitative analysis of proteins, nucleic acids, and carbohydrates due to its high sensitivity, specificity, and rapid testing. *Objective*. To use histochemiluminescence based on lectin conjugated to acridinium ester (AE) for the investigation of glycophenotype changes in cutaneous tumors. *Methods*. Concanavalin A (Con A), Peanut agglutinin (PNA), *Ulex europaeus* agglutinin-I (UEA-I), and *Maackia amurensis* agglutinin (MAA) were conjugated to acridinium ester. Biopsies of cutaneous tumors and normal skin were incubated with the lectins-AE, and chemiluminescence was quantified and expressed as Relative Light Units (RLU). *Results*. Actinic keratosis (AK), keratoacanthoma (KA), squamous cell carcinoma (SCC), and basal cell carcinoma (BCC) showed lower expression of α-D-glucose/mannose and α-L-fucose residues compared to normal tissue. Cutaneous tumors displayed higher expression of Gal-β(1-3)-GalNAc residues than normal tissue. AK and SCC exhibited higher expression of Neu5Ac-α(2,3)Gal residues than normal epidermis. KA and BCC showed equivalent RLU values compared to normal tissue. *Conclusions*. Lectin histochemiluminescence allowed quantitative assessment of the carbohydrate expression in cutaneous tissues, contributing to eliminate the subjectivity of conventional techniques used in the histopathological diagnosis.

1. Introduction

Nearly all types of malignant cells and many types of diseased tissue cells demonstrate alterations in their glycosylation patterns when compared to their normal counterparts [1]. Alterations in glycosyltransferases expression, sugar nucleotide donors, and disruption of the Golgi contribute to the development of diseases, such as hereditary disorders, immune deficiencies, cardiovascular disease, and cancer [2, 3]. In the tumor environment, underexpression, truncation or altered branching patterns of certain glycans, and/or changes in glycosylation allow neoplasm cells to usurp many of the events that occur in development such as receptor activation, cell adhesion, and cell motility, which permits tumor cells to invade and spread throughout the organism [4]. The investigation of lectin-carbohydrates interactions is necessary for the understanding of both the glycophenotype and the behavior of the tumor during its differentiation and confirms the hypothesis that biochemical changes in the cell are events that may signal the cell differentiation [5].

Lectins have been used to evaluate the glycophenotype in skin tissues [6–9]. Lectin histochemistry based on colorimetric detection has been used as a supporting tool for differential diagnosis for skin lesions, but this technique only

permits qualitative or semiquantitative analyses of carbohydrates expression. Thus, quantitative analyses require a sensitive detection that permits an objective and quantitative evaluation of the bound lectin.

Chemiluminescence (CL) analyses represent a powerful tool in life sciences, offering high detectability and specificity [10, 11] and allowing low limits of detection (attomole-zeptomole) and versatility [12]. It is a promising and effective technique for quantitative analysis of proteins, nucleic acids, and carbohydrates [13–15]. Therefore, this approach combined with lectin histochemistry could be valuable to reduce the subjective evaluation.

Acridinium ester (AE) compounds quickly outperformed luminol and isoluminol in chemiluminescence for their low detection limits (attomolar range) and specificity [16]. Exposure of AE label to an alkaline hydrogen peroxide solution triggers a flash of light. AE forms an unstable dioxetane yielding *N*-methylacridone and produces light at a wavelength of 470 nm [17].

The skin tumors comprise mainly basal cell carcinoma (BCC), squamous cell carcinoma (SCC), keratoacanthoma (KA), and actinic keratosis (AK). Nonmelanoma skin cancers are the most common form of cancer and their incidence has been increasing considerably. These tumors are rarely fatal but are considered to be fast growing and if neglected may be locally and functionally destructive [18]. The diagnosis of skin neoplasias becomes inaccurate, in some cases, due to a variety of factors that affect the test accuracy, such as a huge spectrum of tumors and their variants and the lower differentiation [8].

