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Clonal dominance in bone marrow failure syndromes

Pedro Henrique De Lima Prata

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En cotutelle avec l'Université de São Paulo

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Clonal dominance in bone marrow failure syndromes

Par Pedro Henrique de Lima Prata

Thèse de doctorat d'Hématologie

Dirigée par Régis Peffault de Latour
Et Rodrigo Calado de Saloma Rodrigues

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Titre : Dominance clonale dans les insuffisances médullaires

Résumé :

Les insuffisances médullaires sont des maladies rares qui se manifestent principalement par pancytopénie. L'incapacité du tissu hématopoïétique à maintenir l'hématopoïèse peut être acquise, principalement l'aplasie médullaire idiopathique (AM), ou des défauts génétiques, tels que le déficit de réparation de l'ADN de l'anémie de Fanconi (AF). L'hématopoïèse clonale est très fréquente chez les patients atteints d'une insuffisance médullaire et l'évolution vers un syndrome myélodysplasique ou leucémie aiguë myéloïde (SMD/LAM) est un événement préoccupant. Certaines données publiées indiquent que l'hématopoïèse clonale affecterait le risque de progression. Notre travail s'est concentré sur l'émergence de clones avec des mutations somatiques chez AM et AF et leur effet clinique.

Dans la première partie, nous nous sommes concentrés sur l'aplasie acquise. Près d'un tiers des patients AM présente une hémoglobinurie paroxystique nocturne (HPN) concomitante. La HPN est une maladie rare et monogénique qui se manifeste par une hémolyse intravasculaire. Elle est causée par des mutations somatiques du gène *PIGA*, entraînant une susceptibilité cellulaire au système du complément. Outre le gène *PIGA*, les anomalies du HLA de classe I sont fréquentes chez les patients AM. Ces deux groupes de cellules échapperaient à la destruction immunitaire, contribuant ainsi à soutenir l'hématopoïèse. De plus, certains allèles HLA de classe I sont surreprésentés dans l'AM. Cela pourrait indiquer que l'HLA joue un rôle dans la physiopathologie de l'AM. Jusqu'à présent, une prédisposition héréditaire à l'HPN est inconnue. Nous avons constaté que des variantes constitutionnelles du facteur H du complément étaient surreprésentées chez les patients atteints d'HPN. Certaines caractéristiques constitutionnelles semblent donc influencer la dominance clonale chez les patients atteints d'une aplasie acquise.

Nous avons étudié comment les caractéristiques cliniques, cytogénétiques et moléculaires des patients AM/HPN affectent la transformation en SMD/LAM. Nous avons confirmé que l'absence de réponse aux traitements non-greffe, l'âge et les mutations myéloïdes conféraient un risque plus élevé de progression. Par ailleurs, nous avons observé que les mutations *PIGA/HLA*, prédominantes au départ, étaient remplacées par des mutations myéloïdes classiques lors de la transformation.

Pour la deuxième partie, nous avons voulu explorer l'impact du mosaïcisme somatique dans l'AF sur la transformation en néoplasies myéloïdes. Près de 30% des patients atteints d'AF développeront un cancer du sang au cours de leur vie. Globalement, les complications hématologiques représentent près de 70 % des décès dus à l'AF. Divercement, certains patients présentent un phénotype hématologique moins grave : des cellules hématopoïétiques spontanément corrigées coexistent avec des cellules Fanconi, soutenant l'hématopoïèse. Notre équipe a précédemment étudié une cohorte de 350 patients FA, décrivant les caractéristiques cliniques et moléculaires de 17 révertants, et définissant les mécanismes génétiques expliquant la réversion. Jusqu'à présent, nous n'avons pas identifié de patient révertant évolué vers SMD/LAM. La correction fonctionnelle d'un certain nombre de cellules souches hématopoïétiques pourrait conférer un avantage écologique, mitigeant le risque de dominance clonale de clones pré-malins.

Inspirés par le mosaïcisme somatique, des essais cliniques ont montré des résultats prometteurs en utilisant des cellules souches hématopoïétiques corrigées génétiquement – thérapie génique - pour traiter l'insuffisance médullaire chez les patients AF. Cependant, l'effet sur le risque de transformation en SMD/LAM est inconnu pour l'instant. Nous avons travaillé sur le développement d'un modèle de réversion génétique chez des souris AF. Nous avons voulu tester des sous-clones

corrigés génétiquement ou pré-leucémiques dans le contexte de Fanconi et le risque d'évolution.

Ce travail contribuera à la compréhension des mécanismes qui participent de la progression vers les SMD/LAM dans les insuffisances médullaires, pouvant aider à adapter les stratégies de suivi et de traitement, réduisant ainsi la morbidité.

Mots-clefs :

aplasie médullaire, hémoglobinurie paroxystique nocturne, anémie de Fanconi, leucémie aiguë, syndrome myélodysplasique, hématopoïèse clonale

Título: Dominância clonal nas falências medulares

Resumo:

As falências medulares são doenças raras que se manifestam principalmente por pancitopenia. A incapacidade do tecido hematopoiético de manter a hematopoiese pode ser adquirida, como na aplasia imune, ou constitucional, como a deficiência de reparo do DNA da anemia de Fanconi (AF). A hematopoiese clonal é muito comum em pacientes com falência da medula óssea e progressão para síndrome mielodisplásica ou leucemia mielóide aguda (SMD/LMA) é um evento preocupante. Existem evidências de que a hematopoiese clonal pode afetar o risco de progressão. Nosso trabalho concentrou-se no aparecimento de clones com mutações somáticas em aplasia imune e AF e seu efeito clínico.

Na primeira parte, concentramo-nos na aplasia imune. Cerca de um terço dos pacientes com aplasia tem hemoglobinúria paroxística noturna concomitante (HPN). A HPN é uma doença rara que se manifesta principalmente por hemólise intravascular. É causada por mutações somáticas no gene *PIGA*, resultando em suscetibilidade celular ao sistema do complemento. Além do gene *PIGA*, anormalidades do HLA de classe I são comuns em pacientes com aplasia imune. Pensa-se que ambos os grupos de células (HLA e *PIGA*) escapariam do ataque imunológico, ajudando a manter a hematopoiese. Além disso, certos alelos HLA de classe I estão sobre-representados na aplasia imune, podendo indicar que o HLA desempenha um papel na fisiopatologia da aplasia. Por enquanto, uma predisposição constitucional para a HPN é desconhecida. Curiosamente, descobrimos que as variantes constitucionais do fator H do complemento estavam sobre-representadas em pacientes com HPN. Assim, certas características constitucionais parecem influenciar o domínio clonal em pacientes com aplasia adquirida.

Para aprofundar, investigamos como as características clínicas, citogenéticas e moleculares dos pacientes aplasia/HPN afetam a transformação para SMD/LMA. Confirmamos que a resposta sub-ótima aos tratamentos não transplante, a idade ao diagnóstico e mutações mielóides conferiram um maior risco de progressão. Além disso, observamos que as mutações *PIGA/HLA*, que eram predominantes no diagnóstico, foram substituídas por mutações mielóides clássicas durante a transformação.

Na segunda parte, exploramos o impacto do mosaicismosomático na AF na transformação para neoplasia mielóide. Quase 30% dos pacientes com AF desenvolverão um câncer de sangue durante sua vida. Em geral, as complicações hematológicas são responsáveis por quase 70% das mortes de AF. Diversamente, alguns pacientes apresentam um fenótipo hematológico menos grave: células hematopoiéticas corrigidas espontaneamente (revertantes) coexistem com células de Fanconi, suportando a hematopoiese. Nossa equipe estudou previamente uma coorte de 350 pacientes da FA, descrevendo as características clínicas e moleculares de 17 revertantes e definindo os mecanismos genéticos que explicam a reversão. Até o momento, não identificamos nenhum paciente revertante que tenha progredido para SMD/LMA. É possível que a correção funcional de algumas células-tronco hematopoiéticas confira uma vantagem ecológica, reduzindo a probabilidade de que células pré-malignas atinjam dominância clonal.

Inspirados pelo mosaicismosomático, ensaios clínicos mostraram resultados promissores usando células-tronco hematopoiéticas corrigidas geneticamente – terapia gênica - para tratar a falência da medula óssea em pacientes com AF. No entanto, o efeito sobre o risco de transformação para MDS/AML é desconhecido no

momento. Desenvolvemos um modelo de reversão genética em camundongos AF para testar subclones geneticamente corrigidos ou pré-leucêmicos no contexto de Fanconi e o risco de progressão.

Estes trabalhos contribuirão para a compreensão dos mecanismos envolvidos na progressão para SMD/LMA na falência da medula óssea, podendo ajudar a adaptar as estratégias de acompanhamento e tratamento, reduzindo assim a morbidade.

Palavras-chave:

aplasia medular, hemoglobinúria paroxística noturna, anemia de Fanconi, leucemia aguda, síndrome mielodisplásica, hematopoiese clonal

Title: Clonal Dominance in Bone Marrow Failure Syndromes

Abstract:

Bone marrow failure syndromes (BMF) are a diverse group of rare diseases that manifest chiefly with pancytopenia. The incapacity of the haematopoietic tissue to sustain haematopoiesis can be acquired, chiefly immune-mediated aplastic anaemia (AA), or inherited intrinsic cellular defects, such as the DNA repair deficiency in Fanconi anaemia (FA). BMF severely impairs patients' quality of life and complications can be fatal. Besides, clonal haematopoiesis is very frequent among BMF patients, and progression into MDS/AML is a concerning event with a dismal prognosis. Despite a long-known association between BMF and myeloid malignancies, the precise mechanisms involved in clonal dominance are unclear. Some published data indicate that clonal haematopoiesis affects the MDS/AML progression risk. This work focused on the emergence of clones bearing somatic mutations in AA and FA and their clinical effect.

In the first part of this work, we focused on acquired BMF. Nearly a third of AA patients have concomitant paroxysmal nocturnal haemoglobinuria (PNH). PNH is a rare, monogenic disease that manifests as intravascular haemolysis. It is caused by somatic mutations in the *PIGA* gene, resulting in impaired cellular defence against the complement system. Besides *PIGA*, HLA class I abnormalities are frequent among AA patients. Such cells would evade the immune-mediated destruction and expand, helping to sustain haematopoiesis. Moreover, some HLA class I alleles are overrepresented in aplastic anaemia irrespective of ethnic background. The overrepresentation of specific class I alleles among AA patients strongly supports its influence on AA pathophysiology. Thus far, an inherited predisposition to PNH is unknown. We found that rare germline complement factor H variants were overrepresented among PNH patients. Germline characteristics seem to influence clonal dominance among acquired BMF patients.

We investigated how baseline clinical, cytogenetic, and molecular characteristics in AA/PNH patients affected AML/MDS transformation. We confirmed that lack of response to non-transplant treatments, older age, and myeloid driver mutations at marrow failure onset conferred a higher risk of progression. Besides, we observed that *PIGA/HLA*-mutations, predominant at baseline, were replaced by classic myeloid drivers at the transformation.

For the second part of this work, we were interested in exploring the impact of somatic mosaicism in FA on AML/MDS transformation. Nearly 30% of FA patients will develop a myeloid malignancy during their lifetime. Overall, hematologic complications account for almost 70% of FA deaths. Diversely, a few patients present a milder hematologic phenotype: a mosaicism of spontaneously corrected cells coexist with FA-deficient cells. Our team has previously studied a cohort of FA patients, described revertants' clinical and molecular characteristics, and defined the genetic mechanisms that explained reversion. Thus far, revertant patients have not progressed to MDS/AML. The functional correction of a few hematopoietic stem cells might confer an ecologic advantage, mitigating the risk of pre-leukemic clonal dominance.

Allogeneic hematopoietic stem cell transplantation is the only curative treatment for hematologic FA manifestations. This modality carries high treatment-related mortality but abrogates the risk of AML/MDS. Inspired by revertant somatic mosaicism, clinical trials showed promising results using autologous *ex vivo* gene-corrected hematopoietic stem cells – gene therapy - to rescue marrow failure in FA patients. However, the effect on AML/MDS transformation risk is unknown thus far. We worked

on developing a model of genetic reversion in FA mice. We aimed to test the fitness of gene-corrected or preleukemic subclones in the Fanconi context.

This work will contribute to understand the intrinsic and extrinsic mechanisms underlying clonal dominance and progression towards MDS/AML in BMF, helping to adapt follow-up and treatment strategies, reducing morbidity.

Keywords:

aplastic anaemia, paroxysmal nocturnal haemoglobinuria, Fanconi Anaemia, acute leukaemia, myelodysplastic syndromes, clonal evolution, clonal haematopoiesis

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Acronyms and abbreviations

AA: aplastic anaemia

AML: acute myeloid leukaemia

ARCH: age-related clonal haematopoiesis

ASH: the American Society of Hematology

BMF: bone marrow failure

CCUS: clonal cytopenia of undetermined significance

CFU: colony-forming unit

CH: clonal haematopoiesis

CHIP: clonal haematopoiesis of indeterminate potential

CHOP: clonal haematopoiesis of oncogenic potential

DBA: Diamond-Blackfan Anaemia

EBMT: European Society for Blood and Marrow Transplantation

EMA: European Medicine Agency

FDA: Food and Drug Administration

GPI: glycosylphosphatidylinositol

GvHD: graft-versus-host disease

HLA: human leukocyte antigen

HSC: haematopoietic stem cell

HSCT: haematopoietic stem cell transplantation

HSPC: haematopoietic stem and progenitor cells

IBMF: inherited bone marrow failure

ICUS: idiopathic cytopenia of undetermined significance

KO: knock-out

Lin⁻: lineage-negative haematopoietic cells

LSK: lineage-negative, Sca-positive, Kit-positive haematopoietic cells

MDS: myelodysplastic syndrome

NGS: next-generation sequencing

pI:pC: polyinosinic : polycytidylic acid

PNH: paroxysmal nocturnal haemoglobinuria

SDS: Schawcham-Diamond syndrome

TBD: telomere biology diseases

VAF: variant allele frequency

VUS: variant of unknown / uncertain significance

WT: wild-type

Résumé en Français

Le sang est un tissu liquide complexe vital pour la vie des vertébrés. Le compartiment cellulaire du sang est constitué de globules rouges, de globules blancs et de plaquettes. En résumé, les globules rouges, ou érythrocytes, sont responsables du transport de l'oxygène et du dioxyde de carbone des poumons vers les tissus ; les globules blancs, ou leucocytes, sont le pilier du système immunitaire ; les plaquettes ne sont pas des cellules à part entière, mais des fragments d'une autre cellule spécialisée (les mégacaryocytes) et sont des acteurs majeurs de la coagulation sanguine et de la réparation vasculaire.

Les cellules sanguines dérivent d'un type cellulaire unique dans la moelle osseuse : la cellule souche hématopoïétique (CSH). Le processus hiérarchique de différenciation des cellules souches jusqu'aux cellules matures circulantes est connu sous le nom d'hématopoïèse. Lorsqu'une mutation génétique a lieu dans une CSH et que cette mutation confère un avantage écologique à cette cellule de façon à ce que le clone muté contribue de manière disproportionnée et persistante à la formation des cellules sanguines, la condition est appelée hématopoïèse clonale. Cela est un facteur de risque d'évolution en néoplasies hématologiques, notamment des syndromes myélodysplasiques et leucémies aiguës myéloïdes (SMD/LAM).

L'hématopoïèse clonale est très fréquente chez les patients atteints d'une insuffisance médullaire et l'évolution vers un SMD/LAM est un événement préoccupant. Les insuffisances médullaires sont des maladies rares qui se manifestent principalement par une insuffisance globale de production de cellules du sang appelée pancytopenie. L'incapacité du tissu hématopoïétique à maintenir l'hématopoïèse peut être acquise, principalement l'aplasie médullaire idiopathique, ou causée par des défauts génétiques, tels que le déficit de réparation de l'ADN de l'anémie de Fanconi.

Les objectifs de mon travail étaient de présenter l'effet de l'hématopoïèse clonale dans les insuffisances médullaires type aplasie acquise et anémie de Fanconi et leur impact sur l'évolution des SMD/LAM. Les patients atteints d'une insuffisance médullaire et d'une hématopoïèse clonale présentent des histoires naturelles très différentes. Je me suis concentré sur deux questions : l'étude des déterminants de la transformation dans les aplasies acquises et l'impact de la correction génétique spontanée dans le tissu hématopoïétique dans l'anémie de Fanconi (réversion) sur le risque de progression dans un modèle murin.

Dans une première partie, je me suis concentré sur l'aplasie acquise. Près d'un tiers des patients aplasiques présente une hémoglobinurie paroxystique nocturne (HPN) concomitante. La HPN est une maladie rare et monogénique qui se manifeste par une hémolyse intravasculaire. Elle est causée par des mutations somatiques du gène *PIGA*, entraînant une susceptibilité cellulaire au système du complément. Outre le gène *PIGA*, les anomalies du HLA de classe I sont fréquentes chez les patients aplasiques. Ces deux groupes de cellules échapperaient à la destruction immunitaire, contribuant ainsi à soutenir l'hématopoïèse. De plus, certains allèles HLA de classe I sont surreprésentés dans l'aplasie. Cela pourrait indiquer que l'HLA joue un rôle dans la physiopathologie de l'aplasie.

Mon objectif initial était de rechercher si des anomalies d'expression du HLA de classe I dans les cellules hématopoïétiques chez les patients atteints d'une aplasie médullaire acquise étaient associées à un risque augmenté de transformation vers SMD/LAM. Nous avons donc mené une étude pilote et avons séquencé les gènes HLA des classes I et II de dix patients (neuf LAM et une SMD avec excès de blastes) qui avaient transformé à partir d'une aplasie acquise. Nous n'avons pas mis en évidence d'anomalie génomique des gènes HLA (mutation de perte de fonction ou de perte

d'hétérozygotie). Ces résultats n'ont pas conforté notre hypothèse initiale. De ce fait, nous avons établi une collaboration multicentrique (Hôpital Saint-Louis en France, Université de Sao Paulo au Brésil et Clinique de Cleveland aux États-Unis).

