Douglas Rafaele Almeida Silveira

Estudo multicêntrico retrospectivo em leucemia mieloide aguda: abordando de forma abrangente variáveis demográficas, clínicas e genéticas de adultos tratados intensivamente

Tese apresentada à Faculdade de Medicina da Universidade de São Paulo para obtenção do título de Doutor em Ciências

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Área de concentração: Distúrbios do Crescimento Celular, Hemodinâmicos e da Hemostasia

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Douglas Rafaele Almeida Silveira

A multicentre retrospective acute myeloid leukaemia study: approaching comprehensively demographical, clinical and genetic variables of intensively treated adults

Dissertation presented to the Faculdade de Medicina, Universidade de São Paulo to obtain the title of PhD in Sciences

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DEDICATÓRIA

Dedico esse trabalho a minha companheira e esposa Aliny, com quem divido todos os momentos há 20 anos, e a nossa filha Catarina, nosso solzinho, que nos seus 3 anos de existência mudou tão sensivelmente nossas vidas.

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Esta tese está de acordo com as seguintes normas, em vigor no momento desta publicação:

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INTRODUÇÃO: Os desfechos na leucemia mieloide aguda (LMA) dependem das características do paciente, doença, tratamento e fatores socioeconômicos, no entanto, os resultados entre países em desenvolvimento e desenvolvidos nunca foram comparados diretamente. A estratificação de risco genético também vem aumentando complexidade e custos, apesar de ainda indisponível para a maioria dos serviços de saúde. Neste estudo, comparamos o resultado entre realidades economicamente contrastantes, e propomos uma alternativa para estratificação de risco que permita dados citogenéticos ou moleculares ausentes. **PACIENTES E MÉTODOS:** Analisamos dados de 167 pacientes com LMA tratados intensivamente em São Paulo, Brasil (FMUSP) - conjunto de treinamento. Usando ELN2017 como o padrão, o comparamos com nosso AGR e combinamos este com parâmetros prognósticos encontrados em um modelo de Cox para criar um sistema de pontuação (SAMLS) que estratifica pacientes com LMA. Em seguida, usamos duas coortes independentes, FMRP (Brasil, n = 145) e OUH (Reino Unido, n = 157), para validar nossos achados. Finalmente, mesclando os dados da FMUSP e da FMRP em uma única coorte (USP, N = 312), ajustamos o desfecho de sobrevida para variáveis relacionadas ao paciente, tratamento e doença, comparando os resultados de USP e OUH. **RESULTADOS:** AGR foi estatisticamente significativo para sobrevida global (SG) em ambas as coortes de teste (FMRP p = 0.037, OUH p = 0.012). SAMLS foi composto por: AGR, idade (>45 anos), leucócitos (<1.5 ou >30x10e3/mm3) e níveis de albumina (<3.8g/dL). SAMLS mostrou uma diferença significativa em SG na coorte de treinamento (p <0,001) e coortes de teste (FMRP p = 0,0018, OUH p <0,001). A coorte USP teve SG em 5 anos inferior quando

comparada à OUH (29% vs. 49%, P-ajustado = 0,027). Os pacientes da USP apresentam mortalidade precoce mais elevada (23% vs 6% P <0,001) principalmente devido a infecções bacterianas multiressistentes e fúngicas. USP teve uma maior incidência cumulativa de recidiva em 5 anos (60% vs 50%, P = 0,0022), e menor taxa de transplante de células-tronco hematopoiéticas (TCTH) (28% vs 75%, P <0,001), além de espera maior para o TCTH (mediana, 23,8 vs 7,2 meses, P <0,001). A sobrevida de 3 anos em pacientes com recidiva foi pior na USP do que OUH (10% vs 39%, P <0,001). **CONCLUSÕES:** O AGR é uma avaliação de risco citogenético-molecular substituta que lida com algum grau de dados ausentes sem perder a precisão. SAMLS melhora a previsão de sobrevida em LMA independentemente das diferenças nas características basais e de tratamento das populações de LMA. A SG foi menor em pacientes da USP devido à mortalidade precoce, maior incidência cumulativa de recidiva e piores resultados após recaída. Controle de infecções, melhores cuidados de suporte e acesso ao TCTH alogênico ainda são limites que precisam de atenção para superar os resultados desapontadores em nosso país.

Descritores: Leucemia mieloide aguda; Medição de risco; Análise de sobrevida; Análise de regressão; Fatores socioeconômicos; Transplante de medula óssea; Infecções oportunistas.

ABSTRACT

Silveira DRA. *A multicentre retrospective acute myeloid leukaemia study:* approaching comprehensively demographical, clinical and genetic variables of intensively treated adults [thesis]. São Paulo: "Faculdade de Medicina, Universidade de São Paulo"; 2020.

INTRODUCTION: Outcomes in acute myeloid leukaemia (AML) are dependent on patient and disease characteristics, treatment, and socioeconomic factors; nonetheless, AML outcomes between resource-constrained and developed countries have not been compared directly. The genetic risk stratification has also been facing an increase in complexity and costs, but still unavailable for most healthcare centres. In this retrospective multicenter study, we analyse multicentre real-life data to compare the outcome between resource contrasting settings, and we propose an alternative for risk stratification which permits cytogenetic or molecular missing data while retaining prognostic power. **PATIENTS AND METHODS:** We first analysed data from 167 consecutive intensively treated non-acute promyelocytic leukaemia AML patients enrolled in Sao Paulo, Brazil (FMUSP) as our training dataset, using ELN2017 as the standard for comparison with our AGR. Next, we combined our AGR with clinical prognostic parameters found in a Cox Proportional-Hazard Model (CPHM) to create a novel scoring system (survival AML score, SAMLS) that stratifies newly diagnosed AML patients. Then, we have used two equivalent independent test cohorts, FMRP (Brazil, n=145) and OUH (United Kingdom, n=157) for validating our findings. Finally, we merged FMUSP and FMRP data in a single cohort (USP, N = 312), and using CPHM adjusted treating centre survival outcome to patients, treatment, and disease variables comparing USP and OUH results. **RESULTS:** AGR was statistically significant for OS in both test cohorts (FMRP p=0.037, OUH p=0.012) and DFS in FMRP (p=0.04). SAMLS features were: AGR, age (>45 years), WBC (<1.5 or >30.0x10e3/mm3) and low albumin levels (<3.8g/dL). SAMLS showed a significant difference in

OS in the training cohort (p<0.001) and test cohorts (FMRP p=0.0018, OUH p<0.001). USP cohort had inferior 5-year overall survival compared with OUH (29% vs. 49%, adjusted-P=0.027). USP patients have higher early-mortality (23% vs 6% P<0.001) primarily due to multi-resistant gram-negative bacterial and fungal infections. USP had a higher 5-year cumulative incidence of relapse (60% vs 50%, P=0.0022), were less likely to undergo haematopoietic stem-cell transplant (HSCT) (28% vs. 75%, P<0.001) and waited longer for HSCT (median, 23.8 vs 7.2 months, P<0.001). 3-year survival in relapsed patients was worse in USP than OUH (10% vs 39%, P<0.001). **CONCLUSIONS:** Adapted Genetic Risk is a surrogate Cytogenetic-molecular risk assessment dealing with some degree of missing data without losing accuracy. SAMLS improves AML survival prediction regardless of differences in baseline characteristics and treatment of AML populations. Overall Survival was lower in USP patients due to early-mortality, a higher cumulative incidence of relapse, and worse results after relapsing. Control of infections, best supportive care and access to HSCT are still boundaries that need attention to overcome poorer outcomes in our country.

Descriptors: Leukemia, myeloid, acute; Risk assessment; Survival analysis; Regression analysis; Socioeconomic factors; Bone marrow transplantation; Opportunistic infections.

1. Introduction

Acute Myeloid Leukaemia (AML) is a group of hematologic malignant neoplasms where there is an excess of immature myeloid lineage progenitors and precursors cells in bone marrow (BM), peripheral blood (PB), and less frequently, in non-hematopoietic tissues (e.g., skin, central nervous system) (1). The two main clinico-pathological hallmarks of AML clonal expansion and bone marrow failure.

We have made great strides forward in our understanding of AML pathobiology in the last few decades. The advent of new technologies, in particular in flow cytometry and genomics has made it possible to study changes to haematopoietic populations, and the underlying genetic aberrations in AML. More recently, advances have been made to understand how genetic aberrations may cause disease at a more mechanistic level. By studying how genetic abnormalities accumulate over time, either by studying longitudinal patient sample sets or by inferring evolution from clonal structures, we have further insights into how AML develops from normal bone marrow stem and progenitor cells. There are many aspects to abnormal AML cellular physiology of AML. These include genomic instability and acquisition of mutations and chromosomal anomalies, aberrant self-renewal in immature haematopoietic stem and progenitor populations, arrested differentiation and maturation, inappropriate proliferation in the absence of normal growth signalling, dysregulated apoptosis and checkpoint control, and invasion of tissues and organs by leukaemic blasts (2–4).