Here lectins conjugated to AE were used to evaluate the glycophenotype in tissues from cutaneous tumors based on the chemiluminescence approach. The biological relevant carbohydrate residues α-D-glucose/mannose, Gal-β(1-3)-GalNAc, α-L-fucose, and Neu5Ac-α(2,3) were evaluated, respectively, by using the lectins Concanavalin A (Con A), Peanut agglutinin (PNA), *Ulex europaeus* agglutinin-I (UEA-I), and *Maackia amurensis* agglutinin (MAA).

2. Material and Methods

2.1. Reagents. *N*-hydroxysuccinimide-activated dimethyl acridinium ester ((DMAE-NHS)/1966-1-53-2/Organic Lab) was kindly supplied by Dr. H. H. Weetall. Con A, PNA, UEA-I, MAA, N,N-dimethylformamide, trypsin from porcine pancreas, methyl-α-D-mannoside, D-galactose, α-L-fucose, n-acetylneuraminic acid, and Sephadex G-25 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chemiluminescent detection was performed using Siemens Reagent TSH 500T (Siemens Medical Solutions Diagnostics, Malvern, PA, USA) composed of 0.5% H_2O_2 in 0.1 N HNO_3 and 0.25 M NaOH. Xylene and ethanol were obtained from Merck (Darmstadt, Germany). All other chemical reagents used were of analytical grade.

2.2. Samples. Skin fragments were obtained through paraffin-embedded biopsies from the Tissue Bank of the Clinic Hospital at the Federal University of the State of Pernambuco (UFPE), Northeastern Region of Brazil, and diagnosed as basal cell carcinoma (BCC = 17), squamous cell carcinoma (SCC = 13), keratoacanthoma (KA = 13), and actinic keratosis (AK = 9). The diagnosis of skin neoplasms was performed by Dr. Mariana Silva. The normal human epidermis fragments (NE = 14) were obtained from individuals submitted to plastic surgery and supplied from Hospital Santa Clara, Recife, PE, Brazil. The diagnosis of the cutaneous tumors was based on histopathology. This study was approved by the Health Science Centre Bioethical Board from the Federal University of Pernambuco, Brazil (CEP/CCS/UFPE no. 195/09).

2.3. Lectins Conjugation with AE. Lectins (1 mL containing 2 mg of protein) were incubated with 10 μL of acridinium ester solution (0.2 mg diluted in 400 μL of N,N-dimethylformamide) for 1 h at 25°C under rotary stirring. The conjugate (lectin-AE) was applied to a column of Sephadex G-25 (10 × 1 cm), previously equilibrated with 10 mM phosphate buffer, containing 0.15 M NaCl (PBS), pH 7.2, and eluted with this buffer. Aliquots (1 mL) were collected and their protein content was spectrophotometrically determined at 280 nm. The aliquots corresponding to the protein peak had their chemiluminescence assayed. After conjugation, Con A-AE, PNA-AE, and MAA-AE were evaluated regarding the maintenance of their carbohydrate recognition property (hemagglutinating activity) using glutaraldehyde treated rabbit erythrocytes and human erythrocytes for UEA-AE according to Beltrão et al. [19]. Protein concentration was measured according to Lowry et al. [20].