Pour infirmer notre hypothèse, nous avons interprété ensemble les séquences HLA de 10 patients français et de 10 patients américains. Chaque fichier de séquençage a été vérifié par l'autre centre compte tenu de la difficulté d'une telle analyse (quantité de polymorphismes connus et inconnus et de nombreux pseudogènes). Après cette analyse, nous avons confirmé l'absence de telles lésions HLA dans les échantillons de SMD/LAM secondaires à une aplasie médullaire. Nous avons conclu que les anomalies HLA ne jouent pas de rôle majeur dans la progression.

À la suite de ces résultats, nous avons étudié comment les caractéristiques cliniques, cytogénétiques et moléculaires des patients aplasie acquise/HPN affectent la transformation en SMD/LAM. Nous avons confirmé que l'absence de réponse aux traitements, l'âge et les mutations myéloïdes conféraient un risque plus élevé de progression. Par ailleurs, nous avons observé que les mutations *PIGA/HLA*, prédominantes au départ, étaient remplacées par des mutations myéloïdes classiques lors de la transformation. Ce travail, dans lequel je suis co-premier auteur, est publié dans le *Journal of Clinical Oncology* en Septembre 2022. Ce papier est inclus dans son intégralité dans ce manuscrit.

Avec un suivi médian supérieur à 8 ans, aucun patient greffé (n=117) n'a évolué vers un SMD/LAM, ce qui suggère que l'hématopoïèse du donneur l'emporte sur les clones pré-leucémiques du receveur qui pourraient exister au moment de la transplantation. Parmi les patients atteints d'une forme grave d'aplasie médullaire, nous avons confirmé qu'une réponse sous-optimale à l'immunosuppression et l'âge au moment de l'apparition de l'aplasie étaient des facteurs pronostiques indépendants pour la survenue d'une néoplasie myéloïde secondaire.

Les mutations les plus fréquemment trouvées au moment du diagnostic de l'aplasie étaient en accord avec la littérature et se chevauchaient avec l'hématopoïèse clonale liée à l'âge : *BCOR*, *BCORL1*, *TET2* et *ASXL1*. La présence de mutations myéloïdes au diagnostic de l'aplasie prédisait un risque plus élevé de progression par rapport à l'absence de mutations ; les mutations isolées *PIGA/HLA* prédisaient le risque le plus faible.

Nous avons pu comparer le profil moléculaire des patients dont le séquençage a été effectué au diagnostic de l'aplasie et au diagnostic de l'évolution (analyse transversale). Les fréquences alléliques de *PIGA* ou de *HLA* ont eu tendance à augmenter chez ceux qui n'ont pas progressé et à diminuer chez ceux qui ont transformé en SMD/LAM, remplacés par une augmentation des mutations de gènes myéloïdes.

Comme on pouvait s'y attendre, le pronostic des patients transformés était mauvais et comparable aux résultats publiés. Le nombre de blastes au moment du diagnostic de SMD/LAM et l'âge au diagnostic de l'aplasie prédisaient indépendamment une mauvaise survie.

Les SMD secondaires à l'aplasie différaient cytogénétiquement des SMD primaires, présentant fréquemment des caryotypes à haut risque. Les anomalies du chromosome 7 et les caryotypes complexes conféraient un mauvais pronostic. Contrairement au R-IPSS, un caryotype normal au moment du diagnostic de SMD/LAM ne permettait pas de prédire un bon pronostic. L'intervalle entre l'aplasie et le SMD/LAM était plus court et le caryotype normal était associé à des mutations dans des gènes associés aux LAM tels que *NPM1*, *FLT3*, *IDH1/2* et *U2AF1*.

Pour la deuxième partie, nous avons voulu explorer l'impact du mosaïcisme somatique dans l'anémie de Fanconi sur la transformation en néoplasies myéloïdes. Globalement, les complications hématologiques représentent près de 70 % des décès. Près de 30% des patients atteints d'anémie de Fanconi développeront une SMD/LAM au cours de leur vie. L'évolution clonale vers SMD/LAM semble être linéaire dans l'anémie de Fanconi. L'anomalie la plus précoce et la plus fréquente est la trisomie 1q (état pré-malin). La trisomie 3q, des anomalies du chromosome 7 et des mutations du gène *RUNX1* sont plus tardives et prédisent une évolution vers SMD/LAM.

Néanmoins, certains patients présentent un phénotype hématologique moins grave : des cellules hématopoïétiques spontanément corrigées coexistent avec des cellules Fanconi soutenant hématopoïèse – on appelle ce phénotype « révertants ». Parmi 350 patients Fanconi de la cohorte française, notre équipe a identifié 17 cas de réversion : aucun de ces patients n'a évolué vers de SMD/LAM.

Inspirés par la réversion, des essais cliniques ont montré des résultats prometteurs en utilisant des cellules souches hématopoïétiques corrigées génétiquement – thérapie génique - pour traiter l'insuffisance médullaire chez les patients Fanconi. Cependant, l'effet sur le risque de transformation en SMD/LAM est inconnu pour l'instant.

J'ai commencé un projet sur le développement d'un modèle de réversion génétique chez des souris *Fancg*^{-/-}. Nous testons des sous-clones corrigés génétiquement ou pré-leucémiques dans le contexte de Fanconi et le risque d'évolution en SMD/LAM. L'objectif est de comparer l'aptitude des cellules souches et progénitrices corrigées ou pré-leucémiques, en modélisant le phénotype révertant et l'évolution clonale. La correction fonctionnelle d'un certain nombre de cellules souches hématopoïétiques pourrait conférer un avantage écologique, mitigant le risque de dominance clonale de clones pré-malins. Dans l'hématopoïèse, les cellules révertantes écraseraient les cellules pré-malignes (1q+, 3q+).

Pour tester cette hypothèse, j'ai procédé à des expériences de greffe en compétition entre les cellules *Fancg*^{-/-} génétiquement corrigées *ex vivo* et cellules *Fancg*^{-/-} pré-leucémiques (*Fancg*^{-/-} transgéniques sur-exprimant *Mdm4*, comme un marqueur de substitution pour le 1q+ et *Fancg*^{-/-} sur-exprimant *Evi1*, comme un marqueur de substitution 3q+).

La validation de notre hypothèse peut avoir des implications thérapeutiques. Chez les patients atteints de Fanconi qui sont greffés, le tissu hématopoïétique est remplacé par un tissu sain. En plus de réduire la charge de cellules oligoclonales, la surveillance du nouveau système immunitaire allogène détecte et élimine les cellules résiduelles. Cependant, cet avantage a un coût : les patients atteints de Fanconi sont très sensibles aux régimes de conditionnement pour la greffe. Outre la toxicité à court terme du conditionnement, les patients transplantés sont exposés à la maladie du greffon contre l'hôte. Malgré cela, les résultats cliniques de la greffe pour les patients atteints de Fanconi se sont considérablement améliorés au cours des dernières décennies et la survie est excellente pour les jeunes patients transplantés avant la transformation.

L'hématopoïèse clonale fait partie du vieillissement physiologique et est plus fréquente chez les patients atteints d'une insuffisance médullaire que dans la population générale. Cependant, les patients atteints d'une insuffisance médullaire avec hématopoïèse clonale semblent présenter des histoires naturelles très différentes. La présence de mutations des gènes myéloïdes spécifiques ou l'augmentation de la taille du clone semblent conférer un risque plus élevé de transformation en SMD/LAM. Des mécanismes de réversion sont observés dans

l'ensemble des insuffisances médullaires d'origine génétique (autre Fanconi, GATA2, maladie des télomères et Shwachman-Diamond), et la transformation en SMD/AML semble exceptionnelle.

Une surveillance étroite comprenant au moins une numération-formule sanguine, un myélogramme et une cytogénétique conventionnelle doivent être faits à intervalles réguliers pour tous les patients atteints d'une insuffisance médullaire. Nous ne disposons pas encore de suffisamment de preuves pour recommander le séquençage de routine pour tous les cas - une anomalie moléculaire isolée ne changera guère la thérapeutique. Le NGS et les études fonctionnelles doivent être envisagés au cas par cas.

Prologue

I was interested in the mechanism involving the emergence of clones bearing somatic mutations in bone marrow failure syndromes. We started a partnership between my mentors, Rodrigo Calado, Régis Peffault de Latour, and Jean Soulier, through a joint doctoral programme between the Universities of São Paulo (Brazil) and Paris-Cité (France). This work encouraged international and multi-institutional collaborations in bone marrow failure clinical and translational research.

I performed the experiments, data collection and interpretation at the “*Institut de Recherche Saint Louis*” (IRSL) within the team of Jean Soulier. Upon starting this doctoral programme, I had a background and training focused on clinical practice and research. I am most grateful to the lab team and mentors for their will and patience in training me in Science, preclinical models, and wet lab techniques. I am also very grateful to the American Society of Hematology for their financial (ASH Global Research Award) and training support (Clinical Research Training Institute).

We conducted independent, novel, clinical, and translational research to understand the mechanisms contributing to clonal evolution in marrow failure syndromes, from the “protective” somatic mosaicism to premalignant and malignant progression. The results help perceive what underlies clonal selection of different subsets, and may help better understand malignant evolution. An adequate risk assessment of MDS/AML would arguably impact therapy choice, from novel options such as gene therapy in Fanconi Anaemia to adapting the timing of consolidated strategies as allogeneic hematopoietic stem cell transplantation for Aplastic Anaemia.

I have worked full-time in research for the last three years, except for the unprecedented sanitary crisis. At that moment, as a physician, I felt it necessary to resume hospital clinical activities to join the collective effort to assist the overflow of patients for a few months.

My lab journey was a wonderful and rich experience, and my research skills improved significantly from the beginning. During these years, I have learnt how to perform bibliographical research, especially in the PubMed database. I have learnt statistics applied to clinical and preclinical studies. I gained experience in medical and scientific writing and received good feedback from my peers and mentors. I wrote research proposals and applied to grants, some more successful than others. I have learnt about data management, visualisation, and analysis using R. I have submitted all my research projects to institutional review boards and got their approval. I have learnt how to ask scientific questions, test hypotheses, and conduct experiments in the laboratory and animal facility, as I will present in this manuscript. I could lead my projects and collaborate with colleagues’ research. I also had the opportunity to present my research progress to my colleagues periodically in lab meetings in France and Brazil (videoconference) and congresses (EBMT and ASH).

My initial research project was entitled: “Clonal evolution in acquired aplastic anaemia: immune escape and somatic genomic alterations”. Our primary objective was to investigate if the reduction or loss of HLA (HLA⁻ clone) in hematopoietic cells in acquired aplastic anaemia patients was associated with AML/MDS transformation.

As a pilot study, we sequenced HLA classes I and II of ten patients (nine acute myeloid leukaemias and one myelodysplastic syndrome with excess of blasts type 2) transformed from severe aplastic anaemia. We failed to demonstrate any loss of function mutation or loss of heterozygosity at the moment of the myeloid neoplasm diagnosis. This pilot study did not support our hypothesis that the loss of HLA expression in hematopoietic cells in acquired aplastic anaemia patients is linked to

AML/MDS-EB transformation. However, we did not have enough power to affirm negative results and established a collaboration with the Cleveland Clinic to increase the sample size.

To overturn our postulated hypothesis, we have interpreted together the HLA sequences of 10 patients from France and ten from the United States, each FASTQ file cross-checked by the other centre considering the difficulty of such an analysis (massive amount of known and unknown polymorphisms and many pseudogenes). We confirmed the absence of such lesions. We concluded that HLA loss-of-function mutations or 6p copy-neutral loss of heterozygosity is not a major player in the progression to MDS/AML. I present the theoretical basis and the continuation of this successful tripartite collaboration between Saint-Louis Hospital, University of Sao Paulo and Cleveland Clinics in Chapter 2, section 2.1 of this manuscript. I have included a discussion and proposition for a continuation study we are about to start.

Given the preliminary negative results of the work mentioned above, my mentors and I developed an alternative plan to secure an experimental PhD work. Therefore, I worked on developing a murine model of somatic mosaicism (genetic reversion) in Fanconi Anaemia mice (*Fancg*^{-/-} already available at the animal facility). The aim was to test the fitness of gene-corrected or preleukemic stem and progenitor cells, mimicking the revertant phenotype and the early stages of bone marrow progression. I present the theoretical basis, methods, pitfalls and preliminary results in Chapter 2, section 2.2. I have included a discussion and the next steps towards completing this project.

Chapter 1 presents a general introduction to clonal haematopoiesis, preleukemic states, and the use of animal models in the study of blood diseases. Chapter 1 also introduces the subject of bone marrow failures, focusing on acquired aplastic anaemia and Fanconi anaemia. I have included the ancillary and collaborative projects as annexes.

I tried to avoid heavy writing while presenting my progress, reasoning, and steps undertaken. From the many lessons I have learnt from this journey, first and foremost, I have learnt to adapt to new environments and to overcome adversities, both on the professional and personal sides. Ultimately, I have become somatic mosaicism: germline Brazilian and somatically French. I hope you will enjoy reading it.

Chapter 1: Introduction

1.1 The blood and polyclonal haematopoiesis

The blood is a complex, liquid tissue vital for animal life. The blood's cellular compartment consists of red and white cells and platelets. In short, red blood cells, or erythrocytes, are responsible for oxygen and carbon dioxide transportation from lungs to tissues; white blood cells, or leukocytes, are the mainstay of the immune system; platelets are not cells by themselves, but they are fragments of another specialised cell (megakaryocytes) and are major players in blood coagulation and vascular repair.

Despite very diverse functions and structures, blood cells derive from a unique cell type in the bone marrow: the haematopoietic stem cell (HSC).¹ Stem cells can divide and give rise to identical copies of themselves – a process called self-renewal - but at the same time, to differentiated cells that will further differentiate and divide and so on.² One single HSC is the ancestor of thousands of mature cells.^{1,3} The hierarchic process of production and differentiation, from stem to circulating mature cells, is known as haematopoiesis.

Vertebrate haematopoiesis starts with a few active and many quiescent multipotent HSC. The classical representation of haematopoiesis (**Figure 1**) consists of a hierarchical process with many oligopotent intermediates. Downstream intermediates no longer have self-renewal capacities and go through many lineage-committing steps until becoming mature cells. Understanding the process though which multipotent cells generate blood is crucial for the perception of quantitative (e.g. bone marrow failure – BMF) or qualitative diseases (e.g., myelodysplastic syndromes – MDS) of blood cells.

Insufficient or abnormal production of blood might severely impair patients' quality of life because of infections, bleeding, and frequent trips to healthcare facilities for blood transfusions. Depending on the degree of abnormality, marrow neoplastic or failure complications can be fatal.

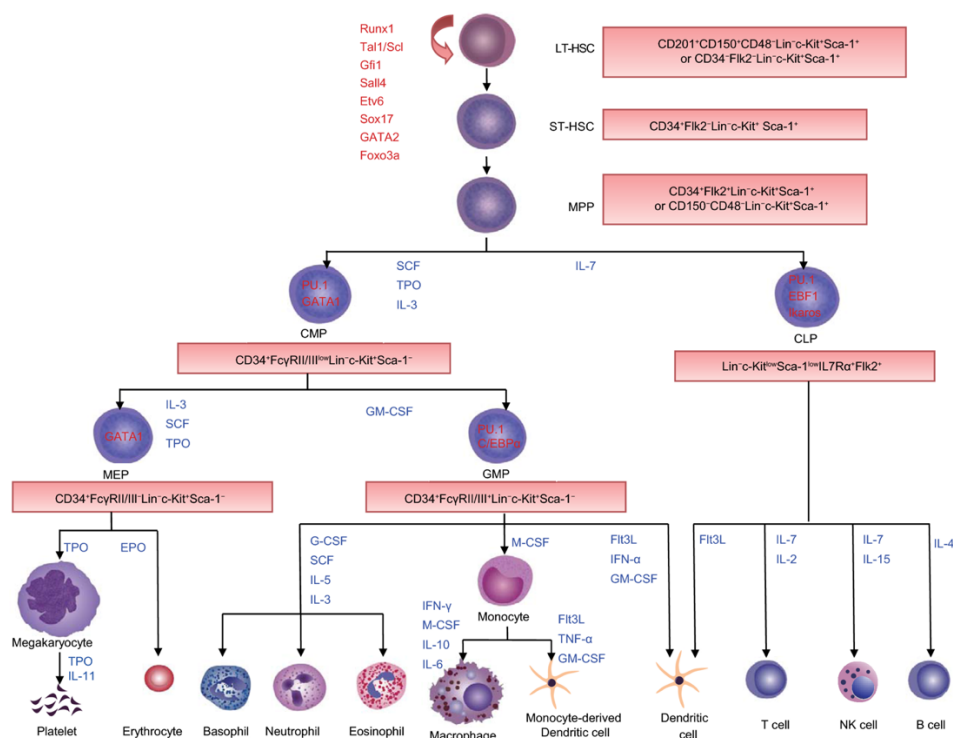


Figure 1: A schematic representation of the hierarchical process of haematopoiesis⁴

LT-HSC: long-term haematopoietic stem cell; **ST-HSC:** short-term haematopoietic stem cell; **MPP:** multipotent progenitors; **CMP:** common myeloid progenitor; **MEP:** megakaryocyte-erythrocyte progenitor; **GMP:** granulocyte-macrophage progenitor; **CLP:** common lymphoid progenitor. In red, intrinsic transcription factors and in blue, extrinsic cytokines that regulate differentiation. The immunophenotypic markers used for population enrichment are inside the pink boxes purple.