This results in bone marrow failure and cytopenias, which most

commonly manifest clinically with symptoms including asthenia, susceptibility to bacterial and fungal infections and bleeding. These, in turn cause, death through multi-organ failure. Severe BM failure and its sequelae is often lethal within weeks to months if normal hematopoiesis is not re-established through therapy (1).

Despite these shared characteristics, our increasingly detailed understanding, particularly of the molecular abnormalities which are the root of this disease, has revealed underlying heterogeneity of AML, which in turn is reflected in differences in clinical outcome in patients. Different subgroups of AML patients, divided using clinical and diagnostic parameters can have highly variable survival: from a 5-yrs overall survival (OS) of 70-80% down to 10-15% (5–7). Based on best available data to date, outcome is dependent on either disease-related characteristics like cytogenetic-molecular risk, white-blood-cell count, de novo versus secondary aetiology, or patient-specific features such as age, performance status, and co-morbidities (1,8). Socioeconomic factors may also have an impact on clinical outcomes in AML patients. Significant disparities in access to healthcare resources have been reported, even in economically developed nations (9,10).

Curative treatment for AML in adults still involves intensive chemotherapy (ICT) which results in prolonged treatment-induced cytopenia. Patients with disease deemed to be at higher risk of relapse are offered consolidative allogeneic haematopoietic stem-cell transplantation (HSCT), which remains the only curative option for patients with the highest-risk disease (1). Throughout the patient's therapeutic journey, supportive care, which includes environmental hygiene, adequate nutrition, safe blood products,

effective antimicrobial prophylaxis, prompt and aggressive treatment of life-threatening infections and access to intensive life-support care are important factors in improving clinical outcomes. Since infection is a major cause of death in AML patients (11), under-funding of supportive care in resource-constrained healthcare systems feeds into adverse clinical outcomes (12,13). By contrast, high-income healthcare systems have invested in, and improved prophylaxis and prompt management of life-threatening infections in an effort to improve patient survival (14).

Although HSCT is recommended as consolidation in first complete response (CR1) for patients with intermediate and adverse genetic risk (15); it is an expensive treatment, with increases inpatient stay that doubles treatment costs (16). Thus it has been reported that only 5% of patients received HSCT in CR1 in a Brazilian multicentre study (17), compared to 50% in a university hospital in France (18). Furthermore, to our knowledge, no published study has directly compared AML outcomes between centres from economically developed countries and those with emerging economies.

Another challenge faced by treating physicians in resource-limited settings is AML risk-stratification and prognosis prediction. The invention and validation of molecular diagnostic tests have led to the refinement of classification and prognostic scoring. For example, the 4th WHO classification included new categories defined by mutation status alone (e.g., *NPM1* mutation, *CEBPa* mutation, *RUNX1* mutation) (19). Furthermore, we now have evidence that the presence of mutations, or combinations of mutations, affects disease biology (e.g., mutated *NPM1* plus *FLT3-ITD* status) (20). Despite these advances, there remains a challenge as the majority of patients are still

categorised within an intermediate-risk group (~50% of AML patients) where there is a wide range in OS, suggesting that further sub-classification of prognosis is required.

Current AML classification and risk assessment depend on cytogenetic and molecular biology findings, which are widely accepted as major factors affecting the outcome. Classification and risk assessment are becoming more complex alongside advances in the understanding of AML biology (6,21–23). The rationale for increasing this complexity is to allow a more precise stratification, thereby further subsetting the intermediate-risk group, and to develop targeted therapies to specific molecular pathways (24).

The first European LeukemiaNet 2017 (ELN2017) risk assessment validation has shown that the intermediate-risk group with 47% of cases is still the biggest in some cohorts (7); however, reviewing therapy advances proves that rationale has at least partially achieved its purposes once genetic targeted therapy is now a reality in AML (25). Nevertheless, there are concerns regarding how socioeconomic disparities can impact implementation of up to date AML risk scoring. Furthermore, as the ELN risk assessment is based on data collected from higher income countries (6), there are as yet no reports which validate the latest iteration, ELN2017, in low-middle income countries (LMIC).

Studies to validate the older ELN2010 classification (21) in LMIC have shown that although genetic markers can predict survival, the clinical outcome was inferior (17) or the classification was of limited accuracy when compared with developed countries (26). A retrospective analysis of 241 patients in two Brazilian centres, using a cytogenetic risk stratification also

reached a similar conclusion, that genetic factors can split patients into different risk categories, but the overall result clinical outcome is below that expected (5,27). Taken together, some genetic risk groups, especially cytogenetics abnormalities, appear to segregate patients by outcome in the LMIC setting, but others, including *NPM1* mutated AML, which accounts for ~25% of AML (22), have not had their prognosis impact studies in such populations.

An alternative approach to using a conventional cytogenetic and limited molecular assessment is to generate statistical models using large, complex retrospective datasets including clinical outcome and detailed molecular mutation profiles to provide an individualized prediction of outcome (28,29). However, these models have not been validated prospectively. Furthermore, the use of extensive mutation panel testing in such scoring systems may not be applicable for many patients who do not have access to comprehensive molecular genetic testing (30), or be irrelevant to patient care when testing is not sufficiently timely. This highlights the importance of validating genetic risk assessment and genetic specific groups in different socioeconomic settings as and when new technologies are implemented.

The acceptance of risk scores formulated and implemented as standard practice in international clinical studies may inadvertently disadvantage and exclude patients in poorer healthcare systems that do not have access to ‘state of the art’ diagnostic tests. Such exclusions lead to data bias, distortions of our understanding of disease pathophysiology, and may adversely affect the ability of clinicians to deliver optimal prognostic information and therapy to patients.

While more recent advances have been in molecular diagnostics,

it is important to consider non-genetic clinicopathological biomarkers that may be relevant, including those that form part of routine clinical laboratory testing even in resource-constrained contexts.

Age and performance status (PS) are often cited as independent prognostic factors for both induction therapy response and overall survival (31,32). Several prognostic scoring systems have combined age and PS together or in association with other variables such as white blood cell and platelet counts, creatinine levels, and LDH to predict early death (i.e. death within 30-days) (8,32,33).

Absolute leukocyte count at presentation is also associated with worse prognosis in both APL and non-APL AML. In non-APL, there is a correlation between count and worse OS outcome, and again different score systems have attempted to define the influence of leukocyte count over clinical outcomes, but as yet no consensus with regard to an absolute threshold that separates good or poor outcomes (8,33–37).

Overexpression of CD25 (a.k.a., Interleukin-2 receptor alpha chain), measured by flow cytometry, is an independent prognostic marker associated with lower OS (38). Inflammatory cytokines, especially tumour necrosis factor-alpha (TNF-alpha), at serum levels <10 pg/mL, was associated with a better outcome. Surprisingly, the influence of values above that threshold on outcomes was not statistically significant, at least not before removing leukocytes from the model, what suggests an expected correlation between leukocytosis and high levels of inflammatory markers (39). Fibrinogen levels, which are another marker of inflammation, with a cutoff of 4.1g/L were also statistically significant related to a better outcome for the lower fibrinogen level

group (40).

Serum albumin is commonly measured in routine clinical practice. It has been shown to affect prognosis in several types of solid tumours (e.g.: gastric, pancreatic, lung cancers) (41), but has not been described as an independent prognostic factor in AML. Albumin was recently found to be an independent factor for overall survival after salvage therapy (42); nevertheless, its role as a predictor for previously untreated AML was relegated to either a low-rank factor in early death score system or a secondarily correlated feature with the primary study variable (8,39,43).

Having reviewed current practice and recent advances in AML risk stratification, it is clear that there is room for improvement. In particular, no study has attempted to combine a cytogenetic-molecular risk assessment together with non-genetic variables in AML patients at diagnosis, which may improve predictive power.

The work presented in this thesis sets out to address those issues, through a comprehensive analysis of genetic and non-genetic variables from two Brazilian centres (both from the University of São Paulo hospitals), compared with a single-centre cohort from Oxford (United Kingdom). All cohorts consist of patients treated within the public health sector, according to standard treatment protocols. This work will assess possible differences in clinical outcome, and where present, correlate them with potential contributory factors. We will also explore the extent to which different parameters impact clinical outcome in each setting. This also presents the first validation of ELN2017 in an LMIC setting. I aim to model parameters to build a score new score which merges both genetic and non-genetic variables, and which can be widely

applied to AML patients in both low and high-income settings.

2. Projeto aprovado pela CONEP/CAPPesq

2.1. Título

Estudo de Mutações Genéticas em Pacientes com Leucemias Mielóide e Promielocítica Agudas e correlação com prognóstico e resposta ao tratamento

2.2. Introdução

A oncogênese consiste em um processo de múltiplas etapas caracterizado pela aquisição sucessiva de lesões genéticas conhecidas como *driver mutations* que levam ao desenvolvimento do fenótipo neoplásico (44). Nas neoplasias mielóides sabe-se que são necessárias pelo menos duas *driver mutations* para que haja desenvolvimento da doença manifesta, sejam elas caracterizadas por eventos catastróficos como as translocações cromossômicas ou pequenas mutações decorrentes de variações de um único nucleotídeo (SNVs) na sequência do gene, dentre outros mecanismos como inserções, deleções, etc (45,46).