2.4. Chemiluminescent Lectin-Histochemistry. Paraffin sections (8 μm) of samples were cut, transferred to glass slides, deparaffinized in xylene, and rehydrated in graded alcohols (3 × 100% and 1 × 70%—10 dips each). Slices were treated with 0.1% (w/v) trypsin solution at 37°C for 2 min and washed (twice, 5 minutes each time) with 10 mM phosphate buffer saline containing 0.15M NaCl (PBS). Afterwards, tissue slices were incubated with lectins-AE (100 μL–100 μg mL^{-1}) for 2 h at 4°C followed by washings (3 × 5 min) with 100 mL of PBS. The area corresponding to the tissue section (square-shaped) was defined as 0.25 cm^2. Then, the tissues were cut and transferred to polypropylene test tubes containing 50 μL of PBS. Finally, solutions of 0.5% H_2O_2 in 0.1 N HNO_3 (50 μL) and 0.25 M NaOH (50 μL) were added for chemiluminescent measurement using a luminometer Modulus Single Tube 9200-001 (Turner BioSystems, USA). The emission intensity was determined as relative light units (RLU) with a counting time of 5 seconds per sample. Triplicate measurements were carried out throughout this study. Lectin binding inhibition assays were accomplished by incubating each lectin solution with 300 mM methyl-α-D-mannoside (Con A), D-galactose (PNA), α-L-fucose (UEA-I), and n-acetylneuraminic acid (MAA) for 45 min at 25°C prior to their incubation with tissues. The following steps were as described previously for the binding protocol.

2.5. Statistical Analysis. The software OriginPro 8 (OriginLab Corporation, One Roundhouse Plaza, Northampton, MA, USA) was used for the statistical analysis, and data were expressed as mean ± standard deviation (s.d.). Obtained data

were compared using ANOVA and parametric statistical test of Tukey ($P < 0.05$) through SigmaPlot (CA, USA).

3. Results

Lectins conjugated to acridinium ester (Con A-AE, PNA-AE, UEA-AE, and MAA-AE) were collected by Sephadex G-25 chromatography and their profiles were identical to those previously reported [15]. The superposition of the protein and chemiluminescence peaks indicated that all studied lectins were labeled with the AE. The lectin derivatives were also capable of recognizing their specific carbohydrates according to the hemagglutinating activity.

These lectin derivatives were used to investigate the glycophenotype of cutaneous tumor tissues and the results are displayed in Figure 1. Lower expression of α-D-glucose/mannose was presented for all tumors compared with the normal tissue. AK, KA, SCC, and BCC showed values of RLU equal to 143,694 ± 28,626 RLU; 140,704 ± 29,009 RLU; 148,238 ± 38,333 RLU, and 134,007 ± 30,537 RLU, respectively, whereas the normal tissue was 271,435 ± 64,329 RLU. The α-L-fucose expression pattern was also lower for cutaneous tumors (RLU values of 11,831 ± 1,935 RLU; 28,773 ± 6,468 RLU; 14,586 ± 3,464 RLU, and 13,880 ± 2,926 RLU for AK, KA, SCC, and BCC, resp.) compared with the normal epidermis (33,743 ± 5,182 RLU). On the other hand, they presented higher expression of Gal-β(1-3)-GalNAc compared with the normal skin tissues. AK, KA, SCC, and BCC presented RLU values of 23,766 ± 2,525 RLU; 32,567 ± 6,127 RLU; 31,172 ± 5,134 RLU; and 18,007 ± 4,618 RLU, respectively, whereas a value of 7,567 ± 1,799 RLU was found for the normal tissue. Actinic keratosis and SCC exhibited higher expression of Neu5Ac-α(2,3)Gal than normal epidermis (35,687 ± 8,226 RLU, 65,370 ± 16,811 RLU, and 44,864 ± 11,644 RLU, resp.). However, KA (26,927 ± 6,942 RLU) and BCC (29,836 ± 6,179 RLU) showed equal RLU values compared with the normal tissue. The standard deviation of all measurements varied from 10.6% to 25.9% of the mean value.

For all lectins, the mean among RLU values of cutaneous tumors and normal epidermis was significantly different, as observed using parametric statistical test of Tukey. However, statistically significant variations were not observed among RLU values of cutaneous tumors labeled with ConA-AE, AK and KA labeled with PNA-AE, AK and SCC labeled with UEA-AE, and BCC and KA labeled with MAA-AE. Lectins-AE inhibition binding assay using specifics carbohydrates (300 mM) resulted in a decrease in RLU values (from 65% to 93%) indicating that unspecific binding between the conjugates and cell surface molecules did not occur.