1.2 Men and mice

Studying normal and pathological haematopoiesis in humans is difficult because the HSC remains an elusive cell. Studies based on different animal models contributed significantly to today's knowledge. A game-changer happened in the aftermath of WWII: James Till and Ernest McCulloch were interested in hematopoietic stem cells as a countermeasure against the toxicity of nuclear weapons. They demonstrated that donor bone marrow cells could rescue haematopoiesis in mice treated with supralethal irradiation.^{1,3} From that moment on, haematopoietic cell transplantation experiments in *mice* became the backbone of studies in haematopoiesis and much of what we know today became from mouse studies.

Mice are so close but far away. Phylogenetically, we are about 50 million years apart⁵ (**Figure 2**). It might seem much, but if we compiled the life history on planet Earth in 365 days (the Big Bang happened on the 1st of January)⁶, the development gap between mice and man would fit in only a few hours. Being so close, it is no surprise that numerous evolutionary mechanisms, including facets of haematopoiesis and DNA repair, are similar.

The human and mouse genomes contain nearly twenty thousand genes, among which nearly 70% are shared between both species. These one-to-one orthologs have, on average, 80% homology.⁷ On the other hand, 20% of human genes have no matching ortholog on mice and vice-versa. The remaining 10% resulted from duplications or further sequence change, rendering the genetic copy number and functions different between species.⁸ The similarities between man and mice enable biomedical research.

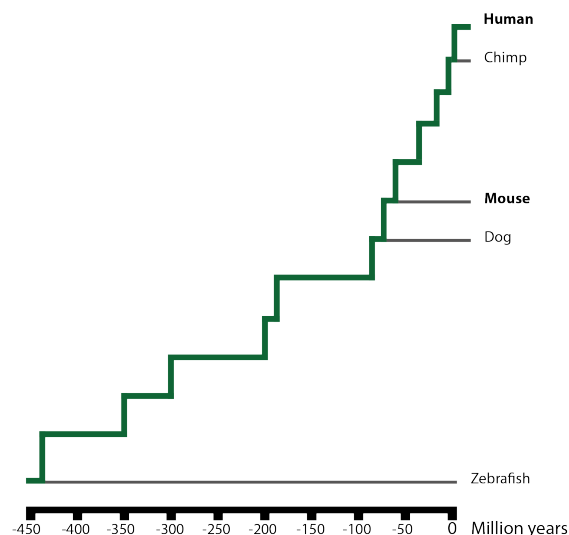


Figure 2: Schematic representation of the last common ancestor between different animal models and humans (adapted from⁵).

The description of such a didactic system of blood cells' differentiation (**Figure 1**) is based significantly on mouse studies. HSCs are divided according their capacities of sustaining haematopoiesis: long and short-term (16 weeks). Multipotent progenitors (MPP), no longer capable of self-renewal, are still ancestors to all mature blood cells. In this model, the first critical commitment step occurs at the stage of common myeloid (CMP) or lymphoid progenitors (CLP): at this stage, progenitor cells are already

lineage-committed. Intrinsic (i.e., transcription factors) and extrinsic (cytokines) regulators strictly control the differentiation steps.⁴ The isolation of each population depends on its surface markers identified using flow cytometry.

Subsequent mice studies have identified more intermediate populations with different self-renewal and differentiation capacities. For example, MPPs can be further divided based on surface markers into MPP1 to 4 with distinct self-renewal, gene expression, and differentiation potentials (**Figure 3**).^{9,10} Also, human MPPs, CMPs, and MEPs are heterogeneous populations.¹¹

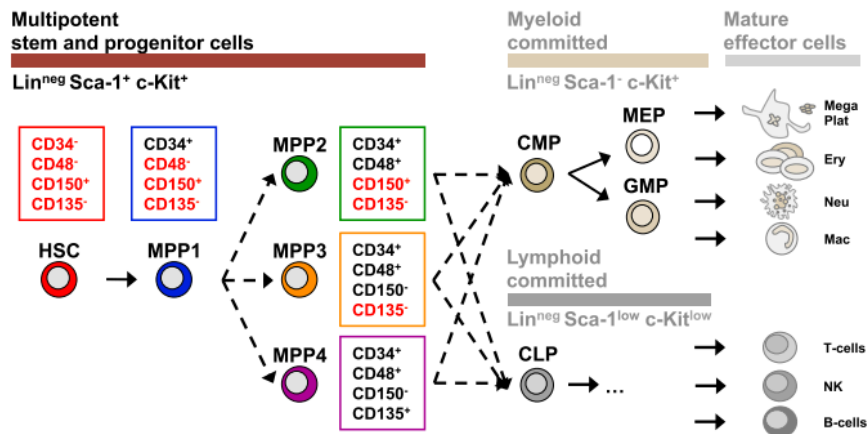


Figure 3: Revised murine haematopoietic tree¹⁰

The red bar represents the LSK population whereas Lin⁻ all but the light gray (mature effector cells) bars.

However, haematopoiesis schematic representations were based on pre-defined surface markers. Along with technological evolution (e.g, multiomics and scRNA-seq), more heterogeneity is uncovered and the model represented in **Figure 1** seems insufficient. Most likely, each cell undergoes its own differentiation process, without hierarchical boundaries.¹² In homeostasis, committed haematopoietic cells would emerge directly from HSCs (**Figure 4**).^{11,12} In special, in both human and mouse, megakaryocytes precursors seem to emerge directly from HSCs, and MEP would generate chiefly RBCs, as represented in **Figure 1**.^{4,11}

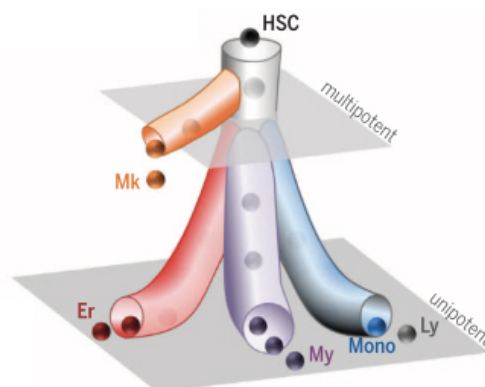


Figure 4: Lineage-committed cells would emerge directly from multipotent cells.¹¹

Although differentiation seem to be a continuous process, to further study haematopoiesis and the characteristics of each cell compartment, researchers need to enrich samples with target populations. In studying diseases, we are more interested in hematopoietic stem and progenitor cells (**Figures 1 and 3**). For practical reasons, many research teams use a pool of cells lacking surface markers of commitment to a

specific mature lineage (lineage-negative¹ cells or Lin⁻, around 2% of marrow's mononucleated cells, **Figures 1 and 3**), or, for more enriched HSPCs, the LSK population (around 0.05% - HSCs and MPP1-4,⁹ **Figure 3**). The true HSCs are very rare and difficult to isolate (less than 0.01%). We can highly enrich samples by selecting a group of cells within the LSK population (SLAM markers, signalling lymphocyte and activation molecule: CD150⁺ CD244⁻ CD48⁻ CD41⁻). I have worked with Lin⁻ and LSK cells for the mice studies I present in this manuscript.

By definition, models are imperfect approximations, often reductionist. Differences exist, and we must not simply extrapolate conclusions from mice models to human diseases. Some species-specific evolutionary needs - different body sizes (70kg vs 20g) and life span (80 vs two years) - affect the daily needs for blood cells (proportionally, the ratio of active and quiescent HSCs are ten times higher in mice than humans).¹³ Hematopoietic stem cells face, then, different selective pressures concerning replication, DNA repair, and tumorigenesis. For instance, while the CD34 marker is helpful for distinguishing stem and progenitor cells in humans, its expression is dim or absent in mice. On the other hand, *sca-1* and SLAM, very useful for mice studies (**Figure 3**), is absent or less useful for human studies. Another significant difference is telomere length, which is much higher in mice.¹³ Therefore, mice studies are a guide, not a mirror for human haematopoiesis.

1.3 Clonal haematopoiesis

During mitosis, the DNA must be duplicated. Cells have complex machinery to copy the nearly three billion base pairs of their genome. Although this replication process is exact, tens of thousands of errors might occur during each mitotic cycle. Therefore, the accumulation of replication errors is an inevitable fate for any ageing organism. Furthermore, some environmental factors such as sunlight, pollutants, radiation, and chemicals increase manifold the occurrence of DNA lesions. Fortunately, evolution granted cells mechanisms to correct such mistakes. In superior vertebrates, the apparatus is diverse and highly efficient. If such systems are dysfunctional – as in some diseases – the accumulation of errors can trigger cells' programmed death (apoptosis) or be a risk factor for cancers due to the accumulation of errors.

However, even with the DNA-repair apparatus in perfect function, some mistakes might slip the proofreading process and be transmitted to daughter cells. In other words, the DNA sequence of daughter cells will be slightly different from mother cells. In most cases, these differences will not affect the cell because they happen to be in places of the DNA that are not needed for the normal functioning of that cell or because the variants do not confer a selective advantage. The American College of Medical Genetics defines mutation as a permanent change in the nucleotide sequence".¹⁴ In current medical practice, the word "mutation" is reserved for changes entailing a deleterious effect on the organism. When the effect is not known, the change is considered a "variant of unknown significance" (VUS). Beneficial changes at an organism level are unusual and less evidently noticed. When a living organism acquires a permanent genetic change, the variants are called *somatic* (from Greek, *sōma* 'body'). These variants are therefore restrained to the tissue where it happened in the body (e.g., the haematopoietic tissue). When the variant is transmitted from one generation to another or occurs in germ cells that become a new individual, they are called *constitutional* or *germline*. These variants are therefore present in all the cells of the individual.

¹ Lineage-negative cells are negative for the following markers: CD5⁻ CD45R⁻ CD11b⁻ CD5⁻ Gr-1⁻ 7-4⁻ Ter-199⁻

Gene abnormalities are detected using molecular biology techniques. In the last twenty years, a significant breakthrough, the next-generation sequencing (NGS), allowed physicians and researchers to study in-depth many genes (a panel) at the same time. NGS enables the detection of somatic and germline genetic abnormalities that would hardly be seen otherwise. Haematology pioneered the use of NGS in research and clinical care chiefly because of the ease of access (blood and marrow samples as opposed to tissue biopsies).

The frequency of detected somatic variants can be expressed as percentages: each cell has two alleles for each gene (but for genes located in X and Y chromosomes in men). The variant allele frequency (VAF) measures the proportion of individual variants in the DNA sample. For example, when a patient inherited one mutated allele from his or her progenitor and another healthy one, the expected VAF for the mutant allele is close to 50% (not precisely 50% because of measurement variation). On the other hand, the replication error can occur in one hematopoietic stem cell and give some replicative or survival advantage. This mutated HSC will self-renewal and differentiate, producing thousands of clones. When the variant is acquired and expands during patients' life, the VAF can assume any value from 0 to nearly 100%. When a mutated HSC clone contributes disproportionately and persistently to the blood cells' formation (frequently defined as a mutation detected in blood with a VAF $\geq 2\%$ - the lower limit detectable using whole-exome sequencing, WES), the condition is called *clonal haematopoiesis* (CH).

As replication errors are part of physiological ageing, clonal haematopoiesis can be detected in about 12.5% of individuals in all age groups (>50% in the elderly)^{15,16} and is not a synonym for disease. Besides errors, base substitutions occur because of thermodynamics: for instance, methylated cytosines can undergo hydrolysis (**Figure 5**), becoming thymidine (C>T transition), i.e., age-associated mutations.¹⁷ Hydrolysis in biology are exergonic processes, therefore, spontaneous. In other words, somatic mutations are an unavoidable fate of all living beings.

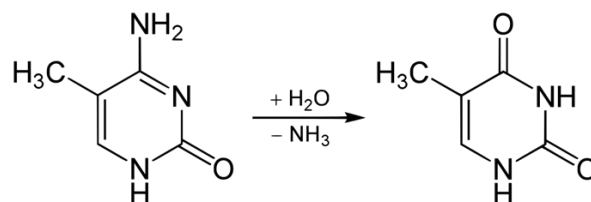


Figure 5: 5-methylcytosine hydrolysis into thymidine¹⁸

Although CH has been linked to a higher risk of coronary artery disease¹⁸ and a tenfold increased risk of blood cancers compared to the general population, the absolute risks are still low.¹⁹ Furthermore, the risks and diseases associated with different somatic variants are unequal, and some CH are secondary to *neutral drift*^{II} instead of an adaptative advantage.¹⁵ For some patients, the presence of CH might be a reflex, a biomarker of stressed haematopoiesis (i.e., the more replication cycles, the more errors) that is a risk factor to malignant transformation and not the cause in itself. Therefore, clinicians must interpret the meaning of CH adequately. Clonal haematopoiesis and non-haematologic disorders²⁰ are out of the scope of this work.

Overall, mutations in epigenetic regulators are frequent in clonal haematopoiesis,^{16,19} and clones steadily expand with age. Faster clone growth rate indicates a higher risk of malignant progression.¹⁶ Some other features of CH might confer a higher risk for myeloid neoplasm. Notably, the diagnosis of CH in the presence

^{II} Neutral drift: variants with neutral effect occur and expand not because of gain in adaptation skills, but because of random genetic drift.

of cytopaenias carries a high risk of progression and deserves close monitoring. Furthermore, specific genes seem to play a more significant role than others. *Splicing factors*^{III}, *TP53*, *IDH1/2*, *PHF6*, *RUNX1*, the total number of mutations, and VAF $\geq 10\%$ conferred a higher hazard ratio to progression than *DNMT3A*, *ASXL1* or *TET2*.^{21,22}

Cancer evolution is a *multistep process*^{IV} in which the accumulation of DNA lesions in tissues' indolent self-renewing stem cells induces clonal expansion. Once sufficient *driver mutations*^V happen in subclones, an overt neoplasm develops. Eventually, additional somatic lesions in neoplastic cells lead to an aggressive disease.

Neoplastic cells evolve from premalignant to malignant. These concepts help to face CH as a multistep process, from a non-malignant predisposing condition to an overt myeloid neoplasm through a pre-myelodysplastic stage (**Figure 6**). Single-cell analysis showed that founder HSC mutations are silent and followed by serial acquisition of clones and mutations, preceding overt myeloid malignancy.²³

Although single-cell studies are not yet included in routine clinical practice, NGS is accessible and used in the workup of cytopaenias. Clinicians needed new nosologic entities to categorise CH patients without sufficient criteria for myeloid neoplasm who are, nonetheless, premalignant:

ARCH: age-related clonal hematopoiesis¹⁹. It is defined as the expansion of an HSC clone harbouring “specific, disruptive, and recurrent genetic variants in individuals without a clear diagnosis of haematological malignancies”²⁴. Its definition overlaps with clonal haematopoiesis of indeterminate potential (CHIP). The absolute risk of malignant transformation is low.

CHIP: identification of a *clonal mutation associated with hematologic neoplasms*^{VI} with a VAF $\geq 2\%$ in an individual with normal or near-normal full blood counts not meeting WHO 2016 criteria for hematologic neoplasm, PNH, monoclonal gammopathy of undetermined significance, or monoclonal B lymphocytosis.²⁵ The risk of progression is nearly 1%/year.²¹

CCUS: clonal cytopaenias of undetermined significance. It is generally assumed as CHIP with at least one cytopaenia.²⁵ However, some authors prefer to use a higher VAF cut-off (20%) to separate from other cytopaenias that co-occur with incidental and not causal small CHIP.²⁶

ICUS: idiopathic cytopenia of undetermined significance. It describes a condition without evidence of clonality not fulfilling the WHO-2016 criteria for MDS.²⁷

^{III} *SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*, *SF1*, *PRPF8*

^{IV} Knudson hypothesis or two-hit theory: Alfred Knudson was the first to hypothesize that cancer develops in a multi-step genetic process. He proposed that sequential genetic “hits” / mutations were needed to the development of cancer¹⁷⁴.

^V Driver mutations: “changes in the DNA sequence of genes that cause cells to become cancer cells and grow and spread in the body”¹⁷⁵ as opposed to passenger mutations, which do not confer any adaptive advantage. Discriminating between driver and passenger mutations can be very difficult and relies on the sequencing method, analytical pipeline, filtering, annotation and *in silico* prediction of pathogenicity²⁶.

^{VI} *ASXL1*, *BCOR*, *BCORL1*, *BRAF*, *CALR*, *CDKN2A*, *CREBBP*, *CBL*, *CUX1*, *DNMT3A*, *EZH2*, *ETV6*, *FLT3*, *GATA2*, *GNAS*, *GNB1*, *IDH1*, *IDH2*, *IRF1*, *JAK2*, *JARID2*, *KDM6A*, *KIT*, *KRAS*, *KTM2A/MLL*, *MPL*, *MYD68*, *NOTCH1*, *NPM1*, *NRAS*, *PHF6*, *PPM1D*, *PTEN*, *PTPN11*, *PRPF8*, *RUNX1*, *SETBP1*, *SETDB1*, *SETD2*, *SF1*, *SF3B1*, *SMC1A*, *SMC3*, *SRSF2*, *STAG2*, *TET2*, *TP53*, *U2AF1*, *WT1*, *ZRSR2*^{21,24,25}. This list is constantly evolving and is not exhaustive.

CHOP: clonal haematopoiesis with substantial oncogenic potential. It is CHIP with high-risk and persistent disease-determining abnormalities (e.g., *JAK2*, *KIT*, *KRAS*, *HRAS*, *FLT3*).²⁸ CHOP is still an evolving definition.