No universo das neoplasias mielóides, a leucemia promielocítica aguda (LPA), um subtipo de leucemia mielóide aguda (LMA) que predomina em pacientes jovens (< 60 anos) é marcada pelo acúmulo de promielócitos anômalos na medula óssea. Caracteriza-se por: baixa contagem leucocitária (< 10.000 células/mm^3) quando comparada a outros subtipos de LMA, exceto em sua variante microgranular; distúrbios de coagulação potencialmente fatais e resposta dramática à terapia diferenciadora com ácido trans-retinóico (ATRA) e indutora de apoptose com trióxido de arsênico (ATO), associados entre si ou à quimioterapia citotóxica convencional. Atualmente é considerada uma

neoplasia de bom prognóstico e altamente curável, se diagnosticada precocemente (47).

A LPA possui como particularidade a presença de um evento oncogênico iniciador bem definido (48). A translocação balanceada envolvendo os cromossomos 15 e 17 é encontrada em quase todos os pacientes (49), e como consequência gera-se o transcrito de fusão entre os genes da leucemia promielocítica e o receptor alfa do ácido trans-retinóico (*PML-RARa*), mecanismo fisiopatológico central responsável pela parada da granulopoiese em fase de promielócito (50). O receptor do ácido trans-retinóico (RAR) pertence à superfamília dos receptores nucleares, e suas isoformas quando heterodimerização com o receptor X do ácido retinóico (RXR) possuem atividade de fator de transcrição ligante-dependente com capacidade de ligar a sequências específicas do DNA (RARE - Retinoic Acid Response Elements) presentes na região promotora de inúmeros genes, influenciando assim o processo de transcrição gênica (51). No que concerne à granulopoiese, o RARa influencia o programa de maturação e diferenciação regidos especialmente pelos *core binding factors* (CBF) (51,52), fatores de transcrição maestros na hematopoiese, e pelos *ccaat enhancer binding proteins* (CEPB) e suas isoformas da superfamília dos fatores de transcrição *basic leucine zipper* (bZIP) (51,53), responsáveis em grande parte pela orquestração do programa de diferenciação final granulocítico.

Aparentemente o gene de fusão *PML-RARa* não só interrompe a função do alelo RARa envolvido no transcrito mutante por torná-lo resistente à ligação ao ácido retinóico em níveis fisiológicos, como também, inibe o RARa selvagem transcrito pelo alelo não envolvido na translocação através do

recrutamento de complexos correpressores (51,54). Raramente, outras translocações podem gerar produtos de fusão RARa com parceiros distintos do PML. Exemplos são os genes *NPM1, NUMA, KMT2A, PLZF, STAT5*, que apesar de possuírem como ponto de convergência a disfunção do fator de transcrição RARa, podem levar ao surgimento de uma LPA com expressão fenotípica distinta, inclusive com resistência à terapia diferenciadora (51,55,56), mostrando que o parceiro de fusão RARa possui influência fisiopatológica.

A despeito do extenso conhecimento acerca do mecanismo oncogênico iniciador do clone pré-leucêmico na LPA, o mesmo não se pode dizer acerca das mutações que levam à progressão para a doença clinicamente manifesta. Modelos em ratos transgênicos mostraram que a presença do *PML-RARA* levava a uma fase pré-leucêmica prolongada sublinhando que mutações adicionais capazes de conferir ao clone vantagem proliferativa seriam necessárias (57–59).

Mutações em genes envolvendo as vias de sinalização intracelular, levando a ganho de função, são encontradas tanto em pacientes com LPA como com outras LMA. Alguns dos mais frequentes são o gene codificante da proteína com atividade GTPase RAS (*NRAS* e *KRAS*) e o gene do receptor com atividade de tirosina quinase *Fms-like tyrosine kinase receptor 3* (*FLT3*), que estão mutados em 10-15% e 30% dos pacientes com LPA, respectivamente (60–66). Essas vias estão envolvidas na regulação dos estímulos proliferativos, de diferenciação e de sobrevivência celular e são potenciais alvos terapêuticos.

Historicamente, as neoplasias mielóides sempre estiveram na vanguarda das descobertas dos mecanismos genéticos de oncogênese. A

primeira anormalidade citogenética recorrente, o cromossomo Filadélfia, foi descrito pela primeira vez em 1961 por Nowell e Hungerford em pacientes com Leucemia Mielóide Crônica (LMC), uma forma de neoplasia mieloproliferativa (NMP). Na década seguinte, estudos pioneiros de citogenética conduzidos pela Janet Rowley demonstraram a presença de translocações genéticas balanceadas em pacientes com LMA, e caracterizaram o cromossomo Filadélfia como sendo a translocação t(9;22)(q34;q11). Os estudos de caracterização cromossômica de neoplasias apresentaram grandes avanços na década de 80 e 90 nas LMAs e nas síndromes mielodisplásicas (SMDs), demonstrando a importância prognóstica que determinadas alterações genéticas tinham no curso dos pacientes. Mais recentemente, com o desenvolvimento de tecnologias de sequenciamento genômico avançadas, iniciou-se a era genômica do câncer em 2008 com a publicação do primeiro genoma de um paciente com LMA.

Em um estudo do grupo da Universidade de Washington (48) foi realizado o sequenciamento do genoma de 12 pacientes diagnosticados com LPA em paralelo com 12 pacientes diagnosticados com LMA não promielocítica e mostrou que nove genes se apresentavam mutados tanto nos casos de LPA quanto de LMA: *FLT3; TTN; NRAS; PKD1L2; CACNA1E; DNAH9; WT1; ANKRD24* e *PHF6*. Enquanto treze genes estavam presentes somente nos pacientes com LMA não promielocítica: *NPM1; DNMT3A; IDH1; TET2; IDH2; RUNX1; ASXL1; PTPN11; DIS3; KIT; SMC1A; SMC3* e *STAG2*, sendo que seis de forma estatisticamente significante: *NPM1*, $p < 0.0001$; *IDH1*, $p < 0.0001$; *IDH2*, $p < 0.01$; *TET2*, $p < 0.001$; *DNMT3A*, $p < 0.0001$ e *ASXL1*, $p < 0.03$. Outro estudo publicado recentemente, amostras de 1540 pacientes foram

analisadas em um painel de mutações por segunda geração para 111 genes, conseguindo categorizar os pacientes em 11 classes genéticas, baseadas em características clínicas, prognósticas e biológicas, de forma mais fidedigna do que a classificação atualmente utilizada (22).

Em um trabalho publicado em 2011 (67), utilizou-se a metodologia de sequenciamento do exoma completo (WES) em 3 pacientes com LPA, além das já conhecidas mutações nos genes *WT1 e KRAS*, as outras 12 mutações encontradas não eram previamente citadas como recorrentes neste subtipo de leucemia. Recentemente, dois estudos também realizaram WES aliado à execução de um painel de mutações por sequenciamento de segunda geração: o estudo publicado por Ibáñez e colaboradores (68) utilizou 30 amostras (5 por WES e 25 por sequenciamento de painel) mostrando repetidamente mutações nos genes *STAG2, U2AF1, SMC1A, USP9X, IKZF1, LYN, MYCBP2 e PTPN11*, além de outras mutações não recorrentes em outros genes, algumas não previamente descritas. Esse estudo também avaliou o conceito de interassoma associado a presença de mutações concorrentes. O segundo trabalho (69), utilizou uma amostra ainda maior (> 200 amostras) mostrando mutações recorrentes nos genes *FLT3, WT1, NRAS e KRAS*, além dos genes *AIRD18 e AIRD19* (previamente não descritos na LPA) associados ao complexo regulador do nucleossoma SWI/SMF. Em suma, sabe-se que além das mutações recorrentes nas vias de sinalização intracelular e modificação/regulação da cromatina, controle da transcrição gênica, metilação do DNA, spliceossoma e supressão tumoral, existe um grande número de mutações randômica associadas ao PML-RARa na oncogênese da doença, no entanto, ainda precisa-se validar e correlacionar

biologicamente muitos desses achados.

Tradicionalmente, a maioria dos estudos avaliando a presença de mutações genéticas nas neoplasias usa abordagens de sequenciamento de DNA de primeira geração (i.e. Sanger sequencing) (70). Embora essa tecnologia seja muito eficaz, ela pode ser muito cara e trabalhosa, particularmente quando há necessidade de sequenciar todos os exons de um gene. Isto limita o número de genes/mutações que podem ser sequenciados em um estudo. Na última década, as tecnologias de sequenciamento de DNA de última geração (“Next-Generation Sequencing”, NGS) revolucionaram o sequenciamento de DNA (71). A tecnologia de NGS se baseia nos princípios de fragmentação do DNA, amplificação por PCR simultânea das várias áreas de interesse e sequenciamento paralelo maciço levando a milhares de segmentos de DNA de curto tamanho (50-200 nucleotídeos) (72,73). Esta tecnologia tornou possível sequenciar o genoma humano completo em poucos dias e com um custo muito reduzido (72). Recentemente, NGS foi utilizada para sequenciar o genoma e exoma (genoma codificante) de diversos tipos de tumores, aumentando muito o conhecimento sobre a patogênese dessas doenças (74–78). Uma possível aplicação da NGS é o sequenciamento direcionado de painéis de genes selecionados (79–81). nesta abordagem o DNA é enriquecido para regiões genômicas selecionadas correspondendo aos genes de interesse, seguido de amplificação por PCR sequenciamento maciço paralelo. O DNA sequenciado é analisado por bioinformática para detectar mutações e outras aberrações genômicas. Comparado ao sequenciamento tradicional Sanger, o sequenciamento de painéis de genes por NGS permite a avaliação simultânea de um número muito grande de genes com um custo

mais baixo, e com uma necessidade limitada de DNA. O sequenciamento de painéis genéticos não é tão amplo como sequenciamento total do genoma ou exoma, porém é menos caro e demanda menos tempo.