4. Discussion

In our laboratory, the acridinium ester was used as a marker to *Trypanosoma cruzi* and *Schistosoma mansoni* antigens in chemiluminescent immunoassay. By this method, small amounts (nanogram scale) of circulating antibodies were detected in the patients' serum [21]. The glycocode of normal, fibroadenoma, and invasive duct carcinoma tissues was investigated using lectins labeled with AE [15, 22]. Anti-HER-2-AE allowed a reduction in the subjectivity in prognostic and predictive information intrinsic to Her-2 status evaluation in breast tumors [23].

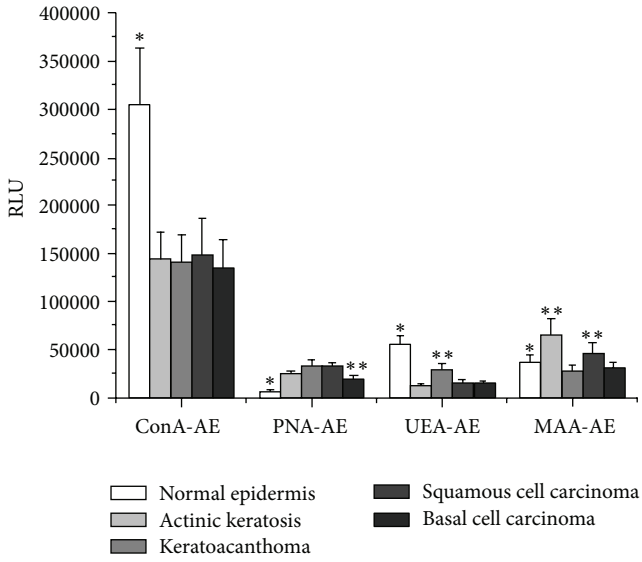

Figure 1: Comparison between the chemiluminescence of normal epidermis (NE, $n = 14$), actinic keratosis (AK, $n = 9$), keratoacanthoma (KA, $n = 13$), squamous cell carcinoma (SCC, $n = 13$), and basal cell carcinoma (BCC, $n = 17$) labeled with Con A-AE, PNA-AE, UEA-AE, and MAA-AE. (∗) Statistically significant variations were observed among RLU values of cutaneous tumors and normal epidermis. (∗∗) Statistically significant variations were observed among RLU values of cutaneous tumors.

Cell surface glycans are highly related to cell-cell communication, host-pathogen interaction, and cell matrix interactions [24]. Tumor invasion is dependent on a loss of intercellular adhesion and transmigration of cells through the basement membrane (BM) as well as the surrounding extracellular matrix (ECM) [25]. Metastasis is a multistep phenomenon, which involves the loosening of the tumor cells from the primary tumor, the degradation of the extracellular matrix, and the invasion of blood vessels at the site of the primary tumor [6]. These steps require adhesive interactions, which are mediated by cell surface glycans and their interactions with endogenous carbohydrate binding proteins (lectins). Aberrant glycosylation is a key feature of malignant transformation and the glycans involved influence the adhesive interactions of cancer cells often providing favorable conditions for tumor dissemination [26]. The molecular organization and the stability of adherent junctions formation are influenced by the presence of altered glycans on the cell surface, phenotypic change commonly observed in malignancy [27].

Alterations in the expression of glycophenotype between benign and malignant skin lesions have been previously reported through lectin histochemistry and computer image analysis [8]. It is worthwhile to register that they did not compare with normal epidermis and included trichoepithelioma and seborrheic keratosis that were not studied in this work.