MDS: myelodysplastic syndromes. CH with cytopaenias and dysplastic changes in at least 10% of cells of one lineage in the bone marrow. Different syndromes confer different risks to progression into acute myeloid leukaemia (AML). MDS diagnostic criteria are defined by the 2016 revision of the World Health Organization classification of myeloid neoplasms and acute leukemia.²⁹ Several tools assess the risk of MDS transformation into AML. The most widely used (Revised International Prognostic Scoring System – R-IPSS) combines clinical and cytogenetic data.³⁰ In 2022, a newer revision of the scoring system included molecular data (IPSS-M).³¹

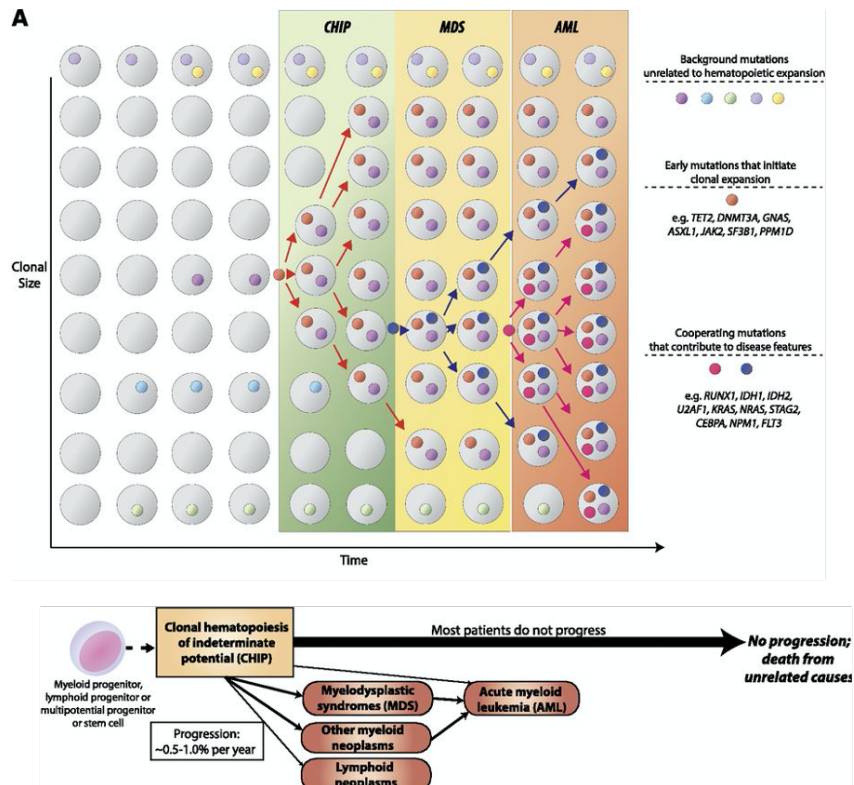


Figure 6: Schematic representation of the stepwise evolution from CHIP to AML through the accumulation of multiple hits in the stem cell compartment²⁶

Thus far, most of the molecular data available derive from bulk analyses and data from single-cell studies for CH/CHIP/ARCH is still scarce – the need for longitudinal samples over a long follow-up being a crucial limitation. scDNA-sequencing confirmed that *DNMT3A* and *TET2* are the most frequent driver mutations and that the number of unique clones in overt myeloid malignancies is higher than in CH.³² On the other hand, the number of mutations within the dominant clone is not different, indicating that the mutation burden is not the main driver of leukemogenesis.^{32,33}

The overrepresentation of *DNMT3A*, *TET2*, and *ASXL1* among CH suggests that epigenetic regulators mediate clonal dominance. Interestingly, the coexistence of such mutations is rare within the same clone in CH and frequent in overt myeloid malignancies.³² For instance, clones with both *DNTMT3A* and *TET2* mutations in CH showed higher VAFs.³⁴ These data support that the combination of some mutations have a synergistic effect, driving clonal dominance from premalignant to malignant states.

1.4 Bone marrow failure syndromes

The hallmark of bone marrow failure syndromes is pancytopenia because of the incapacity of the haematopoietic tissue to sustain haematopoiesis. Historically, this group of diseases was called aplastic anaemia. The first documented cases date from the late XIX and early XX centuries, without any aetiopathological distinction.

Thus far, three main aetiopathologies have been described:

direct cellular toxicity: drugs, radiation, or chemicals.

- Idiopathic: autoimmune.
- Inherited: intracellular biological pathway dysfunctions (DNA repair, ribosome or telomere biology).

I will focus on idiopathic and inherited bone marrow failure syndromes in this work.

1.4.1 Idiopathic aplastic anaemia

This condition is also known as immune / acquired aplastic anaemia, or plainly, aplastic anaemia. Aplastic anaemia (AA) is a rare disease (ORPHA 88), with an incidence of 2 cases per one million inhabitants and a prevalence of 0.0004% in Western countries.^{35,36} For unknown reasons, it is two to three times more frequent in Asia.³⁷

AA affects both men and women equally and has a bimodal age distribution, with the first peak among young adults and a second peak among patients over 50. The classic clinical findings are secondary to pancytopenia: fatigue, bleeding, and infection.

1.4.1.1 Pathophysiology

The first evidence of immune pathophysiology for aplastic anaemia came from a clinical observation: Mathé and Schwarzenberg noted an autologous reconstitution following allogeneic cell transplantation with anti-thymoglobulin (ATG) and concluded that immunosuppression was responsible for the response³⁸.

The first functional demonstration of the immune pathophysiology came from the Louis Wasserman team in 1977: peripheral blood lymphocytes from five out of seven aplastic anaemia patients induced approximately 80% reduction in erythroid colony formation in semi-solid co-culture assay³⁹. The demonstration of cytotoxic T-cell mediated destruction of autologous bone marrow mononuclear cells further strengthened the immune hypothesis⁴⁰⁻⁴². These autoreactive T cells, enriched in the patient's bone marrow environment,⁴³ synthesise TNF- α and IFN- γ , cytokines that inhibit haematopoiesis and upregulate Fas expression in haematopoietic cells⁴⁴, favouring apoptosis. Thus far, the triggers of the immune process leading to bone marrow failure are unknown.

1.4.1.2 Treatment

Aplastic anaemia can be fatal if left untreated. First-line treatment consists of haematopoietic cell transplantation for eligible patients younger than 40 years of age for whom a fully matched HLA sibling is apt to donate^{37,45,46}. However, such a donor is unavailable for nearly one-third of patients. Furthermore, many patients are older or not eligible for upfront transplantation. Triple therapy with horse ATG and cyclosporine^{45,47}, and, more recently, eltrombopag⁴⁸ yielded the best results: nearly two-thirds of overall response.

Relapsed or refractory patients may respond to a second cycle of immunosuppression. However, the results are disappointing. Eltrombopag alone can

restore some degree of haematopoiesis and render 40% of patients transfusion-independent.^{49,50} A matched sibling or unrelated transplantation is the preferred choice if eligible.⁴⁵ If a matched donor is unavailable, an alternative transplant is an option. We and others reported good results using haploidentical transplantation using post-transplant cyclophosphamide for relapsed/refractory patients (**Annexe III**).^{51–55} Prospective phase 2 trials are currently ongoing.

Patients who fail salvage treatment have a poor prognosis, and infection is the leading cause of death.⁵⁶

1.4.1.3 Aplastic anaemia animal models

As an acquired disease of elusive aetiology, the use of animal models is less reliable than for FA. To mimic pancytopenia, some models were based on exposure to chemical agents, but they were insufficient to study the classic mechanism of AA, the immune-mediated destruction of HSCs. Therefore, models based on mismatched hematopoietic cell transplantation resulted in cytopenias and were used to understand marrow destruction better.⁵⁷ However, one confounding factor is graft-versus-host disease. Considering the mouse models available thus far for studying immune AA, we based our study on clonal evolution on direct observation of patient data.

1.4.2 Inherited bone marrow failure syndromes

The inherited bone marrow failure syndromes (IBMF) comprise different genetic diseases, affecting distinct biological pathways that converge to cellular stress and apoptosis. Marrow failure is only one aspect of otherwise systemic syndromes. The most frequent are Fanconi Anaemia (FA), Shwachman-Diamond syndrome (SDS) Diamond-Blackfan Anaemia (DBA), and telomere biology diseases (TBD, *e.g.*, Dyskeratosis Congenita). The true incidence of these diseases is unknown: variable penetrance, limited availability of genetic testing, misleading beliefs that the clinical picture must start at childhood, and somatic mosaicism contribute to underestimate the real epidemiology of IBMF (**Table 1**).

The correct diagnosis of IBMF prior to treatment initiation is of utmost importance, for IST is ineffective; haematologists must adapt HSCT conditioning regimen and donor screening accordingly. The initial work-up includes, at least, chromosomal breakage tests (FA), pancreatic function tests (SDS), and telomere length (TBD). Targeted genetic sequencing to look for germline mutations are becoming widely available and some centres defend haematologists must screen all patients with BMF. Unrecognised IBMF would affect a significant number of transplanted patients and be a risk factor for treatment related mortality.⁵⁸

Table 1: Estimated incidences of different BMF

Syndrome	Incidence (x10 ⁶ newborns)
FA ⁵⁹	11.4
SDS ⁶⁰	6
DBA ⁵⁹	5
TBD ⁶¹	1
AA ^{35,36}	2 (general population)

Some IBMF increase the risk of solid malignancies and all of them are associated with clonal haematopoiesis and progression to MDS/AML, the archetype being Fanconi Anaemia.

1.4.2.1 Fanconi Anaemia

Guido Fanconi described pancytopenia and physical anomalies within a family in 1927.⁶² Besides marrow failure and physical abnormalities, the presenting sign of

FA can also be solid tumours. The clinical picture might be heterogeneous within the family, suggesting other factors might influence disease course (epigenetic, environmental).

FA pathway is crucial for interstrand crosslinks DNA-repair. To date, 22 different proteins and many other associated proteins play a role in this pathway (**Tables 2 and 3**). **Figure 7** represents the FA-core complex, an assembly of eight FA-proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FAAP100),⁶³ that is recruited by FANCM to DNA crosslinks and, in conjunction with FANCT, monoubiquitinates two proteins (FANCD2 and FANCI). This ubiquitinated heterodimer recruits the FA-downstream proteins, that correct the crosslink by nucleotide excision repair and homologous recombination (HR).

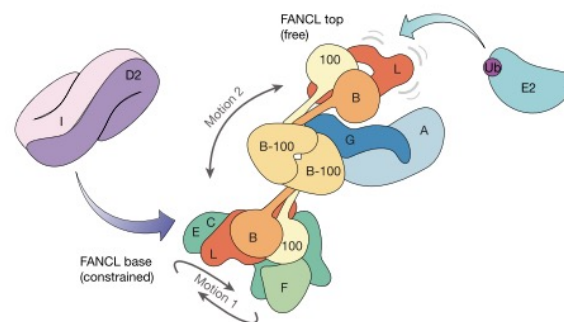


Figure 7: Schematic representation of the FA core complex and I/D2 monoubiquitination process⁶³

Biallelic inactivations in any of the 22 FA genes disrupt the pathway and cause chromosome instability and accelerated cellular aging. It is the most frequent cause of bone marrow failure syndrome in the world. Other hallmarks of the disease are cancer predisposition and malformations. The diagnosis relies on the presence of chromosome breakages in cytogenetic studies performed under genotoxic stress and the sequencing of the FANCA genes in order to identify the causal mutations. *FANCA* is, by far, the most affected gene, accounting for more than 70% of cases of FA.

Nearly all FA patients will present hematologic abnormalities impacting prognosis and quality of life.⁶⁴ Progressive haematopoietic failure starts before birth, and the median age of bone marrow failure diagnosis is eight years.^{65–67} FA hematopoietic cells undergo a p53/p21 response to stress and DNA damage, leading to progressive marrow failure since an very early age.^{67,68} Reactive oxygen species (ROS) and especially aldehydes are an important source of endogenous DNA damage.⁶⁷ Inflammatory cytokines, such as $TNF\alpha$ and $IFN\gamma$, contribute also to marrow failure⁶⁷. Nearly half of non-transplanted FA patients will develop a myeloid neoplasm during their lifetime. The prognosis of FA MDS/AML patients is unfavourable.^{69,70} Overall, hematologic complications account for nearly 70% of FA deaths.⁶⁶

The cumulative incidence of solid tumours by the age of 30 is about 30% and account for significant morbi-mortality.⁶⁶ Solid malignancies in FA are out of the scope of this work.

1.4.2.2 Standard treatment

The sole curative treatment for FA-associated haematologic complications is HSCT.⁷¹ Historically, transplantation in the FA setting resulted in significant early (organ failure) and late (secondary malignancies) because of the conditioning regimen and graft-versus-host disease. However, reduced intensity regimens, adapted donor choice, and better immunosuppression has improved survival.⁷² Transplantation yields better survival if performed before myeloid malignancy and 14 years of age.⁷¹

Androgens are an alternative to patients unsuitable for transplantation or with milder marrow failure⁷³. However, it cause liver adenoma and might boost pre-malignant clones, and progression towards MDS/AML is a risk.⁷⁴ Transplantation replaces recipient's defective haematopoiesis by donors' healthy cells, reducing the risk of progression.⁷⁵

1.4.2.3 Fanconi anaemia models

After discovering the Fanconi anaemia pathway and the genetic aetiology of the disease, many researchers looked for orthologs among other species. Ancestor FA pathways are present since lower eukaryotes, such as fungi⁷⁶ and plants⁷⁷, followed by progressively more advanced systems in nematodes⁷⁸, arthropods⁷⁹, and chordates⁸⁰. Among vertebrates, the FA pathway is strikingly conserved⁸¹ (**Table 2**). The FA-core complex evolved later in the phylogenetic tree.

Table 2: Orthologs of the 22 FA genes in different species

Species	FA core complex	D2/I heterodimer	Downstream proteins
<i>Homo sapiens</i>	A B C D E F G L M T	yes	D1 J N O P Q R S U V W
<i>Mus musculus</i>	A B C D E F G L M T	yes	D2 J N O P Q R S U V W
<i>Danio rerio</i>	A B C D E F G L M T	yes	D2 J N O P Q R U V W
<i>Drosophila melanogaster</i>	L M T	yes	O P Q R U V
<i>Caenorhabditis elegans</i>	T	yes	J O P Q R S
<i>Saccharomyces cerevisiae</i>	M T	no	J O P Q R

Adapted from ⁸² and updated based on <https://www.alliancegenome.org>.

Of the most frequently used animal models, the rodent's FA-pathway is closest to man's. All 22 human *FANC* genes are also present in mice with over 70% homology (**Table 3**). Therefore, scientists developed transgenic knock-out mice for nearly all *FANC* genes to study the FA pathway and disease⁸². Mice studies have been central to the study of FA, allowing not only to grasp the intricacies of protein interactions⁸³, but also the development of newer and promising therapeutics, such as gene therapy⁸⁴. Broadly, although the cells reproduce the FA phenotype (e.g., chromosomal breakage under genotoxic stress and hypersensitivity to mitomycin), most models do not present the classical hallmarks of FA disease (spontaneous bone marrow failure and cancer development). All FA mice models are hypofertile, and many have a high embryonic or perinatal mortality, and it is associated with the background. The C57BL/6 background, the most widely used inbred strain in haematopoiesis studies, is associated with a more severe lethality phenotype⁸⁵. Curiously, FA-mouse models do not present spontaneous, severe, and progressive bone marrow failure in most FA patients, except *Brca2*^{Δ27/Δ27}⁸⁶ and *Fancp*^{-/-}⁸⁷.

Thus far, the precise mechanisms by which FA mouse models sustain normal haematopoiesis and fail to present spontaneous tumorigenesis even upon ageing is unknown. Arguably, the clean environment of research animal facilities might play a protective role. Notwithstanding, our team induced BMF in a C57BL/6J *Fancg*^{-/-} by treating mice with chronic exposure to polyinosinic-polycytidylic acid (pl:pC), a synthetic analogue of double-stranded RNA that stimulates inflammation (Sebert et al., in revision).