Propomos a avaliação genética comparando os dois tipos de leucemia mielóide aguda LPA e LMA não-LPA para detecção de mutações nesses pacientes usando tecnologia NGS. Iremos correlacionar a presença desta mutação com variáveis clínicas disponíveis.

2.3. Objetivos

Objetivos primários:

1. Sequenciar o exoma da célula neoplásica ao diagnóstico e compará-lo ao germinativo de um pequeno grupo de pacientes com LPA e LMA no intuito de identificar novas *driver mutations* que possam contribuir na patogênese da doença.
2. Avaliar um painel de mutações genéticas de um grupo maior de pacientes baseado nos achados do sequenciamento exômico e em dados da literatura.
3. Correlacionar a presença das mutações encontradas com características clínicas, sobrevida e resposta ao tratamento.
4. Avaliar características clínico-laboratoriais e terapêuticas correlacionando com desfechos clínicos.

Objetivos secundários:

5. Realizar outros tese genéticos que se mostrem oportunos, como por exemplo, sequenciamento de RNA e sequenciamento de célula única para avaliação da expressão de genes.

2.4. Critérios de Inclusão e Exclusão

2.4.1. Critérios de Inclusão

1) Diagnóstico de Leucemia Promielocítica Aguda pela classificação OMS

2) Diagnóstico de Leucemia Mielóide Aguda pela classificação OMS

3) Ser maior que 18 anos

2.4.2. Critérios de Exclusão

1) Recusa em assinar termo de consentimento livre e esclarecido (exceto, se paciente vir a óbito)

2.5. Materiais e Métodos

2.5.1. Pacientes

Serão selecionados cerca de 60 casos de pacientes com LPA e 170 casos com LMA do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (HCFMUSP) que tenham amostras de DNA, RNA ou células viáveis armazenadas no biobanco do Instituto do Câncer do Estado de São Paulo (ICESP) ou no laboratório de biologia tumoral da disciplina de Hematologia do HCFMUSP, e que tenham sido obtidas no momento do diagnóstico e/ou seguimento clínico. As amostras armazenadas no laboratório de biologia tumoral foram coletadas com o intuito assistencial (diagnóstico e tratamento da doença de base) e serão incluídas mediante assinatura do termo de consentimento no contexto ético de biobanco. Exceto para os paciente que foram à óbito, para estes solicitaremos isenção de TCLE, abordaremos a questão oportunamente neste mesmo documento.

2.5.2. Desenho do estudo

O estudo tem duas coortes. Na primeira coorte (coorte de descoberta), serão realizados sequenciamentos de exoma/transcriptoma de 10 pacientes com LPA e 30 de LMA que tenham material genômico disponível do momento do diagnóstico e material genômico germinativo (DNA normal). As mutações encontradas que forem definidas como recorrentes ou significativas serão avaliadas em uma segunda coorte de 50 para LPA e 140 de LMA pacientes (coorte de prevalência). Poderão a critério dos pesquisadores ser realizadas outras análises como por exemplo sequenciamento de célula única.

2.5.3. Coleta de amostras

De todos os pacientes vivos, iremos coletar amostras de sangue periférico em

remissão, será realizada centrifugação de sangue com Ficoll-Paque para isolamento de granulócitos. Será realizada extração de DNA da fração de com o kit QIAMP DNA Mini da QIAGEN®. Este será o DNA germinativo de controle, que será utilizado na análise de sequenciamento de exoma como controle e que poderá ser utilizado para confirmar a natureza somática de novas mutações encontradas na coorte de prevalência caso sejam mutações não conhecidas.

As amostras já coletadas ao diagnóstico e/ou seguimento conforme explicitado no item 2.5.2, o foram no contexto assistencial (tratamento da doença de base), e portanto não foram consentidas na ocasião. Estas só serão incluídas no estudo após consentimento para os pacientes vivos ou para os já falecidos, se aprovada a isenção de TCLE solicitada no presente estudo.

Pacientes que na ocasião do diagnóstico assinem o TCLE do biobanco e tenham suas amostras armazenadas neste, também poderão ser utilizados.

2.5.4. Sequenciamento de Exoma

As amostras de DNA extraídas fração de células granulocíticas (DNA germinativo) e DNA obtido ao diagnóstico (de biobanco/biorrepositório ou material genético excedente de análise laboratorial de rotina) serão quantificadas através do método fluorimétrico Qubit da Life Technologies. Aproximadamente 3 µg de DNA com absorbância 260/280 de 1.8-2.0 de cada fração será usada para sequenciamento paralelo maciço de exomas pela plataforma HiSeq2500 da Illumina. Captura de exons será realizada com o kit SureSelectXT Human All Exon 50Mb da Agilent conforme instruções do

fabricante, seguido de preparo da biblioteca. A profundidade do sequenciamento será de 150x.

2.5.5. Sequenciamento de Painel Customizado

Um painel customizado SureSelect contendo todos os genes encontrados como mutados no sequenciamento de exoma, assim como todos os genes previamente relatados como sendo mutados em LPA ou LMA na literatura serão solicitados da empresa Agilent. Enriquecimento de genes e preparo da biblioteca será feito conforme instruções do fabricante. Os 48 pacientes serão sequenciados em uma Lane da plataforma HiSeq 2500, seguindo instruções do fabricante, com uma profundidade de 700-800x.

2.5.6. Análise Bioinformática

A análise bioinformática das sequências produzidas consiste nas seguintes etapas:

1) Avaliação de qualidade das sequências produzidas pelo HiSeq 2500 e filtragem das sequências por qualidade (uso da escala Phred) e tamanho (sequências <30 pb são removidas) utilizando o software FastQC v0.10.1
2) Mapeamento das sequências limpas contra a montagem referência do genoma humano (a versão atual é o hg19/UCSC ou GRCh37/NCBI- vide http://genome.ucsc.edu/cgi-bin/hgGateway) utilizando o software BWA v0.7.5a com parâmetros *default*
3) Conversão do arquivo alinhado SAM para seu formato binário BAM através do SAMTools v.0.1.19 (http://sourceforge.net/projects/samtools/files/)
4) Preparo das sequências alinhadas no format BAM para detecção de

alterações seguindo-se os “best practices” do Genome Analysis Toolkit do Broad Institute (http://www.broadinstitute.org/gatk/guide/best-practices): Sucintamente, para marcar duplicatas de PCR sera utilizado o software Picard, versão 1.94 (http://picard.sourceforge.net). Para realizar realinhamento ao redor de inserções/deleções e recalibramento da qualidade das bases, serão utilizadas as ferramentas do Genome Analysis Toolkit (GATK; versão 2.7-4; http://www.broadinstitute.org/gatk/index.php).

5) Detecção de mutações, inserções e deleções: Para o sequenciamento do exoma (coorte de descoberta), esta etapa será realizada através da análise pareada de exoma de DNA germinativo normal (granulócitos em remissão) e DNA maligno (obtidos de promielócitos malignos ao diagnóstico). Para detecção de mutações somáticas em único ponto (SNVs; single nucleotide variants), serão utilizados o softwares SomaticSniper 1.0.2 (http://gmt.genome.wustl.edu/somatic-sniper/1.0.2/index.html) e Mutect (http://www.broadinstitute.org/cancer/cga/mutect), comparando amostra normal (granulócitos) com amostra tumoral (promielócitos). Para detecção de alterações de numero de cópia, será utilizado o software VarScan 2.3.6 (http://varscan.sourceforge.net/copy-number-calling.html). Para detecção de inserções e deleções será utilizado o software Pindel 0.2.4 (http://gmt.genome.wustl.edu/pindel/0.2.4/index.htm). Na análise de mutações na coorte de prevalência do LCTC, será realizado a busca de mutações (SNVs e Indels) com o software Mutect (http://www.broadinstitute.org/cancer/cga/mutect) e Pindel

(http://gmt.genome.wustl.edu/pindel/0.2.4/index.htm), realizando análise tumor “only sample” (sem amostra controle).