These authors reported higher expression of α-D-glucose/mannose (except in SCC) and D-galactose residues in cutaneous tumors. The chemiluminescent α-D-glucose/mannose detection (Con A-AE) corroborated these findings but they were statistically lower than those found for the normal epidermis. They also found high D-galactose residues (PNA) as well as our results except that lower RLU was detected compared to the normal epidermis. Another contradiction was found as far as fucose is concerned. Here fucose was chemiluminescently detected in the tumors that were statistically lower than that estimated for normal epidermis. Therefore, these lectins can be used as markers for distinction among skin lesions; however, the chemiluminescent approach may be more sensitive than that based on computer image analysis of lectin histochemistry.

Smetana et al. [28] reported that laminins, a principal component of BM, exhibit unique saccharidic epitopes (mannose-rich oligosaccharides). Therefore, aberrant α-D-glucose/mannose expression in this protein may modify the adhesive interactions between transformed cells, contributing for the detachment of cancer cells from the primary tumor mass and the acquisition of a more motile and invasive phenotype.

Several common structural changes occur in tumor glycans and can affect interactions between tumor cell-surface glycans and endogenous lectins, which may determine the metastatic potential of the tumor cell, including increases in the level of truncation and branching of structures as well as an increased expression of unusual terminal sequences [29]. These changes often result in an increased exposure of terminal galactose residues such as those found in the cancer-associated T antigen (Gal β1-3 GalNAc) and the Lewis X trisaccharide (Galβ1 4(Fucα1-3)GlcNAc) [30]. The higher expression of galactose residues observed by high RLU could be related to the potential role of galactose ligands in tumor cell metastasis by the correlation between increased expression of galectin, a family of galactose binding proteins, and the ability of many tumor cell types to metastasize [31].

Fucosylation is one of the most common modifications involving oligosaccharides on glycoproteins or glycolipids. It regulates the biological functions of adhesion molecules and growth factor receptors [32]. In this study, fucosylation pattern changes were observed by the decrease of chemiluminescence signal using UEA-I conjugated to AE for the labeling of cutaneous tumors. AK showed fucosylation pattern similar to malignant lesions and different when compared to benign lesion (KA). This suggests a biochemical similarity with malignant neoplasms and supports the studies claiming that AK and CEC are the same lesion in different degrees of development.

It is known that both integrins and E-cadherin are associated with cancer cells characteristics through regulation of the cell-extracellular matrix interaction and homotypic cell-cell adhesion, respectively [33]. Zhao et al. [34] showed that core fucosylation is essential for the function of $\alpha 3\beta 1$ integrin, a protein that connects many biological functions such as development, control of cell proliferation, and protection against apoptosis and malignant transformation. Osumi et al. [35] suggested a possible core fucosylation role in cell-cell adhesion regulation in cancer. Therefore, based on the information above, it can be concluded that fucose would be involved in the biological behavior of cancer cells through functions regulation of many membrane associated proteins.

Sialic acids (Sias) are typically the outermost monosaccharide units on the glycan chains of glycolipids and glycoproteins and usually occur as a terminal component at the nonreducing end of their carbohydrate chains [36]. Due to its terminal localization on glycans and the variety of linkages to the underlying sugar chain, sialic acid has been utilized by a wide variety of Sia-binding proteins, mainly viral and bacterial pathogens proteins, selectins, and siglecs [37].

The increase in the expression of Neu5Ac-α(2,3)Gal between benign and malignant lesions of skin, AK, and SCC, respectively, was observed by the increase of RLU values. The differences in the sialylation degree of glycoconjugates on a tumor cell surface of SCC may play an important role in the process of cell malignization and metastasis [38]. Vural et al. [39] reported the increase of total sialic acid (TSA) levels between patients with AK and controls, probably related to increased turnover of cells [40]. Plzák et al. [13] observed that differentiated squamous cell carcinoma expresses 2,3-linked-NeuAc residues (recognized by lectin MAA) and the poorly differentiated squamous cell carcinoma 2,6-linked-NeuAc (recognized by *Sambucus nigra* agglutinin).