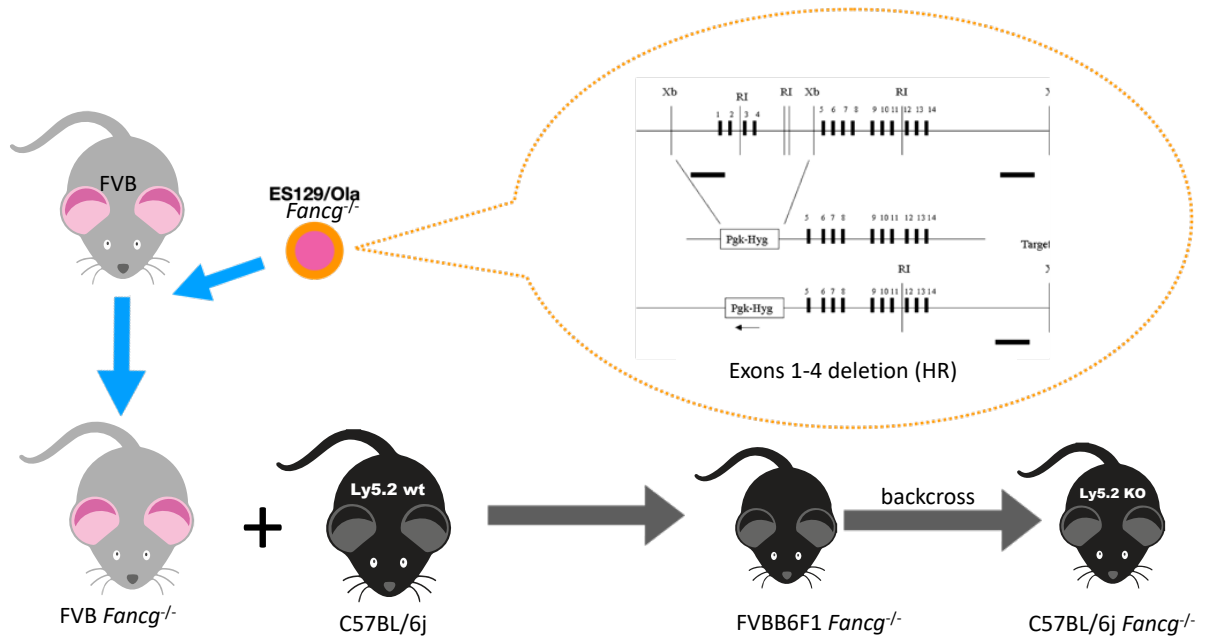
Table 3: High homology between men and mice FANC orthologs

Gene symbol (NCBI ID)		Gene name	FANC name	Chromosomal location		Gene homology*
Human	Mice			Human	Mice	
<i>FANCA</i> (2175)	<i>Fanca</i> (14087)	Fanconi anemia, complementation group A	<i>FANCA</i>	16q24.3	8	84.15
<i>FANCB</i> (2187)	<i>Fanb</i> (237211)	Fanconi anemia, complementation group B	<i>FANCB</i>	Xp22.2	X	71.14
<i>FANCC</i> (2176)	<i>Fance</i> (14088)	Fanconi anemia, complementation group C	<i>FANCC</i>	9q22.32	13	77.56
<i>BRCA2</i> (675)	<i>Brca2</i> (12190)	BRCA2 DNA repair associated	<i>FANCD1</i>	13q13.1	5	71.76
<i>FANCD2</i> (2177)	<i>Fandc2</i> (211651)	Fanconi anemia, complementation group D2	<i>FANCD2</i>	3p25.3	6	80.94
<i>FANCE</i> (2178)	<i>Fance</i> (72775)	Fanconi anemia, complementation group E	<i>FANCE</i>	6p21.31	17	90.32
<i>FANCF</i> (100040608)	<i>Fancf</i> (100040608)	Fanconi anemia, complementation group F	<i>FANCF</i>	11p14.3	7	82.88
<i>FANCG</i> (2189)	<i>Fancg</i> (60534)	Fanconi anemia, complementation group G	<i>FANCG</i>	9p13.3	4	79.5
<i>FANCI</i> (55215)	<i>Fanci</i> (208836)	Fanconi anemia, complementation group I	<i>FANCI</i>	15q26.1	7	82.71
<i>BRIP1</i> (237911)	<i>Brip1</i> (237911)	BRCA1 interacting helicase 1	<i>FANCI</i>	17q23.2	11	80.52
<i>FANCL</i> (55120)	<i>Fancl</i> (67030)	Fanconi anemia, complementation group L	<i>FANCL</i>	2p16.1	11	81.6
<i>FANCM</i> (104806)	<i>Fancm</i> (104806)	Fanconi anemia, complementation group M	<i>FANCM</i>	14q21.2	12	81.91
<i>PALB2</i> (79728)	<i>Palb2</i> (558923)	Partner and localizer of BRCA2	<i>FANCN</i>	16p12.2	7	77.47
<i>RAD51c</i> (5899)	<i>Rad51c</i> (114714)	RAD51 paralogue C	<i>FANCO</i>	17q22	11	87.47
<i>SLX4</i> (84464)	<i>Slx4</i> (850826)	SLX4 structure-specific endonuclease subunit	<i>FANCP</i>	16p13.3	16	69.86
<i>ERCC4</i> (2072)	<i>Ercc4</i> (50505)	ERCC excision repair 4, endonuclease catalytic subunit	<i>FANQP</i>	16p13.12	16	80.59
<i>RAD51</i> (5888)	<i>Rad51</i> (19361)	RAD51 recombinase	<i>FANCR</i>	15q15.1	2	87.85
<i>BRCA1</i> (672)	<i>Brca1</i> (12189)	BRCA1 DNA repair associated	<i>FANCS</i>	17q21.31	11	72.57
<i>UBE2T</i> (29089)	<i>Ube2t</i> (67196)	Ubiquitin conjugating enzyme E2 T	<i>FANCT</i>	1q32.1	1	81.32
<i>XRCC2</i> (7516)	<i>Xrcc2</i> (57434)	X-ray repair complementing defective repair in Chinese hamster cells 2	<i>FANCU</i>	7q36.1	5	78.45
<i>MAD2L2</i> (10459)	<i>Mad2l2</i> (971890)	Mitotic arrest deficient 2 like 2	<i>FANCV</i>	1p36.22	4	89.42
<i>RFWF3</i> (55159)	<i>Rfwf3</i> (234736)	Ring finger and WD repeat domain 3	<i>FANCW</i>	16q23.1	8	77.56

*per cent identity in <https://blast.ncbi.nlm.nih.gov/>

Both human and murine *FANCG* have 14 exons, present nearly 80% homology, and encode the FANCG protein (622 and 623 amino acids, respectively). Both human and mouse FANCG are ~65kDa proteins with four tetratricopeptides (TPRs, protein-protein interaction sites).⁸⁸ The murine model I used in this project (*Fancg*^{-/-}) was previously described and backcrossed into C57BL/6J Ly5.2 over 20 times at the IRSL. In short, the exons 1-4 were deleted, creating a *Fancg* knock-out model (**Figure 8**).⁸⁹ These mice have reduced fertility, and heterozygous couples give rise to less than 10% KO instead of the expected 25% - KO mice are infertile. This submendelian ratio can be explained by gonadal abnormalities and perhaps high embryonic mortality between E12.5 and E14.5. Besides, we observed that C57BL/6J *Fancg* KO are smaller and have craniofacial and ophthalmic abnormalities at birth (**Figure 9**). Haematology-wise, these mice, like most FA mouse models, do not present spontaneous cytopaenias, or any kind of neoplasm, up to the age of 24 months. However, they do become cytopenic under chronic inflammation (Sebert M et al., in revision) as the *Fanca*^{-/-} model.⁹⁰

Although this *Fancg*^{-/-} presents normal blood and marrow counts, it has 30% less LT-HSC than its wild-type counterparts. *In vitro*, *Fancg*^{-/-} CFU-assays demonstrated a proliferative defect (smaller and fewer colonies). *In vivo*, competitive transplantation experiments (*Fancg*^{-/-} vs WT) injected into lethally irradiated mice showed that WT outcompeted KO cells at 24 weeks.⁹¹ Together, these data indicate a haematopoietic defect of *Fancg*^{-/-} mice.



Koomen et al. Hum Mol Gen, 2002

Domenech et al. Stem cell reports, 2018 4

Figure 8: Schematic representation of the steps for obtaining the *Fancg* KO mouse model

Fancg exons 1-4 were deleted by homologous recombination in a 129/Ola embryonic stem cell. The ES129/Ola *Fancg*^{-/-} was implanted in pseudopregnant females and inbred in the FVB background.⁸⁹ Subsequently, FVB *Fancg*^{-/-} were outcrossed with C57BL/6j and more than 20 backcrosses for obtaining coisogenic strains.⁹²

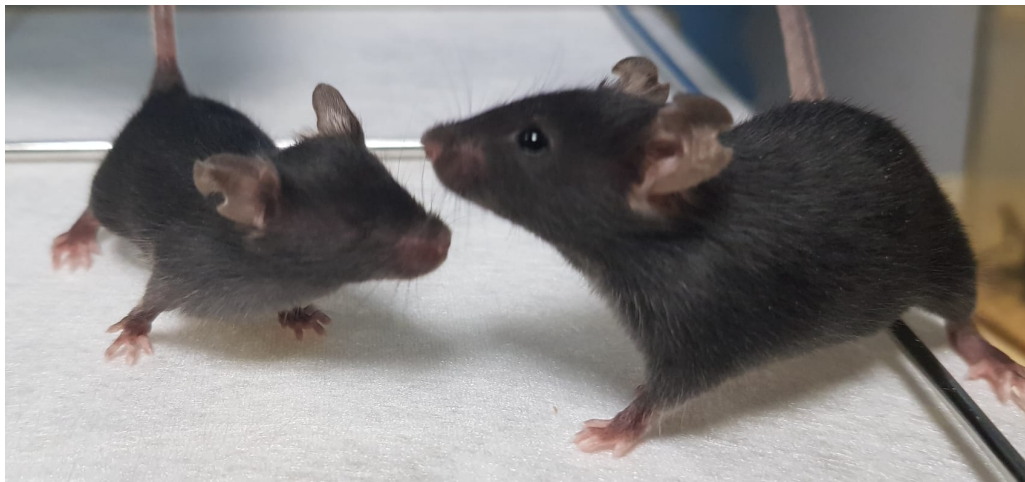


Figure 9: Two sisters

Note the smaller size and absence of the right eye in *Fancg*^{-/-} mouse (left) compared to her wt sister (right) – both are four weeks old.

1.5 Clonal haematopoiesis in aplastic anaemia

1.5.1 The Dameshek's Riddle

Clonal evolution into MDS/AML is the most concerning late event in severe acquired aplastic anaemia, and it occurs in approximately 15% of long-term responders to immunosuppressive therapy^{93,94}. In 1967, William Dameshek asked: "What do Aplastic Anemia, Paroxysmal Nocturnal Hemoglobinuria, and Hypoplastic Leukemia Have in Common?". Despite a long-known association, the precise mechanisms involved in malignant clonal evolution are not fully elucidated. While chromosomal abnormalities such as monosomy 7 and trisomy 8 are considered drivers of transformation,^{95,96} the potential role of somatic genetic variants is unclear to date.

Half to a third of acquired aplastic anaemia patients have a detectable mutated clone at SAA diagnosis^{48,96,97}. As expected, the prevalence of mutations increased with age. At baseline, the most frequent mutations are among *PIGA*, *BCOR*, *BCORL1* or HLA genes (**Figure 10**). Among genes associated with myeloid neoplasms, *ASXL1*, *RUNX1*, and *DNMT3A* are frequently found with VAF $\leq 10\%$.²¹ Data suggest that *PIGA*, *BCOR* or *BCORL1* would be associated with a better response to immunosuppressive treatment.⁹⁶ On the other hand, the univariate analysis indicated that baseline myeloid disease-associated gene mutations would be associated with worst survival in a fashion similar to the aged bone marrow found in ARCH/CHIP.^{97,98}

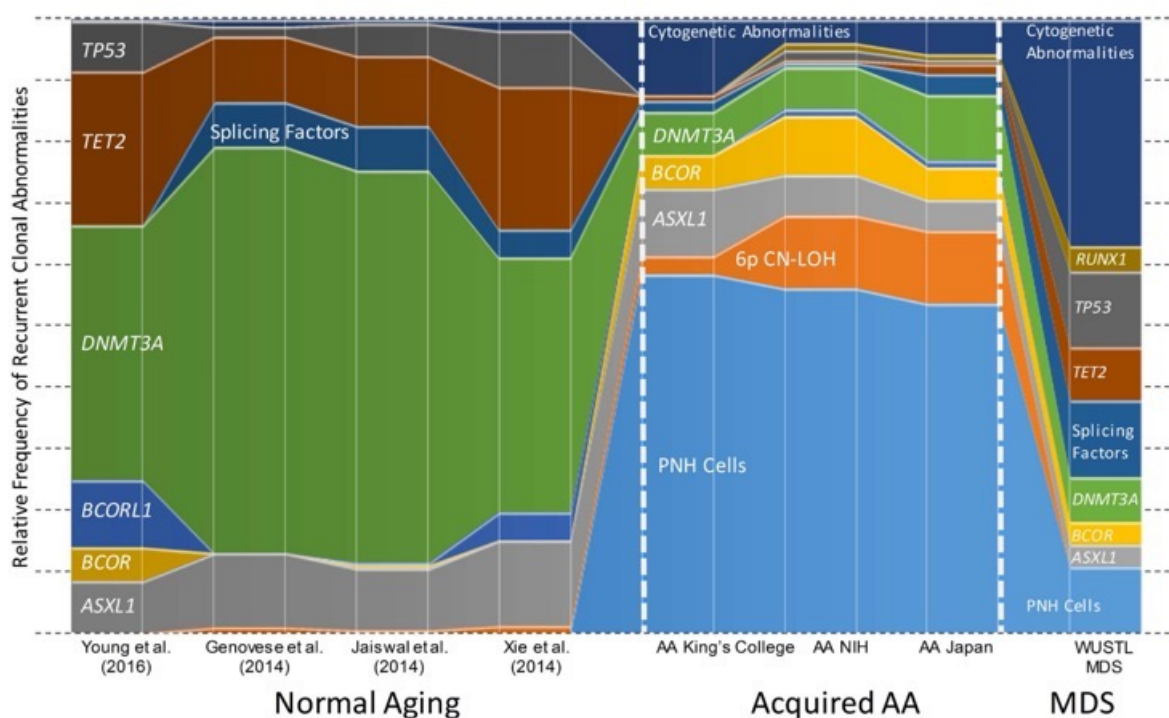


Figure 10: Distribution of the most frequently found genomic abnormalities across ARCH, AA, and MDS⁸⁰

Less than a half of AA patients treated with the state-of-the-art immunosuppression (horse ATG + cyclosporine + eltrombopag) present a complete remission.⁴⁸ Therefore, for most AA patients, some degree of cytopenia will persist for many years. The absence of complete response is a risk factor for progression.⁹⁴ Stress haematopoiesis and chronic inflammation of the marrow's microenvironment might profit myeloid-disease-associated mutants, as demonstrated in animal models.⁹⁹⁻¹⁰³

Because of the rarity of SAA, populational studies are not feasible. Thus far, no study confirmed nor rejected the findings for progression in CHIP (near-normal

haematopoiesis, analogy to complete response) nor CCUS (persistent cytopenias, analogy to absence of complete response). Among CCUS patients, the 5-year risk of progression for patients with mutations among *U2AF1*, *ZRSR2*, *SRSF2*, *JAK2*, or *RUNX1* can be as high as 80%.¹⁰⁴ For other CHIP/ARCH-associated mutations (*TET2*, *DNMT3A*, or *ASXL1*), the risk is inferior to 50%.¹⁰⁴ Therefore, CCUS and non-transplanted AA patients must regularly be screened for MDS/AML.

Cytogenetics-wise, monosomy 7 is much more frequent among transformed AA patients than primary MDS and confers a high risk of evolution to AML.^{95,97} Other cytogenetic abnormalities confer different risks of AML transformation between primary MDS and progressed AA: del(13q), a WHO 2016 MDS-defining cytogenetic abnormality confers intermediate risk for MDS patients,²⁹ whereas it seems favourable among AA patients and linked to PNH and immune escape.^{31,105} On the other hand, del(7q) seems to confer a poor prognosis among progressed AA patients (Cleveland Clinic, personal communication, manuscript to be submitted), whereas it is classified as intermediate for primary MDS.³⁰

1.5.2 Clonal evolution and immune escape: the Luzzatto-Rotoli hypothesis

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare, monogenic disease that manifests classically as intravascular hemolysis, bone marrow failure, and thrombosis. It is caused by somatic mutations in the *PIGA* gene, resulting in impaired glycosylphosphatidylinositol (GPI) biosynthesis.¹⁰⁶ GPI anchors many proteins on cells' surfaces, including the decay-accelerating factor (CD55) and the membrane inhibitor of reactive lysis (CD59) – modulators of the alternative and terminal complement pathway. Impaired GPI synthesis (GPI⁻) leads to complement-mediated hemolysis, the hallmark of PNH.¹⁰⁷

Nearly 30% of AA patients have a detectable PNH clone, and *PIGA* mutation can also be found among healthy individuals. In other words, acquiring a *PIGA* mutation alone is insufficient for PNH disease. The mechanisms underlying GPI⁻ clone expansion are unclear since GPI deficiency does not seem to confer any survival or proliferative advantage in animal models¹⁰⁸.

The most widespread hypothesis to justify GPI⁻ clonal dominance is the *bottleneck effect*^{VII}: GPI⁻ cells would resist the immune-mediated haematopoietic cell destruction seen in AA. The remaining *PIGA*-mutated clones would therefore expand, helping to sustain haematopoiesis.^{97,109} This immune escape theory is known as the Luzzatto-Rotoli hypothesis (**Figure 11**).¹¹⁰

Although the immune escape explains the clonal dominance of GPI⁻ in the AA micro-environment, it is less satisfactory to explain classic PNH patients (haemolysis *without* AA), since there is no evident bottleneck effect. Classic putative intrinsic drivers of clonal evolution as telomere shortening¹¹¹ and somatic mutations are more frequent in GPI⁻ clones¹¹² and might help contribute to GPI⁻ clonal dominance. Thus far, other factors such as environmental or inherited predisposition to PNH are unknown.

^{VII} Bottleneck effect: the way in which a reduction and subsequent increase in a population's size affects the distribution of genetic variation among its individuals¹⁷⁶.

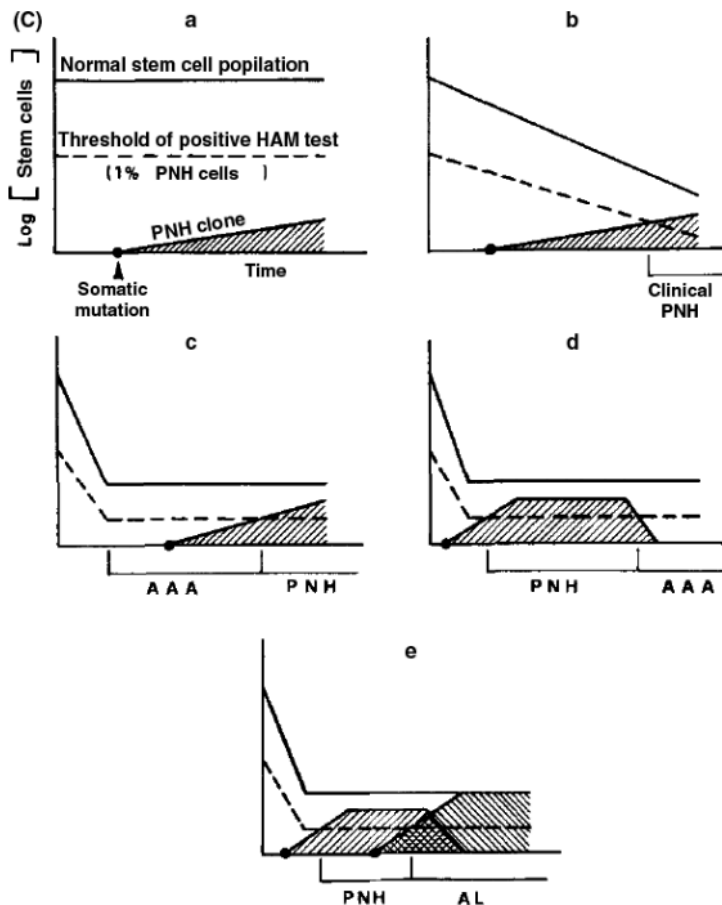


Figure 11: The Luzzatto-Rotoli hypothesis

Panel A: normal individuals might bear a small PNH clone without clinical significance. Panels B, C, and D represent different scenarios of PNH clones in the context of BMF. Panel E represents acute leukaemia emerging following BMF.¹¹⁰

We described for the first time that rare complement factor H (*CFH*) variants were overrepresented among PNH patients and that such variants were associated with suboptimal response to anti-C5 treatment. Factor H is a negative regulator of the alternative pathway.¹¹³ Specific *CFH* variants may downsize factor H activity, upregulating the alternative pathway. The functional consequences of the reported *CFH* variants and their possible role in GPI^{-} clones clonal dominance are incompletely known to date. Our data supported that *CFH* plays a pathophysiological role in PNH, and its protection is necessary for the positive selection of GPI^{-} cells. I submitted this work to Blood (brief communication), which is currently under revision. I include a non-peer-reviewed version of the manuscript in Annexe I. Noteworthy, none of the patients with a rare *CFH* variant evolved to a myeloid malignancy (median follow-up of 5.8 years).