6) Anotação das variações encontradas: Após a identificação das variantes o resultado é convertido para *Variant Call Format* 4.1 (VCF) (http://www.1000genomes.org/wiki/Analysis/Variant%20Call%20Format/vcf-variant-call-format-version-41) que é o formato de entrada para anotação funcional das variantes com o software ANNOVAR (http://www.openbioinformatics.org/annovar/)

7) Filtragem *in-house* das alterações detectadas na etapa 5: Após a identificação e anotação dos diferentes tipos de variantes foi desenvolvido um programa *in-house* para filtrar e selecionar os melhores SNVs/Indels de cada paciente. Os critérios aplicados foram: genes anotados pelo ANNOVAR; cobertura total da base ≥ 8 na amostra germinativa e na amostra tumoral; total de alelos variantes no tumor ≥ 2; ausência de “strand bias” (detectada por teste estatístico de Fisher > 0.05), ausência nos bancos de mutações dbSNP137, ESP6500 e 1000 genomas.

8) Anotação funcional das variantes filtradas na etapa 7: A lista de mutações (SNVs/Indels) potencialmente somáticas obtidas na etapa 7 será enriquecida com uma anotação funcional que tem o objetivo de identificar genes “drivers” (genes com papel importante na patogênese da doença). Isto será realizado através de três etapas: (1) identificando genes com mutações que causem impacto significante na estrutura da proteína; (2) identificando genes que estejam participando de uma mesma via biológica celular. Para a etapa (1), será utilizado o software

Intogen (www.intogen.org); para a etapa (2), será utilizado o software MutSigCV (http://www.broadinstitute.org/cancer/cga/mutsig).

2.5.7. Validação de mutações encontradas no sequenciamento de exoma

As mutações "drivers" encontradas na análise bioinformática do sequenciamento de exoma serão validadas na coorte de descoberta. Isto será realizado através de sequenciamento de Sanger (sequenciamento de 1ª geração) com desenho de primers para reação de PCR com amplificação do ponto de mutação seguido de sequenciamento do DNA. As mutações que forem encontradas no sequenciamento de exoma e confirmadas no sequenciamento de Sanger serão consideradas como validadas e serão utilizadas para análise final e como gene candidatos para análise na coorte de prevalência.

2.5.8. Confirmação de natureza somática das mutações encontradas no sequenciamento de painel customizado

As mutações encontradas no sequenciamento em painel customizado caso sejam mutações previamente estabelecidas na literatura médica como sendo mutações "drivers" serão consideradas como mutações somáticas. Mutações encontradas no sequenciamento em painel customizado que não sejam previamente conhecidas serão validadas por PCR e sequenciamento em Sanger de material genético germinativo ou genotipagem em PCR por tempo real, caso este esteja disponível. Se não houver material genético germinativo disponível, uma mutação nova encontrada no sequenciamento de painel customizado será considerada como "driver" caso não seja encontrada em um banco de dados interno de sequenciamento de exoma germinativo ("normal") de 140 pacientes brasileiros com neoplasias mielóides (obtidos em outro

estudo) e caso a mutação siga um padrão esperado para a literatura (ex.: mutação inativadora em gene supressor tumoral).

2.5.9. Coleta de dados clínicos

Dados a respeito de informações clínicas, resposta ao tratamento, sobrevida global e recidiva serão extraídos de prontuários e bancos de dados. Dados clínicos a serem extraídos estão sumarizados na **tabela 1**. Os dados serão coletados em formulários de papel e passados para uma tabulação em CSV para posterior análise estatística.

2.5.10. Análise Estatística

Análise estatística será feita com o software R v3.4.1. A análise de qui-quadrado será utilizada para comparar características clínicas dos pacientes e taxa de resposta ao tratamento por estado mutacional. Devido à múltiplas comparações, consideraremos como estatisticamente significante um p-valor <0.001 apenas. A sobrevida será estimada pelo método de Kaplan-Meier, e recidiva será estimada pelo método de incidência cumulativa considerando morte não leucêmica como um risco competidor. O teste de logrank e o teste de Grey serão utilizados para comparação de sobrevida global e analisar incidência cumulativa de recidiva, respectiva. Um modelo de regressão logística multivariado será utilizado para definir associações entre múltiplas características clínicas e resposta ao tratamento. Um modelo de Cox será usado para definir variáveis associadas com sobrevida global. O modelo de Grey será usado para definir variáveis associadas com incidência cumulativa de recidiva.

2.6. Centros Parceiros

O HCFMUSP será a instituição coordenadora do estudo. Pesquisadores responsáveis: Prof. Dr. Israel Bendit e Prof. Dr. Vanderson Rocha.

O biobanco do ICESP sob a responsabilidade da pesquisadora Miyuiki Uno, PhD e do Prof. Dr. Roger Chammas fornecerá as amostras de DNA/RNA.

As amostras serão enviadas para o Instituto Weatherall de Medicina Molecular da Universidade de Oxford (Reino Unido), onde realizaremos os sequenciamentos e análises de bioinformática sob a supervisão do aluno de doutorado Douglas Rafaele Almeida Silveira e Prof. Dr. Paresh Vyas (Universidade de Oxford) e Prof. Dr. Vanderson Rocha (HCFMUSP e Universidade de Oxford).

2.7. Considerações éticas

Alguns paciente já possuem material genético coletado que fora utilizado para correta classificação prognóstica e tratamento, esses se ainda vivos deverão assinar o termo de consentimento livre e esclarecido (TCLE) autorizando o armazenamento e estudo de suas amostras. Para o pacientes que já faleceram com o material coletado previamente no mesmo contexto (iniciais e registro no complexo HC destes pacientes estão sumarizadas na tabelas 2 e 3 abaixo) solicitaremos dispensa da assinatura do TCLE. Outros pacientes que já assinaram o TCLE do biobanco poderão também ser incluídos se julgarmos as análises com células viáveis cabíveis e elucidativas para responder à pergunta biológica à qual o estudo se propõe. A participação dos pacientes no estudo é voluntária; dados que possam identificar os pacientes serão mantidos

confidenciais. Cada paciente incluído no estudo receberá um número individual para identificação. Informações sobre os resultados de sequenciamento genético poderão ser compartilhados em parte com bancos públicos existentes de dados genômicos (ex.:SRA- Sequence Read Archive- http://www.ncbi.nlm.nih.gov/sra). Não serão compartilhados dados com instituições financeiras, e ou terceiros. A qualquer momento, o paciente pode desistir de participar do estudo. Caso isso ocorra, seus dados não serão mais analisados, o material genético será desprezado e os dados de sequenciamento que foram eventualmente depositados em bancos públicos de dados genômicos serão retirados.

O material genético será enviado por serviço especializado em transporte de amostras biológicas para o Reino Unido. Em caso de material excedente, esse será descartado conforme declaração em anexo a este protocolo, o representante da Universidade de Oxford se compromete em compartilhar proporcionalmente os dados referentes às utilizações destes materiais, assim como conhecer e obedecer às regulamentações técnico-científicas e éticas da pesquisa no Brasil, assim como também se compromete a não utilizar esse material comercialmente ou em criação de patentes.

Novamente, para pacientes que já tiverem falecido, será solicitado a isenção de obtenção do TCLE. Solicitamos a isenção do TCLE para estes pacientes uma vez que será realizada análise de DNA apenas para sequenciamento de genes específicos com o objetivo único e isolado de buscar alterações somáticas que possam estar relacionadas com a patogênese da doença. Não será realizado cultivo de células desses pacientes em meio de cultura. A análise estatística irá buscar associação das mutações com

sobrevida, e para isso é essencial a inclusão de pacientes que já tenham falecido. Também será solicitada isenção de TCLE caso o paciente ao assinar TCLE do biobanco tenha optado por não ser informado caso novos estudos fossem realizados com suas amostras. Os pacientes já falecidos do estudo estão com suas iniciais e registro do Hospital das Clínicas sumarizado nas **tabelas 2 e 3** abaixo.

2.8. Plano de execução do estudo e cronograma

O projeto será desenvolvido em cinco etapas distintas em um período de dois anos. A primeira fase, com quatro meses de duração, será destinada à aprovação no comitê de ética, inclusão dos pacientes no protocolo, à coleta de material biológico. A segunda, destinada a extração de DNA/RNA e envio de amostras ao Reino Unido e análise de qualidade tem uma duração prevista de 2 mês. Na terceira fase, com duração de 4 meses, serão realizados os experimentos para enriquecimento e sequenciamento dos genes. A quarta fase será destinada à análise computacional dos resultados obtidos e se iniciará tão logo termine o primeiro sequenciamento. Sua duração prevista é de 12 meses. Na quinta e última fase (que ocorre em certa concomitância com a quarta) será realizada a análise estatística dos dados obtidos e comparação com análise de mutações. Sua duração aproximada é de quatro meses.

Tabela 1. Dados clínicos a serem extraídos dos pacientes – variáveis do momento do diagnóstico ou realização biópsia de medula óssea.