Sialyl-glycoconjugates may regulate the interaction of cancer cells with other cells and with the cell matrix. These molecules may be responsible for adhesion as well as antiadhesion and for extending the survival time of cancer cells in the blood stream. Moreover, sialic acid may also be involved in growth regulation [41].

From the results presented in this work one can assume that the chemiluminescent lectin-histochemistry approach allows the glycophenotype direct quantitative evaluation of skin tumors combining the specificity lectin-carbohydrates interaction and sensitivity of chemiluminescent assay. Despite that the number of analyzed samples (n values) does not permit a definitive assumption as far as diagnosis is concerned the statistical analyses showed glycophenotype alterations according to the cutaneous tumors. Furthermore, although paraffin-embedded skin fragments were obtained from different individuals and sections were cut from them, acceptable mean dispersion values (standard variation range of 10.6%–25.9%) were observed. Higher expression of β-Gal (1-3)-GalNAc and Neu5Ac-α(2,3)Gal as well as lower expression of α-D-glucose/mannose and α-L-fucose residues was evidenced by differences in RLU values. These results confirm the importance of lectins as potential biomarkers for detection of changes in glycophenotype of skin neoplasms. Furthermore, this procedure is an effective tool to eliminate subjective analyses of microscopic preparations.

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PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: ESTUDO DO PADRÃO DE METILAÇÃO DA REGIÃO PROMOTORA DOS GENES BRCA1 E BRCA2 EM PACIENTES COM LEUCEMIA MIELÓIDE AGUDA

Pesquisador: Matheus Filgueira Bezerra

Área Temática:

Versão: 1

CAAE: 30229114.1.0000.5208

Instituição Proponente: LABORATÓRIO DE IMUNOPATOLOGIA KEISO ASAMI

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 664.427

Data da Relatoria: 30/05/2014

Apresentação do Projeto:

Indicado na relatoria inicial.

Objetivo da Pesquisa:

Indicado na relatoria inicial.

Avaliação dos Riscos e Benefícios:

Indicado na relatoria inicial.

Comentários e Considerações sobre a Pesquisa:

Indicado na relatoria inicial.

Considerações sobre os Termos de apresentação obrigatória:

Indicado na relatoria inicial.

Recomendações:

Recomenda-se:

a)No cronograma o mês deve ser informado por extenso ou abreviado;

b)Nos caso de pesquisados analfabetos, o TCLE deve apresentar o seguinte: A ROGO DE.....

QUE É ANALFABBETO EU.....ASSINO O PRESENTE DOCUMENTO;

Endereço: Av. da Engenharia s/nº - 1º andar, sala 4, Prédio do CCS
Bairro: Cidade Universitária **CEP:** 50.740-600
UF: PE **Município:** RECIFE
Telefone: (81)2126-8588 **Fax:** (81)2126-8588 **E-mail:** cepccs@ufpe.br

Conclusões ou Pendências e Lista de Inadequações:

Aprovado com recomendação.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

O Colegiado aprova o parecer do protocolo em questão e o pesquisador está autorizado para iniciar a coleta de dados.

Projeto foi avaliado e sua APROVAÇÃO definitiva será dada, após a entrega do relatório final, na PLATAFORMA BRASIL, através de "Notificação " e, após apreciação, será emitido Parecer Consubstanciado .

.

RECIFE, 28 de Maio de 2014

Assinado por:
GERALDO BOSCO LINDOSO COUTO
(Coordenador)

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: IMPACTO PROGNOSTICO DE ALTERAÇÕES MOLECULARES EM PACIENTES COM LEUCEMIA MIELOIDE AGUDA DO ADULTO

Pesquisador: Antonio Roberto Lucena de Araujo

Área Temática:

Versão: 2

CAAE: 50987015.3.0000.5208

Instituição Proponente: CENTRO DE CIÊNCIAS BIOLÓGICIAS

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.400.457

Apresentação do Projeto:

Projeto tem por título: “Impacto prognostico de alterações moleculares em pacientes com leucemia mieloide aguda do adulto”, o qual será desenvolvido pelo Prof. Dr. Antonio Roberto Lucena De Araujo da UFPE. O estudo será do tipo coorte prospectivo. Serão utilizadas amostras provenientes do Hospital do Câncer de Pernambuco, sendo elas aspirados de medula óssea ou sangue periférico. O trabalho pretende analisar em torno de 150 pacientes diagnosticados com leucemia mielóide aguda. Serão utilizadas amostras coletadas para o diagnóstico, não sendo necessária a coleta de material extra, evitando transtornos ao paciente. Serão direcionadas à pesquisa, somente as amostras de pacientes que assinarem o termo de consentimento livre e esclarecido antes da realização do procedimento. Serão incluídos no estudo pacientes diagnosticados com leucemia mielóide aguda de novo tratados e acompanhados no Hospital do Câncer de Pernambuco. O processamento das amostras será realizado no setor de Hematologia do Laboratório Central (LabCen) da UFPE. Para obtenção dos dados clínicos, serão utilizados os prontuários médicos do Hospital do Câncer de Pernambuco.

Objetivo da Pesquisa:

a. Objetivo Geral

Investigar a ocorrência de mutações nos genes FLT3, NPM1, DNMT3a, ASXL1 e KRAS em pacientes com leucemia mielóide aguda de novo do estado de Pernambuco. Associar o perfil molecular dos indivíduos com dados clínico-laboratoriais dos pacientes.

b. Objetivos Específicos

1 Determinar a incidência de mutações nos genes FLT3, NPM1, DNMT3a, ASXL1 e KRAS em pacientes com LMA de novo do estado de Pernambuco;

2 Associar estes achados com os dados clínico-laboratoriais;

3 Analisar a influência do status mutacional dos genes estudados diante do desfecho clínico (sobrevida global, sobrevida livre de doença, taxa de recaída, mortalidade precoce e remissão completa).

Avaliação dos Riscos e Benefícios:

Riscos:

Visto que o material biológico será o mesmo utilizado na rotina laboratorial do Hospital do Câncer de Pernambuco e será encaminhado à pesquisa somente após seu uso na rotina, o único risco será a possibilidade de causar uma situação desconfortável ao paciente devido ao convite para participar da pesquisa, que caso aconteça, será rapidamente contornada, visto que a participação ou não do paciente na pesquisa não influenciará seu tratamento. Em nenhuma ocasião será coletado material extra para uso da pesquisa.

Benefícios:

O estudo visa entender melhor o desenvolvimento da doença, tendo o objetivo de, juntamente com outros trabalhos publicados, possibilitar uma maior acurácia no estabelecimento do prognóstico. Apesar de a pesquisa de algumas destas mutações serem preconizadas pela OMS, o alto custo destes exames muitas vezes inviabiliza sua realização nas instituições públicas ou por custeio próprio do paciente. Nesse contexto, o projeto propõe realizar os testes em questão e fornecer os laudos à equipe do Hospital do Câncer e aos pacientes.

Comentários e Considerações sobre a Pesquisa:

O projeto está bem escrito e elaborado. Cientifica e tecnicamente é viável e há coerência entre o tempo e o número de pacientes. Os resultados obtidos serão importantes no diagnóstico e provavelmente irá contribuir de forma positiva na qualidade de vida dos pacientes envolvidos na

pesquisa.

Considerações sobre os Termos de apresentação obrigatória:

O pesquisador atendeu as exigências formuladas.

Recomendações:

Nenhuma.

Conclusões ou Pendências e Lista de Inadequações:

Nenhuma.