PNH is a risk factor for myeloid malignancies, with more than 100-fold risk compared to an age-matched population¹¹⁴. A PNH clone can be found in up to 20% of patients at primary MDS diagnosis¹¹⁵ AA/PNH had a much higher risk than classic PNH patients (HR 48.8 vs 2.2, $p < 0.001$). The overall 10-year cumulative incidence of MDS/AML was 3.8% (95% CI; 0.1-7.5).¹¹⁴

Based on the data mentioned above (association of PNH with myeloid malignancies and putative drivers of clonal evolution in GPI^{-} cells), one should expect that the premalignant clone arises directly from PNH cells. Surprisingly, the evidence goes otherwise: cytogenetic abnormalities found among PNH patients occur in GPI^{+} cells,¹¹⁶ GPI^{-} and MDS/AML clones seem to originate from distinct founders,¹¹⁷ and the

presence of PIGA-mutated clone grants a better progression-free and overall survival.⁹⁶ So far, the data supports that the leukemic cells derive from a *PIGA*-wt (GPI+) clone (**Figure 12**). In other words, premalignant and PNH clones co-exist, and the former eventually outcompete the latter.

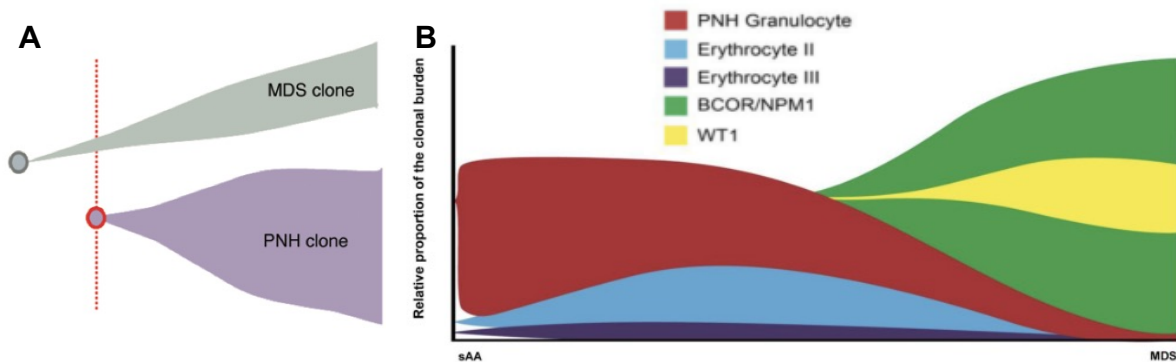


Figure 12: Clonal dynamics in patients' natural history

A) Schematic representation of MDS and PNH clones' co-existence. MDS and PNH originate from different founders¹¹⁸ B) the malignant clone outcompeted PNH cells¹¹⁷

1.5.3 The Luzzatto-Rotoli hypothesis: beyond GPI-deficient cells

Besides PNH, the immune escape hypothesis also explains another frequent clonal evolution in AA. Nearly one-third of AA patients present clones with loss of HLA class I expression (HLA⁻) either by chromosome 6p loss of heterozygosity or loss-of-function mutations at diagnosis (**Figure 13**).^{119,120} The HLA⁻ cells would similarly escape T cell-mediated cytotoxicity as GPI⁻ cells, explaining clonal haematopoiesis. Moreover, some HLA class I alleles are overrepresented in aplastic anaemia irrespective of the genetic background (HLA*B 40:02, for instance).¹²⁰⁻¹²² The overrepresentation of specific class I alleles among AA patients consists of strong evidence of its influence on AA pathophysiology.¹²³

The link between HLA expression and cancer is known for solid malignancies such as melanoma, and lymphoid neoplasms, such as Hodgkin lymphoma and chronic lymphocytic leukemia.¹²⁴⁻¹²⁶ Also, researchers have described HLA class I loss in *de novo* AML/MDS, and this was allegedly related to progression from MDS to AML.¹²⁷ Vago and colleagues identified AML relapses after a haploidentical hematopoietic stem cell transplantation who have lost the non-identical HLA haplotype.¹²⁸ These leukemic cells would have a selective advantage, escaping immunologic pressure from alloreactive donor T cells. In like manner, Christopher and colleagues identified patients with chronic myeloid leukaemia who relapsed after transplantation that have a down-regulated HLA class II expression at relapse.¹²⁹

It was initially proposed that such HLA alleles or abnormalities could be associated with a more severe clinical course and evolution to MDS in the context of AA.^{121,130} Although GPI⁻ and HLA⁻ clones can expand through the natural history of AA patients, driver mutations are hardly seen among HLA⁻ cells.¹²² Nakao's team studied 633 patients with AA and found that 127 had HLA⁻ clones. Interestingly, none of these patients progressed to a myeloid malignancy after a follow-up of 4.2 years. In contrast, 17 patients without HLA⁻ clones transformed to MDS/AML. HLA⁻ was an independent negative predictor for progression (HR: 2.82×10^{-5} , p-value < 0.001).¹³¹

In summary, HLA⁻ or GPI⁻ somatic mosaicism^{VIII} can expand in the context of AA, help rescue blood counts, and are probably not drivers of transformation (**Figure 13**).

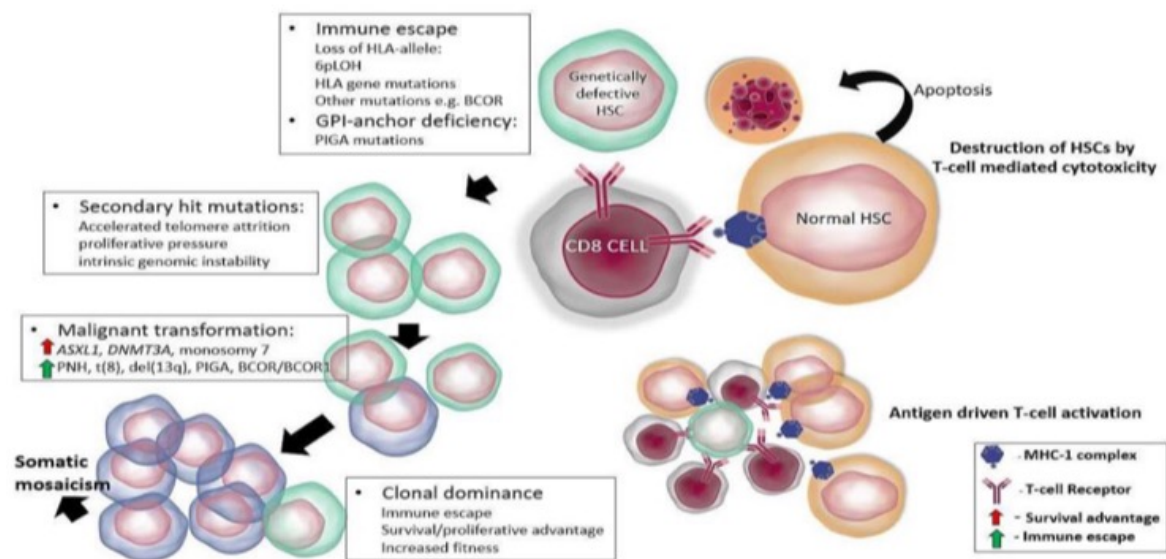


Figure 13: The immune escape hypothesis

HLA⁻ or GPI⁻ HSCs would escape T-cell mediated cytotoxicity and achieve clonal dominance because of a bottleneck effect (Luzzatto-Rotoli hypothesis). The immune escape clones would co-exist with premalignant clones (intrinsic proliferative advantage) that could or not progress to myeloid malignancy.¹³²

1.6 Clonal haematopoiesis in Fanconi Anaemia

1.6.1 Different pathways: reversion, premalignancy, and MDS/AML

Clonal haematopoiesis is frequent among IBMF and patients have an elevated risk of progression to MDS/AML. However, as for acquired marrow failures, CH confer different risks. Each syndrome has its own particularities, depending on the germline biological deficit. Cell competition can sometimes be protective: some patients might spontaneously acquire a genetic correction of the abnormal cellular pathway (a reversion)¹³³ or another somatic event can attenuate the cellular impact of the genetic deficit (a compensation).¹³³ Clones acquiring mutations that restore cellular function might be less susceptible to DNA error accumulation, *i.e.*, lower risk of progression. Therefore, CH is not synonym to cancer.

CH in DKC frequently occurs without driver mutations, perhaps reflecting neutral drift or unknown adaptative mechanisms.¹³³ Indeed, the most frequent events detected in TBD are reversion or compensation (e.g., *TERT* promoter mutations leading to enhanced telomerase activity).¹³⁴

In SDS, patients frequently acquire i(7q) or del(20q). The first encompasses *SBDS*, increasing transcription, and the latter induces *EIF6* haploinsufficiency.¹³⁵ In both cases, the near normal ratio *SBDS*:*EIF6* ameliorates HSPCs fitness. i(7q) and del(20q) seem not to be pre-malignant in SDS; somatic *TP53* drive transformation in SDS.¹³⁶

The risk of MDS/AML is extremely high in non-transplanted FA patients.¹³⁷ Leukemogenesis seems to be somewhat linear.⁷¹ The earliest, and most frequent,

^{VIII} Somatic mosaicism: the occurrence of two genetically distinct populations of cells derived from a somatic mutation within an individual affecting a single tissue (e.g., the blood)¹⁷⁷.

cytogenetic abnormality is trisomy 1q (pre-neoplastic state). Trisomy 3q, chromosome 7 abnormalities, and *RUNX1* mutations occur later in the disease course and harbinger myeloid malignancies (Sebert M. et al, in revision, 2022).¹³⁸ On the other hand, a minority of patients present a milder hematologic phenotype: a mosaicism of spontaneously corrected cells coexist with FA-deficient cells.¹³⁹ Our team has previously studied a cohort of 350 FA patients, described revertants' clinical and molecular characteristics, and defined the genetic mechanisms that explained reversion.^{140,141} Thus far, revertant patients not only have subnormal full blood counts, but also have not progressed to MDS/AML (**Figure 15**).

The diagnosis of functional reversion in FA is straightforward: the chromosomal breakage test correction proves that the pathway is restored.¹³⁹ Furthermore, demonstrating FANCD2 monoubiquitination in a FA-core patient proves the functionality of the pathway.^{140,141} FA-core patients are unable to monoubiquitinate FANCD2 and, the presence in blood and the absence in cultured fibroblasts of monoubiquitinated FANCD2 demonstrates the somatic mosaicism (**Figure 14**).

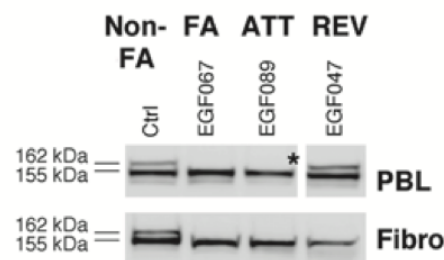


Figure 14: FANCD2 western blot of FA patients

Non-FA patients monoubiquitinate FANCD2 constitutively and the 162kDa band is present in both the haematopoietic tissue and fibroblasts. FA-core patients fail to monoubiquitinate FANCD2 and the 162kDa band is absent. On the other hand, revertant (REV) patients are capable of monoubiquitinating FANCD2 within the haematopoietic tissue but fail elsewhere. The compensation mechanisms underlying some attenuated phenotypes (ATT) are unclear¹⁴²

For some FA patients, the underlying biological mechanism of phenotype attenuation is still unknown. In FA cell lines, SLFN11 depletion reduced cell cycle arrest and lower sensitivity to mytomicin.¹⁴³ Likewise, haematopoietic cells from patients with attenuated phenotype present abrogation of the G2 arrest and have lower expression of CHK1 and p53, protecting cells from apoptosis. Diversely from revertants, the attenuated patients evolve to MDS/AML.¹⁴² Our team is currently identifying 8 patients with an attenuated phenotype for pairwise whole genome sequencing (blood and cultured fibroblasts), trying to identify a genomic mechanism of compensation / attenuation.

In mice models of FA, TBD, or GATA2, constitutive cells were capable of sustaining haematopoiesis during homeostasis. However, in competitive transplantation with wt cells, constitutive cells lost clonal competition and were eliminated.^{86,91,133,144} The functional correction of a few hematopoietic stem cells might confer an ecologic advantage, mitigating the risk of pre-leukemic clonal dominance.

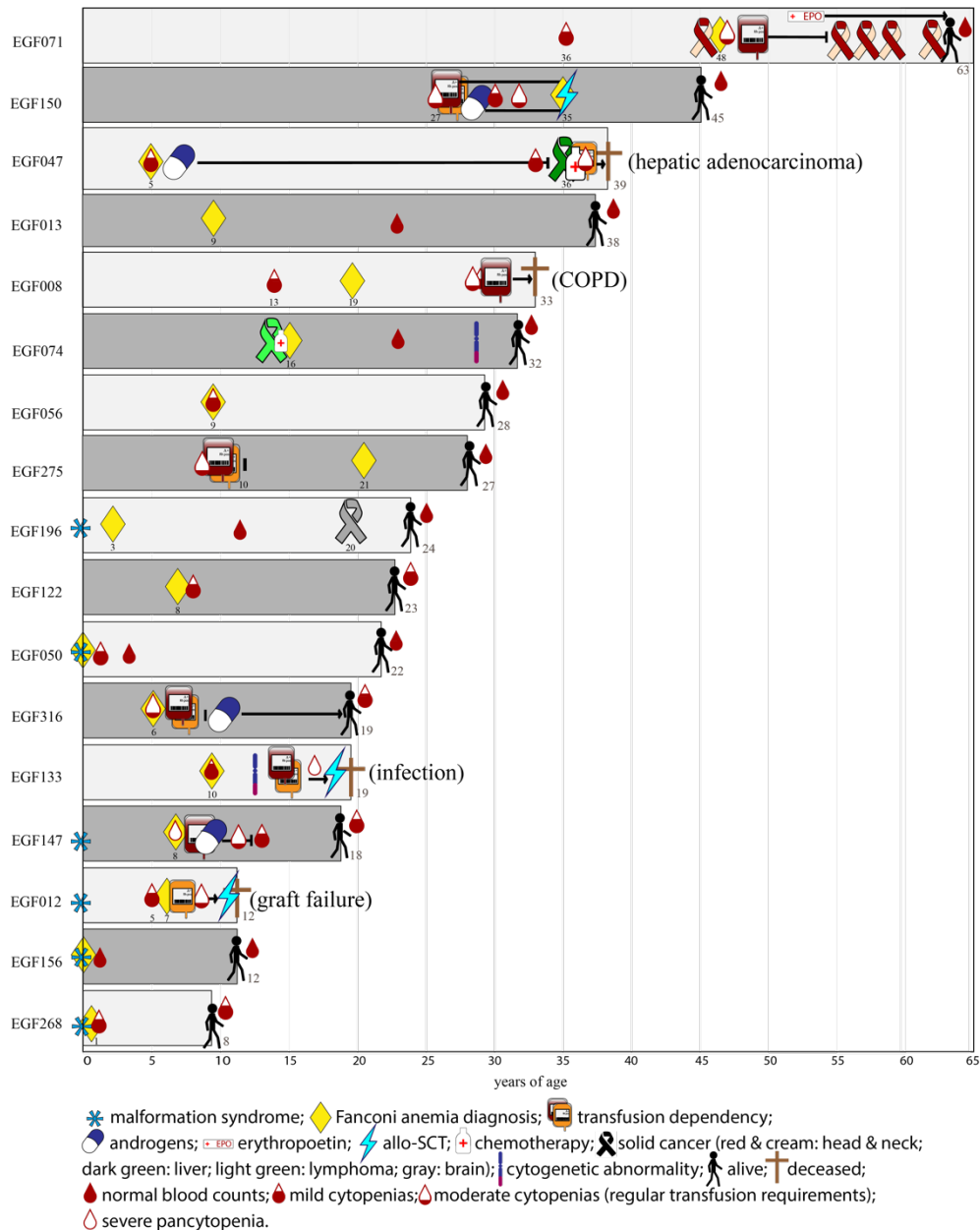


Figure 15: Clinical representation of revertant patients' natural history - French cohort

1.6.2 Gene therapy mimics somatic mosaicism in Fanconi anaemia

Inspired by Nature's somatic mosaicism, gene therapy is an advanced therapy medicinal product (ATMP) with very promising results for monogenic diseases. The use of an autologous cellular source overcomes the burden of graft-versus-host disease, rejection, and the need of long-lasting immunosuppression. The FDA and EMA have already approved ATMPs for thalassemia, adenosine deaminase severe combined immunodeficiency, and metachronic leukodystrophy.