Variavel
Nome (Iniciais)
Prontuário
Centro (HIAE ou HCFMUSP)
Idade, anos
Sexo (masculino vs. feminino)
Data do diagnóstico
Subtipo de Leucemia Promielocítica microgranular (sim vs nao)
Presença de hemorragia (sim vs. não)
Hb, g/dL
Leucócitos, $x10^9$/L
Contagem absoluta de neutrófilos, $x10^9$/L
% de promielócitos no sangue periférico, %
Plaquetas, $x10^9$/L
% de promielócitosna medula óssea, %
DHL, UI/L
Cariótipo (laudo descritivo)
Realizou transplante de medula óssea (sim vs. não)
Data transplante de medula óssea
Vivo (sim vs. não)
Data da última visita ou data da morte
Para pacientes com Leucemia Mielóide Aguda
• Tratamento com quimioterapia intensiva (sim vs. não)
• Protocolo quimioterápico utilizado:
• Remissão completa (sim vs. não)
• Data remissão completa
• Recidiva (sim vs não)
• Data recidiva LMA

Tabela 2. Paciente com LPA que faleceram e portanto, solicitaremos isenção da aplicação do TCLE, amostras de DNA e RNA.

Tabela LPA óbitos		
Nº	Iniciais do paciente	RGHC
1	AG	60027836E
2	AMS	13899510G
3	APS	5292480G
4	ARMS	14157427J
5	AS	2449576E
6	ASB	60035808E
7	ACAA	13902638E
8	AF	90874108B
9	CGF	13657214H
10	CACE	13950149A
11	DGL	14014478G
12	DPO	13844140C
13	ECWP	13965580F
14	ESS	14135559F
15	EFR	14042987J
16	FAGO	13921514A
17	HLS	14047684K
18	JDGL	13948455K
19	JJS	120128005327
20	LFO	14055781C
21	LRBC	60024565B
22	MCSR	14089168J
23	MPAS	60035753J
24	MCSS	14004441G
25	MJBR	14076766I
26	MBSG	14160405C
27	NGD	13855572I
28	PRM	60006060G
29	TMS	60011973F
30	VSO	14035904F

Tabela 3. Paciente com LMA que faleceram e portanto, solicitaremos isenção da aplicação do TCLE, amostras de DNA e RNA

Tabela LPA óbitos		
Nº	Iniciais do paciente	RGHC
1	ASS	33587609A
2	AYS	14036830K
3	ARMS	14157427J
4	ACM	2929190I
5	ALS	14120743E
6	AASL	13476791G
7	ACL	2870302D
8	AFS	14083866C
9	AHF	14037866B
10	ARS	14125449H
11	AJS	14065384J
12	AF	90874108B
13	AMS	2315246E
14	BDFA	60035893E
15	CSAA	91058266K
16	CAC	55542575J
17	CSS	91094467I
18	CCC	14021112H
19	CAC	14102858F
20	CCN	14058831J
21	CS	2263749B
22	CJA	2967421C
23	DMM	14136674G
24	DSG	13955370A
25	DAN	90817686C
26	DPBS	14107848F
27	DAS	14066809D
28	DPF	60033034I
29	ENL	14169827K
30	ESP	13444405D
31	EFS	60026671K
32	ERS	14048117K
33	EVL	55394516A
34	ECO	13990916A
35	ESP	55707544E
36	EGG	55500950F
37	EEM	90525588H
38	FPCF	14162054F
39	FFR	14054252C
40	FFC	91004263I
41	FDS	14038524A
42	FCTA	3373418C

43	FMS	2395507K
44	FMMF	14117996E
45	FEN	13970505K
46	FGF	13911973G
47	GJ	13842937J
48	GAO	14092814H
49	GJO	90875740B
50	HAS	44137158B
51	ILO	91113038B
52	ICS	14095643D
53	IRSTA	14107585I
54	ISM	60035041J
55	JFB	13996994D
56	JOS	14084363J
57	JPAT	14167423H
58	JAS	14038204H
59	JAP	5358637E
60	JAR	55516690C
61	JCMS	14025545D
62	JCB	13774836E
63	JGPD	14168315H
64	JRAS	13939444E
65	JNK	14152601F
66	JGO	13985856G
67	JSG	13802722F
68	JMF	14152773A
69	KACR	2466932H
70	KCBWG	13991183J
71	LMR	14031150C
72	LRL	14064248E
73	LTA	14178768A
74	LMVA	13902409J
75	LACB	13948747K
76	MS	14006403G
77	MAS	33470511C
78	MAFB	55527691B
79	MB	3243914A
80	MAP	13793619I
81	MASS	14076864G
82	MAAS	2895647D
83	MAJL	14004715K
84	MAPS	2271142D
85	MGMS	13559042F
86	MFPA	14117456D
87	MFVM	13585534K
88	MLLS	14110717E
89	MLPS	14046628H

90	ML	14031477K
91	MDCO	14118297E
92	MCGA	14133323F
93	MIF	55418222B
94	MMO	14001292C
95	MOS	14142057D
96	MPS	14084993J
97	MAH	90998079K
98	MPSN	5287896G
99	MRAG	14113638I
100	MZRS	13980801H
101	MAS	13996010B
102	MAOS	14013164I
103	MC	55307061F
104	MSS	14074047H
105	MAG	90727695B
106	NBA	13969653J
107	NPR	13962388J
108	NGSO	13847470K
109	OBO	14042884F
110	OMS	2497333H
111	OT	55489217H
112	PL	13920407J
113	PCRG	14098699A
114	PS	13995201A
115	RQP	90897825J
116	RMA	90807885B
117	RML	91075292I
118	RPC	33609884E
119	RSS	13965570I
120	RSSP	13971368D
121	RPS	3215189D
122	RVT	14039107D
123	SSM	60016251F
124	SSBF	2225126E
125	SRPS	14036714F
126	SSS	14030347G
127	SMJ	14041843B
128	SSAM	14068679C
129	SPQ	13993055K
130	TCF	90950661G
131	TJI	90778630A
132	TKM	14068446A
133	UFG	13993913K
134	WPJ	14067715D
135	WM	13993303E
136	WB	14006479B

137	WJS	13900341I
138	WLBM	13948297E
139	WCS	14082465C
140	YFM	14097894C

3. Published Manuscripts

3.1. Integrating clinical features with genetic factors enhances survival prediction for adults with acute myeloid leukemia

Citation: **Douglas R. A. Silveira**, Lynn Quek, Itamar S. Santos, Anna Corby, Juan L. Coelho-Silva, Diego A. Pereira-Martins, Grant Vallance, Benjamin Brown, Luciana Nardinelli, Wellington F. Silva, Elvira D. R. P. Velloso, Antonio R. Lucena-Araujo, Fabiola Traina, Andy Peniket, Paresh Vyas, Eduardo M. Rego, **Israel Bendit**, Vanderson Rocha; Integrating clinical features with genetic factors enhances survival prediction for adults with acute myeloid leukemia. *Blood Adv* 2020; 4 (10): 2339–2350. doi: https://doi.org/10.1182/bloodadvances.2019001419

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3.2. A Multicenter Comparative Acute Myeloid Leukemia Study: Can We Explain the Differences in the Outcomes in Resource-constrained Settings?

Citation: **Douglas R. A. Silveira**, Juan L. Coelho-Silva, Wellington F. Silva, Grant Vallance, Diego A. Pereira-Martins, Maria I. A. Madeira, Lorena L. Figueredo-Pontes, Elvira D. R. P. Velloso, Belinda P. Simões, Andy Peniket, Robert Danby, Eduardo M. Rego, Paresh Vyas, Fabiola Traina, **Israel Bendit**, Lynn Quek & Vanderson Rocha (2020) A multicenter comparative acute myeloid leukemia study: can we explain the differences in the outcomes in resource-constrained settings?, Leukemia & Lymphoma, DOI: 10.1080/10428194.2020.1827252

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4. Discussion

In our paper, ‘Integrating clinical features with genetic factors enhances survival prediction for adults with acute myeloid leukemia’, we analysed data from three cohorts with a classical statistical approach where FMUSP data was used as the training dataset, and the other two (FMRP and OUH) as validation or test datasets. Firstly, we validated the European LeukemiaNet 2017 (ELN 2017) risk assessment (6) for the first time in a cohort of AML patients from a developing country. We showed that ELN2017 stratifies AML patients into three distinct risk groups. In our FMUSP cohort, the intermediate-risk group was the smallest, which is consistent with a previous report (82).

Current trends in AML risk stratification involve inclusion of ever more complex genetic data (83). However, at least in the near to medium-term future, such approaches will be not applicable to the majority of AML patients worldwide. Reliance on risk stratification tools developed using data from, and for, resource-replete healthcare settings has several caveats. Firstly, unless these tools are validated using local data, they may not be applicable to local patients. Secondly, diagnostic parameters used in risk stratification tools may not be available locally, and lack of such data would impair predictive accuracy. Thirdly, patients from poorer countries are often excluded from international studies, and this skews our understanding of the disease. Therefore, we need to have an AML prognostic scoring system that is fit-for-purpose in local healthcare settings.

The Adapted Genetic Risk (AGR) was designed using the same biological basis as ELN 2010 (21). It stratified patients into three prognostic

categories: favourable-risk (FR), intermediate-risk (IR) or adverse-risk (AR). The novelty of AGR is that it allows for some degree of missing data. AGR is a cytogenetic-molecular risk assessment where patients can be risk-stratified using minimal information. For example, a patient with mutant *NPM1* whose sample failed cytogenetic testing and whose *FLT3-ITD* status is unknown would be classified as IR (i.e. a 'worst-possible' scenario is presumed). Alternatively, an AML patient with deletion of chromosome 5q is considered AR regardless of the presence or absence of other molecular or cytogenetic findings, based on the observation of an association between 5q aneuploidy with complex monosomal karyotype and *TP53* mutations in AML (22).