Considerações Finais a critério do CEP:

As exigências foram atendidas e o protocolo está APROVADO, sendo liberado para o início da coleta de dados. Informamos que a APROVAÇÃO DEFINITIVA do projeto só será dada após o envio do Relatório Final da pesquisa. O pesquisador deverá fazer o download do modelo de Relatório Final para enviá-lo via "Notificação", pela Plataforma Brasil. Siga as instruções do link "Para enviar Relatório Final", disponível no site do CEP/CCS/UFPE. Após apreciação desse relatório, o CEP emitirá novo Parecer Consubstanciado definitivo pelo sistema Plataforma Brasil.

Informamos, ainda, que o (a) pesquisador (a) deve desenvolver a pesquisa conforme delineada neste protocolo aprovado, exceto quando perceber risco ou dano não previsto ao voluntário participante (item V.3., da Resolução CNS/MS Nº 466/12).

Eventuais modificações nesta pesquisa devem ser solicitadas através de EMENDA ao projeto, identificando a parte do protocolo a ser modificada e suas justificativas.

Para projetos com mais de um ano de execução, é obrigatório que o pesquisador responsável pelo Protocolo de Pesquisa apresente a este Comitê de Ética relatórios parciais das atividades desenvolvidas no período de 12 meses a contar da data de sua aprovação (item X.1.3.b., da Resolução CNS/MS Nº 466/12).

O CEP/CCS/UFPE deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (item V.5., da Resolução CNS/MS Nº 466/12). É papel do/a pesquisador/a assegurar todas as medidas imediatas e adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e ainda, enviar notificação à ANVISA – Agência Nacional de Vigilância Sanitária, junto com seu posicionamento.

Continuação do Parecer: 1.400.457

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_549282.pdf	26/01/2016 11:41:08		Aceito
Outros	termoassinado.pdf	26/01/2016 11:37:29	Antonio Roberto Lucena de Araujo	Aceito
Outros	carta_resposta.docx	12/01/2016 23:08:58	Antonio Roberto Lucena de Araujo	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.docx	12/01/2016 23:04:41	Antonio Roberto Lucena de Araujo	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_CEP.docx	12/01/2016 23:04:07	Antonio Roberto Lucena de Araujo	Aceito
Outros	JuanLuizCoelhodaSilva.pdf	08/11/2015 19:06:03	Antonio Roberto Lucena de Araujo	Aceito
Outros	AntonioRobertoLucenadeAraujo.pdf	08/11/2015 19:05:33	Antonio Roberto Lucena de Araujo	Aceito
Outros	MarcosAndreCavalcantiBezerra.pdf	08/11/2015 19:03:42	Antonio Roberto Lucena de Araujo	Aceito
Outros	MarinusdeMoraesLima.pdf	08/11/2015 19:02:48	Antonio Roberto Lucena de Araujo	Aceito
Outros	ReijaneAlvesdeAssis.pdf	08/11/2015 19:02:15	Antonio Roberto Lucena de Araujo	Aceito
Outros	MatheusFilgueiraBezerra.pdf	08/11/2015 19:01:09	Antonio Roberto Lucena de Araujo	Aceito
Outros	MayaraMatiasdeOliveira.pdf	08/11/2015 19:00:36	Antonio Roberto Lucena de Araujo	Aceito
Folha de Rosto	digitalizar0015.pdf	22/10/2015 15:47:17	Antonio Roberto Lucena de Araujo	Aceito
Declaração de Instituição e Infraestrutura	IMG_4036.JPG	07/10/2015 22:14:26	Antonio Roberto Lucena de Araujo	Aceito
Declaração de Instituição e Infraestrutura	carta_de_anuencia.JPG	07/10/2015 22:14:08	Antonio Roberto Lucena de Araujo	Aceito
Declaração de Instituição e Infraestrutura	anuencia_setor.jpg	07/10/2015 22:12:47	Antonio Roberto Lucena de Araujo	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

Continuação do Parecer: 1.400.457

RECIFE, 02 de Fevereiro de 2016

Assinado por:
LUCIANO TAVARES MONTENEGRO
(Coordenador)