The first platform used for gene-therapy in haematologic disorders used γ retroviral vectors (γ RV derived from Moloney murine leukaemia virus – MLV) for gene delivery. This strategy was rather genotoxic (insertional mutagenesis^{IX}) and was deemed causal for 19 acute leukaemias / MDS.¹⁴⁵ γ RV integrates non randomly, preferentially

^{IX} Insertional mutagenesis happens when viral cDNA integrates in host genomic DNA, deregulating neighbour genes. When it deregulates oncogenes or tumour suppressor, insertional mutagenesis can lead to haematologic malignancies.¹⁷⁸

close to *cis*- regulatory elements (enhancers and promoters).¹⁴⁶ Therefore, HIV-derived lentiviral vectors (LV) are more frequent in haematology gene-therapy trials. LV vectors are less genotoxic and,¹⁴⁷ thus far, have not been causal to secondary leukaemia.¹⁴⁵ Notwithstanding, at least two patients developed AML following LV-gene therapy with busulfan conditioning regimen for sickle cell disease, and neoplastic cells contained the LV insert in one of these cases (close to the vesicle-associated membrane protein 4, *VAMP4* – no known association with myeloid malignancies). Strikingly, both cases presented similar features (-7, *PTPN11*, and *RUNX1* mutations), but the abnormalities were not present at the time of gene therapy. In the case that leukaemic cells contained the viral insert, the genomic and transcriptional data were not in favour of gene-therapy causality.¹⁴⁸ The main hypothesis so far is that the leukaemia developed independently from the gene therapy and might be secondary to busulfan conditioning regimen.

Thus far, three clinical trials studied the safety and efficacy of gene therapy in FA: NCT01331018 (Fred Hutchinson, n=3), NCT03157804 (Madrid, n=9), and NCT03814408 (Rocket Pharmaceuticals, n=2). All included patients received *ex vivo* gene-corrected autologous CD34⁺ using self-inactivating PGK^X lentivirus and no conditioning regimen – no reported deaths thus far. The Spanish team reported progressive engraftment of gene-edited HSPCs on 4/4 patients and a halt on the marrow failure process; they did not observe any genotoxic events (**Figure 16**).¹⁴⁹

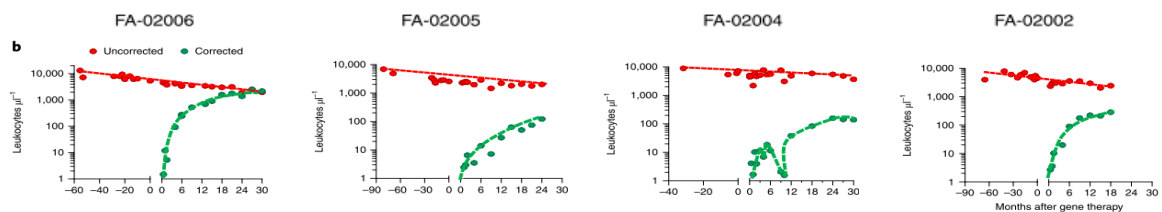


Figure 16: Engraftment dynamics following gene therapy¹⁴⁹

The best timing for gene-therapy in FA is not yet defined. The FA patients treated with gene-therapy had already some degree of marrow failure at the time of treatment. However, FA haematopoietic dysfunction starts early in the ontogenesis, *i.e.*, the multistep leukaemogenic process of clonal haematopoiesis is likely already ongoing at the BMF onset even if not detected with conventional cytogenetics. We do not know if the functional correction of a few hematopoietic stem cells confers an ecologic advantage, in other words, if the gene-corrected cells would outcompete the pre-existing undetected preleukemic clones, mitigating the risk of pre-malignant clonal dominance.

Gene-therapy arguably offers many advantages over HSCT for FA patients, notably reduced toxicity, no risk of GvHD, and perhaps standard risk of solid tumours. However, transplantation undeniably reduces the risk of evolution to MDS/AML. It is unclear whether gene-therapy would be the best therapeutic option to all FA patients considering the current good results of HSCT. Therefore, it is crucial to demonstrate that the gene-therapy's low treatment related mortality is followed by an also low risk of MDS/AML (**Figure 17**).

^X Phosphoglycerate kinase is major player in the glycolytic pathway. This promoter, as housekeeping, allow for constitutive gene expression.

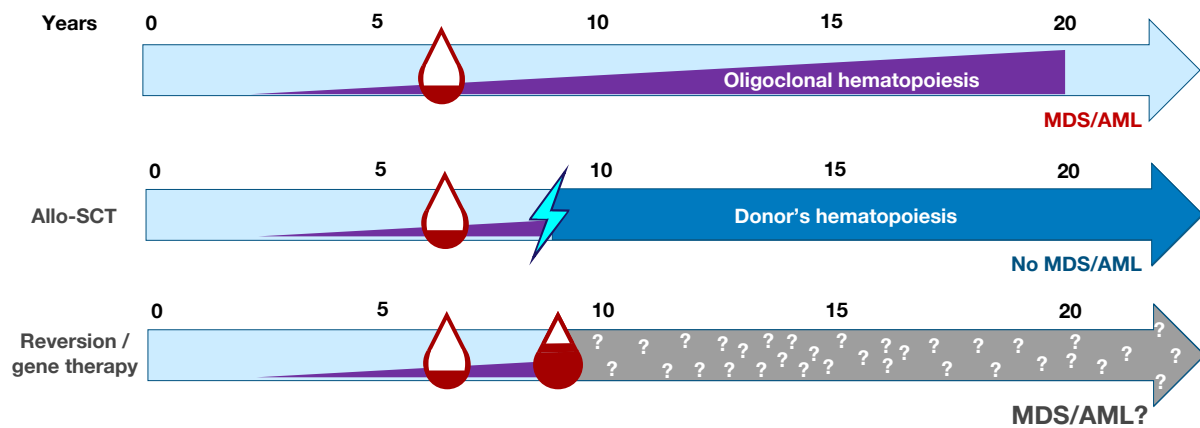


Figure 17: Schematic representation of clonal dominance in untreated, transplanted, and revertant / gene-therapy FA patients.

1.7 Thesis aims

I was interested in mechanisms explaining the emergence of clones bearing somatic mutations in bone marrow failure syndromes. From completing my clinical fellowship to starting the doctoral programme, I had many changes in my life, objectives, and thesis subject. All the proposals I present below resulted from discussions with my mentors.

My first project was entitled: “*ER-stress: a potential therapeutic target in paroxysmal nocturnal haemoglobinuria*”. GPI-deficient cells, normally synthesise these proteins, though the endoplasmic reticulum does not adequately fold and use them; the exact mechanism of these proteins’ disposal is unknown, but they may be either ubiquitinated and metabolized by the proteasome, processed by lysosomes or exported to the extracellular matrix. We hypothesized that the GPI- anchored proteins that are not anchored are processed by the ubiquitin-proteasome pathway, and we would evaluate whether the proteasome blockade induced apoptosis in human GPI-deficient cells.¹⁵⁰ Understanding what happens to the unused GPI-AP in GPI-deficient cells could allow us to understand better how these cells strive instead of being driven to apoptosis. Moreover, showing that GPI-deficient cells are more susceptible to apoptosis induced by proteasome blockade than non- GPI deficient cells could be of therapeutic interest, since new targets could be addressed in this pathway towards eradicating the PNH clone. We did not carry on this proposal because it was outside the scope of host laboratories.

My second proposal was to study the “*Emergence of TERT promoter somatic mutations to rescue haematopoiesis in bone marrow failure syndromes*”. Acquired *TERT* promoter mutations have been initially described in solid tumours and mantle cell lymphoma. They are the most common non-coding acquired mutation observed in cancer. These mutations increase telomerase function, elongate telomeres in cancer cells, and modulate tumour proliferation and aggressiveness. Acquired *TERT* promoter mutations have been detected in leukocytes of approximately 5% of patients with idiopathic pulmonary fibrosis carrying a constitutional telomerase gene mutation. The study aimed to screen bone marrow failure patients carrying constitutional pathogenic telomere-associated gene mutations and their family members for somatic mutations in the *TERT* promoter. We planned to correlate the presence of somatic *TERT* promoter mutations with bone marrow cellularity, peripheral blood counts, telomere length, and evolution to MDS/AML. This proposal was completed by a collaborating team.¹⁵¹

I continued to be interested in the emergence of clones bearing somatic

mutations to rescue haematopoiesis in bone marrow failure syndromes. In the light of papers Nakao's team had recently published (2017 / 2018), I have written the proposal entitled *Clonal evolution in acquired aplastic anaemia: immune escape and somatic genomic alterations*.

Hematopoietic cells that lost HLA class I expression in AA patients, by chromosome 6p loss of heterozygosity (6pLOH) or loss-of-function mutations in HLA genes, especially in *HLA-B*40:02* are frequent among acquired aplastic anaemia patients.^{119,120} These cells (HLA⁻) would escape T cell cytotoxicity, helping sustain haematopoiesis. In consonance with their findings, Babushok *et al.* showed that *HLA-A*68:01*, *HLA-A*33:03*, *HLA-B*14:02*, and *HLA-B*40:02* alleles are overrepresented among AA patients and associate with a more severe clinical course and evolution to myelodysplastic syndromes (MDS).⁸ Together, these results pointed to immune selection as central in the pathogenesis and clonal progression of acquired AA. Whereas chromosomal abnormalities such as monosomy 7, telomere attrition, and mutations are drivers of evolution from AA to AML/MDS, the potential role of immune escape was unknown.^{10–12}

The link between HLA and cancer is known for solid cancers and lymphoid neoplasms^{13–15}. Researchers have described the HLA class I lost in *de novo* AML/MDS, allegedly related to progression from MDS to AML.¹⁶ Vago *et al.* identified AML relapses after an HSCT that lost the non-identical HLA haplotype or deregulated T-cell recognition pathways.^{17,18} These leukaemic cells would escape immunologic pressure from donor T cells. Likewise, Christopher and colleagues identified patients with chronic myeloid leukaemia who relapsed after HSCT that have a down-regulated HLA class II expression at relapse.¹⁹

Altogether, we hypothesized that HLA played a role in malignant clonal evolution in AA. Our primary objective was to investigate whether the reduction or loss of HLA expression in hematopoietic cells in acquired aplastic anaemia patients is associated with AML/MDS transformation. As secondary aims, I would:

- identify the HLA alleles that were overrepresented among AA patients in our participating centres;
- identify risk HLA alleles that were linked to malignant evolution in AA patients;
- determine which AML-associated genes were somatically mutated in the AML cells occurring after AA, in addition, or alternate to the known frequent events such as monosomy 7;
- determine the gene expression profile of AML cells after AA, looking for immune-escape mechanisms (frozen samples).

Therefore, we conducted a pilot study in collaboration with the Histocompatibility laboratory of Saint-Louis Hospital, sequencing HLA classes I and II (NGSgo®-MX11-3, GenDx, Utrecht, The Netherlands - methods are in Supplementals of the JCO paper) of ten patients (nine acute myeloid leukaemias and one myelodysplastic syndrome with an excess of blasts type 2) who transformed from aplastic anaemia. Using the existing HLA pipeline, no mutation or loss of heterozygosity was found at the transformation into myeloid neoplasm. Lacking power to any conclusion, we collaborated with the Cleveland Clinic to increase the sample size and work out the analysis pipeline. Altogether, we have interpreted the HLA sequences of 10 patients from France and 10 from the United States. Each centre cross-checked the results: we confirmed that no mutation nor LOH was found among blastic patients. The hypothesis that the loss of HLA expression would not play a significant role in progression was further corroborated by Nakao's team's research published when we found our results.¹³¹

Following these results, we have adapted our proposal, and I present the study in Chapter 2, section 2.1.

Given the negative results of the pilot study, my mentors and I developed an alternative plan to secure experimental work. I would develop a mice model of somatic mosaicism (genetic reversion) in Fanconi Anaemia. I aimed to test the fitness of various revertant or preleukemic subclones in Fanconi, mimicking *in vivo* the early stages of bone marrow progression. I present this project and the experiments I carried out in Chapter 2, section 2.2.

My proposals changed and evolved for various reasons, but always within the scope of clonal haematopoiesis in marrow failure syndromes and their impact on the evolution of MDS/AML. BMF patients with CH present very different natural histories, and I have focused on two questions: looking into the determinants of transformation in SAA/PNH and the impact of gene correction in FA on the risk of progression.

Chapter 2: Methods and Results

2.1 Clinical and molecular determinants of clonal evolution in aplastic anemia and paroxysmal nocturnal hemoglobinuria

2.1.1 Aim of the work

We and others studied the genomic landscape of patients with malignant evolution, focusing on genomic lesions known to have a role in myeloid neoplasms and bone marrow failures. A sub-analysis concerned the mutational study of the HLA region for all samples already sequenced for class I and II HLA genes. The potential role of immune evasion secondary to HLA loss and the evolution of myeloid malignancies was unknown.

2.1.2 The scientific question

In aplastic anaemia (AA)/paroxysmal nocturnal haemoglobinuria (PNH) patients, how do baseline clinical, cytogenetics, and molecular characteristics influence the risk of secondary myeloid neoplasm?

2.1.3 Results

We have established a collaboration between Cleveland Clinics in the USA, Saint-Louis Hospital, Paris, and University of Sao Paulo, Ribeirao Preto, Brazil. All three Institutional Review Boards approved this work.

I have included a non-edited version of the manuscript, in which I am co-first, with its supplementals in this chapter. This manuscript was accepted for publication in the Journal of Clinical Oncology on the 6 of July 2022 and published online on the 2nd of September 2022.

Clinical and Molecular Determinants of Clonal Evolution in Aplastic Anemia and Paroxysmal Nocturnal Hemoglobinuria

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The symbols “#” and “*” indicate equal contribution

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

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ABSTRACT

PURPOSE: Secondary myeloid neoplasms (sMN) remain the most serious long-term complications in patients with aplastic anemia (AA) and paroxysmal nocturnal hemoglobinuria (PNH). However, sMN lack specific predictors, dedicated surveillance measures and early therapeutic interventions.

PATIENTS AND METHODS: We studied a multicenter, retrospective cohort of 1008 patients (median follow-up 8.6 years) with AA and PNH to assess clinical and molecular determinants of clonal evolution.

RESULTS: Whereas none of the patients transplanted upfront (n=117) developed clonal complications (either sMN or secondary PNH), the 10-year cumulative incidence of sMN in non-transplanted cases was 11.6%. In severe AA, older age at presentation and lack of response to immunosuppressive therapy were independently associated with increased risk of sMN whereas untreated patients had the highest risk among non-severe cases.

Elapsed time from AA to sMN was 4.5 years. sMN developed in 94 patients. Five-year overall survival reached 40% and was independently associated with bone marrow blasts at sMN onset. Myelodysplastic syndrome with high-risk phenotypes, del7/7q and *ASXL1*, *SETBP1*, *RUNX1* and *RAS* pathway gene mutations were the most frequent characteristics. Cross-sectional studies of clonal dynamics from baseline to evolution revealed that *PIGA*/HLA lesions decreased over time, being replaced by clones with myeloid hits. *PIGA* and *BCOR/L1* mutations carriers had a lower risk of sMN progression whereas myeloid driver lesions marked the group with a higher risk.

CONCLUSIONS: The risk of sMN in AA is associated with disease severity, lack of response to treatment and patients' age. sMN display high-risk morphological, karyotypic and molecular features. The landscape of acquired somatic mutations is complex, and incompletely understood, and should be considered with caution in medical management.

Key Objective

Secondary myeloid neoplasms (sMN) are concerning late events in non-transplanted acquired aplastic anemia (AA) or paroxysmal nocturnal hemoglobinuria (PNH) patients. This retrospective, multinational study (n=1008) reports how clinical, cytogenetic, and molecular characteristics influence the risk of sMN, suggesting rational diagnostic approaches.

Knowledge Generated

sMN presented high-risk morphological, cytogenetic, and molecular features. Cross-sectional analyses revealed an inversion in clonal dynamics: *PIGA*/HLA-mutations, predominant at baseline, were overwhelmed by myeloid drivers at transformation. Lack of response to non-transplant treatments, older age, and myeloid driver mutations at AA/PNH onset conferred higher risk of progression.

Relevance

Patients with AA or PNH need close, prolonged monitoring with marrow smear and cytogenetics. Morphological progression to sMN or occurrence of monosomy 7 should prompt clinicians to consider transplantation in eligible patients. The landscape of somatic mutations is complex and incompletely understood. Clinicians should carefully interpret the molecular profile with morphological and cytogenetic data for therapeutic decisions.