AGR was as accurate as ELN2017, and more accurate than the MRC cytogenetic classification (5) for three independent cohorts in predicting OS, including a UK cohort (OUH) where patients had much higher rates of HSCT than Brazilian cohorts. This indicates that AGR is independent of clinical outcome *per se*, and is applicable for AML patients managed with different post-remission approaches. Furthermore, AGR can be applied to more patients as it enables the inclusion of 25% more patients than ELN2017, and from 10-30% more than MRC into our survival analysis.

We postulate that AGR can also be applied in other settings (e.g. clinical trials) with similar prediction accuracy since it is based on the same biological concepts as standard cytogenetics and molecular evaluations. As AGR is based on characteristics of disease at diagnosis, its discriminatory power for DFS was affected by a high rate of HSCT in the OUH cohort. Censoring for HSCT restored the predictive performance of AGR.

AGR was designed to strengthen statistical modelling in real-life,

retrospective studies, not only by enabling the inclusion of more patients into the model when compared with both versions of ELN (2010 and 2017), but also by eliminating the need of adding individual gene mutation (e.g.: *NPM1*, *FLT3*, and *CEBPA*) as model variables, thus decreasing model complexity and enhancing its fitness. For example, in a cohort where ELN application is not feasible, using a cytogenetic only classification such as MRC, will require the addition of at least two additional variables (*NPM1* and *FLT3-ITD* status) for this model would be considered valid. One of the drawbacks of AGR is that its relative permissiveness increases the proportion of patients in the intermediate-risk group. This is ameliorated by using a combined AGR-SAMLS strategy which is discussed later. We propose that AGR is a valid substitute where ELN 2017 cannot be applied and would be useful for ~60% of AML-treating physicians worldwide (some of whom are in developed countries) who do not have access to complete cytogenetic-molecular testing for their patients (84).

From our analysis, serum albumin was a strong independent OS predictor for previously untreated AML. A single-centre French study recently found that very low albumin (<3.0g/dL) had a negative impact on survival in untreated AML patients (85). In another single-centre Chinese study, a albumin level above 3.5 g/dL has shown association with overall survival (HR = 0.375, $p < .001$) and leukaemia-free survival (HR = 0.411, $p < .001$) (86). In our study, the cutoff of 3.8 g/dL makes our scoring system relevant also to cohorts in high-income countries where higher baseline albumin levels are expected (87).

The effect of hypoalbuminemia on survival is likely to be due to its role as a surrogate inflammatory marker (39), and a measure of nutritional state and fitness (88). Furthermore, drug binding to albumin makes it an important

factor in pharmacokinetics in AML therapy (89). In patients who are borderline in fitness for intensive treatment, availability of effective non-intensive regimens now mean that even these patients have a realistic chance of durable remission or even cure (90,91). Thus, albumin may serve as an objective surrogate marker of fitness, such that clinicians may be advised to offer attenuated induction therapy to reduce treatment-related mortality (TRM). However, the utility of albumin here would need to be tested in a prospective clinical trial.

Using surrogate measures of patients' performance status (age, albumin) together with leukaemia biology variables (very low or very high WBC) and combining these with the AGR, we devised a novel score named Survival AML Score (SAMLS). SAMLS improved overall survival prediction by up to 37% compared with genetic risk assessment alone. Our data shows that SAMLS could model early (1-year) and late (5-years) survival differences. We have now validated SAMLS in two clinically independent cohorts, demonstrating its robustness despite variation in baseline patient characteristics, treatment settings (especially post-remission therapy), and the economic conditions in healthcare delivery.

Our data suggest SAMLS can help clinicians decide on therapy. As an example, our subset analysis suggests LR SAMLS would not benefit from HSCT in CR1, yet emphasizing some AGR-IR will be classified as LR SAMLS, indicating the importance of not relying on cytogenetic-molecular parameters alone. Since SAMLS has been developed using data from patients <65 years old with de novo AML treated with ICT, it would be strengthened by prospective validation for specific therapy regimens, and by validation in older AML patients, including those with secondary AML. Prospective validation may also

incorporate additional clinical features into SAMLS to improve its prediction accuracy. However, it is already a proof of principle that missing diagnostic molecular data is not a barrier to effective risk stratification and enables evaluation of patients in studies who would otherwise be excluded due to missing data. We believe the flexibility of SAMLS will be helpful to better risk stratify AML patients in countries where resources are scarce.

In our second paper, ‘A Multicenter Comparative Acute Myeloid Leukemia Study: Can We Explain the Differences in the Outcomes in Resource-constrained Settings?’, we aimed to study potential differences in survival between USP cohorts and OUH, and their causes.

We found a significant difference in survival rates of AML between two cohorts of intensively-treated patients from Brazil and the United Kingdom. Our findings showed that differences in outcomes were not primarily due to disease biology or treatment. We observed heterogeneity in patients' baseline characteristics between two cohorts - patients from Brazil were younger and had lower albumin levels. Consistent with differences in the median age, patients in Brazil had a more favourable genetic risk profile and fewer cases of secondary AML (6,92,93). Age and albumin differences are likely due to demographic and socio-economic factors (87,94). After adjusting OS for patient and disease confounders, and rates of allo-HSCT, OUH still has a 31% hazard-ratio reduction, suggesting that other factors were important in influencing patient outcomes.

There was no difference between the cohorts in the rate of refractory disease following ICT. This suggests that intrinsic biological characteristics of the disease are unlikely to be the reason for differences in

outcome between cohorts. However, induction death without refractory disease was significantly higher in Brazil (19%) compared to the UK (3%) and data previously reported in other developed countries (95). Although we did not specifically address this in our study, we suggest that obstacles access healthcare centres to seek urgent medical attention and higher rates of comorbidity explain this disparity (10,96).

Excess mortality due to infection identified in this study is potentially preventable by improved patient education, access to adequate healthcare in patients with symptoms of infection and prompt administration of antibiotics with activity against causative organisms (12). Although antimicrobial guidelines for febrile neutropenia are in place in most treatment centres in Brazil, we propose that regular audits should be undertaken to enforce robust implementation of best practice.

The anti-bacterial prophylaxis has been recommended for neutropenic patients to reduce the risk of sepsis, yet evidence with regard to its efficacy has been mixed. In the UK, fluoroquinolones are recommended based on a meta-analysis (97); however, implementation is variable across centres. There remain concerns about the risk of *Clostridium difficile* and multi-resistant bacterial species. Recently, it was demonstrated that gut colonization by multi-resistant GNB reduces survival in leukaemia and HSCT patients (98), and we suspect that since most of the identified organisms were enterobacteria, the gut is the likely source of the majority of MDR GNB sepsis in our patients. Use of appropriate antibiotics with reference to and local resistance profiles and appropriate access to isolation nursing, high dependency and intensive care support would also be helpful (99).

Deaths also could be reduced by the use of antifungal prophylaxis for both candidemia and aspergillosis, which was not standard practice in Brazilian centres during the study period. Although established in the literature (100), primary prophylaxis against aspergillosis remains suboptimal in Brazil. Also, anti-candidal prophylactic regimens were adopted from 2016 in both Brazilian centres, this is likely to have had little impact on our cohort as the majority of FUP time would have been without prophylaxis. A prospective multicentre survey conducted in Brazil found a high cumulative incidence of invasive fungal disease among AML and HSCT patients (101), which reinforces the urgent need to review fungal prophylaxis policies.

In addition, as environmental exposure to fungal pathogens are likely to be different in different countries, we need to carefully evaluate epidemiological and microbial evidence when choosing appropriate prophylactic antifungal agents. Importantly, prospective studies to follow-up implementation and outcomes are required to ensure cost-effectiveness and best practice.

Cumulative incidence of relapse (CIR) was higher in USP. After adjustment for confounders, the treatment centre per se did not impact DFS, suggesting that HSCT factors rather than disease biology were negatively impacting USP outcomes. Effectiveness of post-remission consolidation itself was not a factor since patients undergoing the same consolidative treatment had similar CIR and similar overall NRM mortality in both cohorts.

Allogeneic HSCT is the only curative option for many AML patients (102), and the effectiveness of Matched-related donor HSCT delivered in CR1 is similar in OUH and USP. However, our analysis strongly suggests that low HSCT rates and long wait times affect survival of AML patients in USP.

We found that USP centres transplanted fewer patients than OUH (28% vs. 75%). Furthermore, the time to transplant was prolonged and failed to reach the predicted optimum number of cases. Compared with OUH, USP is more likely to transplant patients in CR2 even when HSCT is indicated in CR1, and despite poorer outcomes of CR2 HSCT (103). USP patients also waited longer for CR2 HSCT in patients at high risk of relapse. Differences in OS include death due to relapse, are therefore likely to be compounded by poorer rates and delayed timing of consolidative HSCT.

Disease relapse is associated with abysmal prognosis in Brazilian centres (104), with 90% of relapsed patients dying within 3 years. Nonetheless, allogeneic HSCT rates for haematological disease in Brazil are low, and the majority of stem cell transplantation (21-100 individuals per 10 million inhabitants) are autologous. In contrast, approximately half of transplants in the UK (>500 individuals per 10 million) are allogeneic, the major indication for which is AML (105). An important barrier is decreased availability of matched unrelated donors in Brazil compared to the UK. According to reports from the USA, AML patients of Hispanic and African-Americans ancestry are less likely to receive an HSCT due to both socioeconomic factors and donor availability (106–108). However, greater use of haploidentical donors, which have been shown to give good outcomes, should encourage greater allogeneic HSCT activity (109).

To our knowledge, this is the direct comparison between real-life outcomes for AML patients in contrasting healthcare settings. We find that the main drivers of inferior AML outcomes were higher rates of fatal infections and poorer rates of HSCT. Despite being based on retrospective data, we have

performed robust statistical analysis to address potential sources of bias. However, we were unable to analyze some factors directly related to the care of the patients e.g. length of hospital stay, time taken for presentation to hospital in emergencies, prior antibiotic exposure, occupational exposure, patient's household income, which may impact health outcomes. Nonetheless, we are confident that better results can be achieved in LMIC by implementing better patient and staff education, appropriate antimicrobial prophylaxis based on locally microbiology and susceptibility profiles, robust implementation of febrile neutropenia protocols and infection control measures to isolate and prevent the spread of MDR organisms. Furthermore, investment in infrastructure, training and alternative donor sources are needed to increase the frequency and improve the timeliness of HSCT.

5. Conclusions

The primary aims of my thesis were performing next-generation sequencing of Acute Myeloid Leukaemia samples and correlating this mutational data with outcome and treatment response so that this work could be the first comprehensive AML study approaching genetic and clinical data in a developing country.

My thesis was the first study to validate ELN2017 in a developing country cohort, and it was also the first to validate the diagnostic serum albumin as a prognosis biomarker for *De novo* AML in independent cohorts. It is worth citing that we only could apply ELN2017 in FMUSP as we performed our extended Myeloid gene panel (targeted gene resequencing for mutation detection).

I have devised Adapted Genetic Risk, a flexible but robust cytogenetic-molecular risk assessment, which was validated against the current ‘gold standard’, ELN2017, and in two independent patient cohorts.

AGR was then incorporated together with patients' performance (age and albumin) and disease biology (white-blood-cell counts) to create SAMLS, a new scoring system. SAMLS increased survival prediction accuracy and was also validated in two independent cohorts demonstrating robustness against baseline characteristics and heterogeneity in post-consolidation treatment.

Finally, I analysed available multicentre datasets to ascertain why patients treated in Brazil were having a worse outcome compared with their counterparts treated in the United Kingdom.

Although in this thesis I am using slightly different technologies to what was included in the original project plan (see Addendum section), I have achieved the project goals. I have used a robust approach to generate locally relevant prognostic scoring systems that best serve the needs of local patients and clinicians. In addition, my thesis has provided important conceptual advances in understanding AML as a disease in Brazil.

Future work will include prospective validation of my thesis findings. Furthermore, I intend for this work to be a springboard for improvement to local practices.

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Addendum

In my thesis, I have deviated from and adapted some of the original project objectives. The initial plans were to perform whole-exome sequencing (WES) in patient samples to look for new driver mutation which may be specific for our patient population. However, due to the nature of our DNA samples (extracted from total bone marrow and therefore potentially 'contaminated' with variable proportions of wild type, non-leukaemic cells), WES was not feasible. The secondary objective of the original thesis plan was to perform RNAseq or single-cell sequencing. Again, these were not achievable due to lack of ability to flow-sort and purify leukaemic populations from stored total bone marrow samples.

Importantly, I took advantage of targeted sequencing gene panels and clinical-laboratory data from FMUSP patients to generate a Brazilian multicentre cohort, aimed at studying the real-life impact of DNMT3A mutations and AML with a triple-mutated status (*NPM1* + *FLT3-ITD* + *DNMT3A*). Although this study has not filled the inclusion criteria (i.e.: the PhD student was not its first author) for this thesis with articles compilation, I have nonetheless cited this in the Appendix section below (Appendix A. Other Published Manuscripts - 'Co-occurrence of *DNMT3A*, *NPM1*, *FLT3* mutations identifies a subset of acute myeloid leukemia with adverse prognosis') since it demonstrates concordance with the project objectives.

The initial project plan also included genotyping Acute Promyelocytic Leukemia (APL) cases. However, stored DNA samples were insufficient as our biorepository does not routinely store APL DNA samples, but only prepare total bone marrow RNA samples instead). Furthermore, there was insufficient funding so that I decided to prioritise samples where there was a higher chance of yielding interesting results. Thus, I focused on AML DNA samples for myeloid gene panel targeted sequencing. Nonetheless, we have used clinical-laboratory data from APL patients in a retrospective work to study why our results were inferior to expected according to literature. Appendix section (Appendix A. Other Published Manuscripts - 'Real-life Outcomes on Acute Promyelocytic Leukemia in Brazil – Early Deaths Are Still a Problem').

Finally, there are ongoing studies involving genetic and clinical-laboratory data from my thesis project in national multicentre collaborations which will be cited in an Appendix section (Appendix B. Ongoing Studies and Future Perspectives). I continue to be part of ongoing international collaborative projects, which although are in the field of AML biology, are not directly related to the initial thesis project.

Appendix

Appendix A. Other Published Manuscripts

Below we cited other publications which were directly* related to the original project.

*Using cohort data and project ethical approval.

I. Co-occurrence of *DNMT3A*, *NPM1*, *FLT3* mutations identifies a subset of acute myeloid leukemia with adverse prognosis

Citation: Matheus F. Bezerra, Aleide S. Lima, Maria-Riera Piqué-Borràs, **Douglas R. Silveira**, Juan L. Coelho-Silva, Diego A. Pereira-Martins, Isabel Weinhäuser, Pedro L. Franca-Neto, Lynn Quek, Anna Corby, Mayara M. Oliveira, Marinus M. Lima, Reijane A. de Assis, Paula de Melo Campos, Bruno K. Duarte, **Israel Bendit**, Vanderson Rocha, Eduardo M. Rego, Fabiola Traina, Sara T. Saad, Eduardo I. Beltrão, Marcos A. Bezerra, Antonio R. Lucena-Araujo; Co-occurrence of *DNMT3A*, *NPM1*, *FLT3* mutations identifies a subset of acute myeloid leukemia with adverse prognosis. *Blood* 2020; 135 (11): 870–875. doi: https://doi.org/10.1182/blood.2019003339

Journal: Blood

JCR IF: 17.543

II. Real-life Outcomes on Acute Promyelocytic Leukemia in Brazil – Early Deaths Are Still a Problem

Citation: Wellington F. da Silva, Lidiane Inês da Rosa, Gabriel Lacerda Marquez, Lucas Bassolli, Luciana Tucunduva, **Douglas Rafaele Almeida Silveira**, Valeria Buccheri, **Israel Bendit**, Eduardo Magalhães Rego, Vanderson Rocha, Elvira D.R.P. Velloso,
Real-life Outcomes on Acute Promyelocytic Leukemia in Brazil – Early Deaths Are Still a Problem, Clinical Lymphoma Myeloma and Leukemia, Volume 19, Issue 2, 2019, Pages e116-e122, ISSN 2152-2650, https://doi.org/10.1016/j.clml.2018.11.004.

Journal: Clinical Lymphoma Myeloma and Leukemia

JCR IF: 2.298

III. Salvage treatment for refractory or relapsed acute myeloid leukemia: a 10-year single-center experience

Citation: Silva, W. F. , Rosa, L. I. , Seguro, F. S., **Silveira, D. R. A., Bendit, I.**, Buccheri, V., Velloso, E. D. R. P., Rocha, V., & Rego, E. M. (2020). Salvage treatment for refractory or relapsed acute myeloid leukemia: a 10-year single-center experience. *Clinics (São Paulo)., 75*, e1566. https://doi.org/10.6061/clinics/2020/e1566

Journal: Clinics

JCR IF: 1.435

Appendix B. Ongoing Studies and Future Perspectives

I. IDH mutation impact on AML outcome in a real-life setting: A multicentre study.

Description: Retrospective study to evaluate the impact of IDH mutation in a real-life multicentre AML cohort.

Leading centre: University Federal of Pernambuco

Status: Manuscript writing

II. Impact of additional cytogenetics abnormalities and *NPM1* allelic ratio in *NPM1*-mutated AML: A multicentre study.

Description: Retrospective study to evaluate the impact of additional cytogenetics abnormalities and *NPM1* allelic ratio in a real-life multicentre AML cohort.

Leading centre: University of São Paulo - FMRP

Status: Data collection

III. AML genomic classification single-centre validation.

Description: A single-centre cohort with 140 patients sequenced with extended genotyping (94 genes targeted sequence panel) aiming to validate Papaemmanuil et al. NEJM 2016 genomic classification.

Leading centre: University of São Paulo - FMUSP

Status: Data analysis