Aplastic anemia (AA) is a prototypic bone marrow (BM) failure syndrome manifesting with pancytopenia of autoimmune origin.¹ While hematopoietic stem cell transplant (HSCT) represents a curative option for younger cases, older patients and those lacking a suitable donor are treated with immunosuppressive therapy (IST).² Particularly in such patients, the refinement of IST during the last decades has dramatically improved survival outcomes.³

Nevertheless, with increased numbers of survivors, a higher risk for clonal evolution to both myeloid neoplasia (MN) and paroxysmal nocturnal hemoglobinuria (PNH) emerged, corroborating the hypothesis described by Dr. Dameshek's riddle 50 years ago as to the intertwining relationship of such entities: "what do aplastic anemia, paroxysmal nocturnal hemoglobinuria and "hypoplastic" leukemia have in common?".^{4,5} Indeed, retrospective studies with long follow-up revealed that up to 20% of patients with AA treated with IST may develop either myelodysplastic syndromes (MDS) or acute myeloid leukemia (AML).^{6,7} These events are distinguished by dismal outcomes and absence of obvious clinical predictors. Furthermore, as it has happened for G-CSF in the past^{8,9}, the use of thrombopoietin-receptor agonists has recently raised concerns as to the increased incidence of clonal evolution, further stressing the importance of studies aiming at the identification of predictors of such deadly complication.¹⁰

SNP array karyotyping, and more recently deep massive parallel sequencing (NGS) revealed somatic mutations with a wide range of frequencies (5% up to 72%) in patients with AA and PNH according to the testing timing (diagnosis vs. follow-up), the number of interrogated genes, and technique (targeted vs. whole exome/genome sequencing).^{6,11-17} In addition to *PIGA* mutations, responsible for the emergence of PNH clone(s), the most frequently mutated genes are *DNMT3A*, *ASXL1*, and *BCOR/L1*, whose effects on AA and PNH clonal dynamics are far from being completely understood.^{5,11} In the paucity of hematopoietic stem cells (HSC) surviving after AA immune attack, mutations generating a survival advantage such as *PIGA* or myeloid driver hits (*e.g.*, *RUNX1*, *SETBP1*) may arise, or acquire clonal dominance if already present.¹⁸

These observations suggest that the pathogenesis of secondary MN (sMN) may be linked to clonal hematopoiesis (CH), characterized by somatic mutations typical of MN with a peculiar MDS-related signature.^{6,11,12,17} However, low burden mutations may be transient events and not contributory to later evolution while others may persist, thereby heralding

progression. This is reminiscent of CHIP (CH of indeterminate potential) found in healthy individuals, or CCUS (clonal cytopenia of undetermined significance).¹⁹⁻²¹

Unlike leukemic driver mutations, *PIGA* hits produce an escape phenotype (“blessing in disguise”) in the context of AA immune-mediated attack.²² The theory of “clonality as escape”²³ posits that *PIGA*-mutant HSCs do not significantly sustain BM hematopoiesis unless the T cell-mediated immune attack selectively targets the *PIGA*-wild-type HSCs.²⁴ Likewise, somatic hits in the human leukocyte antigen (HLA) genomic region have also been identified in AA and tentatively assigned to an immune escape of the stressed BM, paralleling findings observed in cancer.^{16,17,25-27}

The rarity of AA and the timing of clonal evolution have made prospective controlled studies aiming to characterize factors associated with evolution difficult. Perhaps the long-term follow-up of the recently completed RACE trial³ may be able to do so; but it will take years to get the full picture. Thus, retrospective analyses including large numbers of patients followed for extended periods of time are the sole able to decipher this complex field. Herein, we performed a comprehensive study of late clonal complications in 1008 consecutive patients with AA and PNH from a multicenter international cohort in order to define predictors of malignant progression. Furthermore, we examined clinical and molecular features of progression, being able to track the dynamics of clonal evolution in a subgroup of patients in a cross-sectional and longitudinal fashion.

PATIENTS AND METHODS

Study design and patients We devised a retrospective, multicenter study to investigate the influence of baseline characteristics of patients with AA and PNH on the risk of late clonal complications (*e.g.*, sMN and secondary PNH) and to characterize the clinical and genomic features of evolution. All consecutive patients diagnosed with acquired AA and/or PNH between 1972 and 2020 at the three participating centers were enrolled in this study after obtaining written informed consent (**Table 1, Figure 1**). The review of medical records was approved by the internal Institutional Review Boards of each center in agreement with the Declaration of Helsinki. Full details on study design, patients and definitions are available in the **Supplementary Appendix**.

Genomic Studies Samples were collected and subjected to multiple NGS platforms as previously described²⁸⁻³⁰ and according to the manufacturer's protocols. Full details are provided in the **Supplementary Appendix (Table S1, Figure S1)**.

Statistical analysis Cumulative incidences of clonal complications were calculated in a competing risk setting, where death and HSCT were considered competing events. Non-adjusted effect was evaluated using Log-Rank and Gray's tests, respectively for overall survival (OS) and cumulative incidence outcomes. Multivariate impact of baseline variables was evaluated using multivariate Cox-cause specific model. The risk proportionality of the Cox regression models was tested and the assumption was not violated (i.e., no global test was significant) while a potential center effect was taken into account using a random effect term (method also known as frailty).

All statistical tests were two-sided, and a p-value <0.05 was considered statistically significant. All analyses and data visualization were generated using the statistical computing environment R (4.0.0 R Core Team, R Foundation for Statistical Computing, Vienna, Austria), Excel Microsoft Office 365 and GraphPad Prism (8.4.0). Further details on statistics are provided in the **Supplementary Appendix**.

RESULTS

Determinants of late clonal complications and survival

A total of 1008 patients were assessed for inclusion. Because of absence of follow-up data (n=3) and overt diagnosis of MN at PNH presentation (n=6), 9 patients were excluded from further analysis (**Table 1, Figure 1**). In agreement with our previous report⁷, none of the patients undergoing HSCT upfront (n=117, **Table S2**) experienced late clonal complications (either sMN or secondary PNH) at a median follow-up time of 8.2 years (IQR, 5.7 - 10.6), registering a 10-year OS of 86.7% (77.8 - 92.1).

We thus studied non-transplanted patients with AA, AA/PNH overlap, and classical hemolytic PNH (n=882, **Table S3**). Their 10-year OS reached 76.4% (median follow-up time 8.6 years, **Figure S2**) and the 10-year cumulative incidence of sMN was 11.6% (**Figure S3**). A trend towards a higher risk of malignant progression was observed in AA (12.8%) and AA/PNH (13.1%) as compared to classical hemolytic PNH (3.4%, both p=0.06, **Figure 2A**). Consequently, classic PNH cases had the best OS

with 95.2% alive at 10 years from diagnosis vs. 72.1% ($p<0.001$) and 88.8% ($p=0.99$) in patients with AA and AA/PNH, respectively ($p<0.001$ globally by Log-Rank, [Figure S4A](#)).

Besides the presence of PNH hemolytic clones, outcomes of AA were also affected by disease severity. While showing similar rates of sMN progression (13.6 vs. 12.4%, $p=0.86$; [Figure 2B](#)), non-severe cases had better OS (81.3 vs. 71.0%, $p<0.001$) but a slightly higher risk of secondary PNH (15 vs. 9%, $p=0.12$) when compared to patients with severe disease ([Figure S4B,S5](#)).

In severe AA ($n=504$, [Table S4](#)), the risk for malignant evolution was higher in older (>35 years) cases (20 vs. 6.6% in younger patients; $p<0.001$) and in those with partial or no response to IST (15.7 vs. 8.5% in complete responders; $p=0.02$). Older age at presentation (HR=1.37 [95%CI 1.20 - 1.56]; $p<0.001$) and poor response to IST (HR=2.60 [95%CI 1.39 - 4.84]; $p=0.003$) were independent predictors of sMN progression, and survival ([Figure 2C-D](#), [Table 2](#)). When studying patients with available molecular information ($n=302$), overall the presence of myeloid mutations at disease onset did not seem to affect evolution ([Table S5](#)). The presence of a small PNH clone characterized patients with better survival outcomes (83.9 vs. 70.7% at 10 years; $p=0.02$) and was strongly associated with evolution to secondary hemolytic PNH (19.8 vs. 4.7%; $p<0.001$; [Table S5](#)). A lower incidence of secondary PNH evolution was instead observed in poor IST responders (5 vs. 15.8%, $p<0.001$) and in male patients (6.0 vs. 12.1% in females; HR=0.49 [95%CI 0.27-0.89]; $p=0.02$ in a multivariate setting).

A similar analysis on non-severe AA ($n=192$, [Table S6](#)) identified non-treated patients (accounting for 34% of cases) as the group with the highest risk of sMN evolution (21.9 vs. 8.9 and 8.3% at 10 years in non-treated, poor and good IST responders, respectively; $p=0.04$; [Figure S6](#)). A slightly increased risk was also observed in AA patients with hemolytic PNH (16.4 vs. 11.5%; $p=0.03$). It is noteworthy that in non-severe cases the risk of sMN progression was not affected by age, which instead still influenced survival outcomes as well as did the presence of PNH clones ([Table S7](#)).

Clinical and molecular features of secondary myeloid neoplasms

After a median time of 4.5 years (IQR 1.8 - 7.7), 94 patients (M:F ratio 1.61; median age 61 years, IQR 34 - 69) evolved to sMN. The majority of progressed cases were patients with severe AA or those not registering complete responses to either

first or salvage therapies. Of note is that nearly all patients undergoing second-line treatment (excluding cases rescued with HSCT) showed poor responses, with only one patient achieving a complete response (Table S8-9). MDS (75%) was the most frequent diagnosis at evolution, followed by AML (18%) and MDS/myeloproliferative neoplasm (7%; Figure S7). With a median follow-up of 4.7 years, the 5-year OS after sMN diagnosis reached 40% (Figure S8) and was independently influenced by blast $\geq 5\%$ (HR= 3.64 [95%CI 1.82 - 7.30]; $p < .001$; Figure 3A-B; Table S10-11).

In contrast to the usual characteristics of the MDS population, the majority of MDS cases had higher R-IPSS scores due to the overrepresentation of poor cytogenetic risk groups. Overall, myeloid mutations (N=82/94) were found in at least 80% of sMN with 61% of mutants harboring ≥ 2 lesions (Figure 3C). Apart from cases uneligible/unfit for active treatment (44.4%), patients received chemotherapy (12.2%), hypomethylating agents (18.9%) or HSCT (36.2%), either upfront (21.1%) or as a consolidation strategy (15.1%).

Analysis of molecular characteristics resulted in the distinction of four different sMN groups (Figure S9-10). In particular, cases harboring del7/7q and complex karyotype (CK) had a similar mutational burden and survival outcomes to those with normal karyotype (NK), differing instead from patients carrying other cytogenetic alterations. *ASXL1*, *SETBP1*, *RUNX1* and *RAS* pathway gene mutations constituted the unique signature of del7/7q carriers, classical leukemogenic drivers such as *DNMT3A*, *FLT3*, and *NPM1* were typical of NK, whereas cases classified as "others" and CK had diverse molecular configurations (Figure 3D-E).

To further establish the discrete peculiarities of myeloid disorders arising from AA, we then applied a propensity score matching to reduce any possible confounding effects between our sMN cases (n=94) and an internal control cohort (n=94 matched patients with a 1:1 ratio from 3599 *de novo* cases) by using age, gender, bone marrow blast percentage and disease type as relevant baseline covariates (confounders). As expected, we found that higher-risk R-IPSS scores (54% vs. 37%) and chromosome 7 aberrations (53% vs. 11%) as well as *ASXL1* (24% vs. 12%) and *RUNX1* (21% vs. 8%) mutations were more frequent in sMN as compared with *de novo* cases, emphasizing that MN arising post-AA constitutes a distinct nosologic entity (Figure S11).

Immuno-molecular dynamics of clonal evolution

In a cross-sectional study of clonal dynamics from baseline (n=302, [Figure S12](#)) to evolution, overall 18% of patients harbored myeloid mutations at primary disease onset with a higher frequency of mutant cases found among patients with AA as opposed to PNH ([Figure 4A](#)). After exclusion of *PIGA*, mutated in up to one-third of cases, the most recurrent alterations at baseline were found in *BCOR/L1*, *ASXL1*, and *TET2* genes ([Figure 4B](#)).

In cases sequenced at baseline, follow-up and evolution, the frequency of *PIGA* mutations dramatically decreased at sMN progression, consistent with a reciprocal expansion of PNH clones and evolution to secondary hemolytic PNH in non-progressors. The same trend was observed for somatic HLA aberrations that decreased during follow-up and at progression, whereas an opposite effect was noticed for myeloid lesions. These quantitative changes were paralleled by dynamics of mutational burden ([Figure 4C,S13](#)).

We then classified our patients based on features (myeloid driver mutations only =Group 1; *PIGA*/PNH clone presence and *BCOR/L1*=Group 2, no mutation=Group 3) previously hypothesized to be associated with sMN evolution, with the exclusion of pure hemolytic PNH, which we have shown to behave as a distinct clinical entity with regards to sMN progression. Accordingly, a Cox-proportional hazard model showed that Group 1 had a higher risk of sMN ($p<0.0026$). Of note, this latter category of patients was characterized by older age at AA presentation when compared with the most favorable group carrying *PIGA* and *BCOR/L1*, while no relationship with IST response was observed (median age 37 [IQR 27 - 73] vs. 26 [19 - 48] years in Group 1 and 2, respectively; $p=0.04$; [Figure 4D, S14](#)).

The results of previous cross-sectional analysis have been confirmed longitudinally in 25 serial cases (AA onset and sMN evolution; [Figure S15](#)). As expected, when compared to baseline we found higher mutational (median number of mutations 0 [IQR 0-1] vs. 2 [1-2], $p<0.001$) and clonal burdens (median VAF 20% [IQR 5-27] vs. 30% [19-44]; $p=0.01$) at myeloid progression ([Figure S16A](#)). Interestingly, the dissection of the genomic architecture of such cases showed that 10% of mutations, which constituted the bulk of disease at evolution, were ancestral events already present at the time of BM failure onset or early in its course. Conversely, we noticed that *CUX1* mutations disappeared at progression with concomitant acquisition of deletions affecting chromosome 7 ([Figure S16B-C](#)). Nevertheless, no difference in time to progression was observed in patients with and without mutations shared at both time points.

Over the course of 10 years from diagnosis, one-fifth of patients with AA treated with IST will experience late clonal complications (e.g., sMN, secondary PNH). Early studies on X-chromosomal inactivation and karyotyping already indicated that CH may be present in a substantial number of AA cases, possibly as a prodromal sign of the increased risk for clonal evolution.^{17,31} Among these clonal cytogenetic aberrations, partial or total loss of chromosome 7 represents the most frequent and worrisome alteration.³¹ Subsequently, a systematic application of NGS further underscored how widely spread CH occurs in AA.^{3,11} To that end, diverging theories on AA oligoclonality have been proposed, ranging from contraction of normal HSC pool and bystander CHIP-like phenomena to immunologic tumor surveillance reactions triggered by abnormal clones.⁵ Despite progress, these issues remained unresolved due to the rarity of AA, and the long latency of sMN evolution, whose today study is amenable to only retrospective analyses of large cohorts of patients, waiting for the long-term follow-up of the randomized RACE trial in which prospective sampling has been performed.³

In the present study, we have now precisely estimated the incidence, timing and clinical associations of late clonal complications including response to IST. Furthermore, the availability of a large cohort of patients followed for a median of 8.6 years enabled us to characterize the clinical and genetic features of sMN evolution, by compiling the largest cohort of cases so far described.

Our results confirm that older age at diagnosis is an independent risk factor for malignant progression.^{7,32} An interesting implication on this association stems from the correlation between age and CHIP in healthy individuals and the lower incidence of CH in pediatric AA cases, paralleling findings on age-related CH.^{17,40} For instance, AA cases presenting at an older age may already carry somatic age-related mutations, which could be positively selected after multiple rounds of IST because of the inherent increased fitness advantage of oligoclonal over normal HSCs. On the other hand, the differential diagnosis with hypoplastic MDS (without excess blasts) occurring in older age is not trivial, even in expert centers. Whatever the scenario is, this shares mechanistic analogies to therapy-related MN, where pre-existing clones are preferentially selected, and is also reminiscent of anecdotal PNH clones expansion/contraction under specific environmental conditions.^{30,33} In line with previous studies, we found that myeloid hits were present in older patients and

heralded sMN progression.¹¹ Indeed, the acquisition of mutations in *ASXL1* and *RUNX1* genes may provide increased HSC self-renewal capabilities offering a fertile soil for clonal sweeping in the context of an “immunologically stressed” hematopoiesis.^{11,34}

Besides age, poor response to standard IST also was found independently associated with an increased risk for myeloid evolution, typically accompanied by a history of failing multiple lines of treatment.^{7,35} In either case, progression of CHIP, age-related CH acceleration, or Darwinian selection phenomena in the context of AA immune responses may be invoked to explain the relationship of age and IST responses with regards to myeloid evolution. One could even speculate that the same mutation may hold a diverse prognostic significance when occurring in two patients with different ages at disease onset (*e.g.*, 20 vs. 60 years). Besides biological implications, in such a scenario therapeutic choice would be strongly influenced by age, with patients undergoing HSCT possibly protected from the development of sMN because of the complete subversion of BM hematopoiesis (*e.g.*, from oligoclonal in AA to normal, polyclonal after HSCT). This is the reason why sMN is a problem typical of the non-transplant setting, where clonal trajectories are sculpted by the re-expansion of the individual patient’s hematopoiesis after IST (so-called bottleneck effect), which not only increases the number of HSCs replications with a non-negligible BM stress/telomere attrition but generates processes of clonal dominance if mutations are already present (selection of the fittest clone).

Our results show that AA progression to sMN has unique molecular characteristics with the distinction of different subgroups characterized by discrete cytogenetic and molecular features. The high frequency of chromosome 7 abnormalities, *ASXL1*, *RUNX1* and *SETBP1* mutations, and their dynamics with other lesions putatively deriving from AA immune-pressure (*PIGA*, HLA mutations) indicate a peculiar signature of clonal evolution, different from that of other MN subtypes.⁵ It is noteworthy that sMN cases acquiring monosomy 7 or del(7q) have been reported to have a shorter time to progression and a lower tendency towards association with del(5q) or *TP53* disruption as opposed to primary or treatment-related MN.⁶ Indeed, we found that the majority of our cases who progressed to sMN within 5 years from the initial diagnosis carried chromosome 7 alterations. Remarkably, the aforementioned deletion 7 gene signature (*ASXL1*, *RUNX1*, *SETBP1*) has been recently found associated with sMN arising in children with *SAMD9/SAMD9L* syndromes³⁶ and

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in other congenital BM failure syndromes, MN developing post-autologous HSC transplantation or chemotherapy, and recently in the context of expanding hematopoiesis following gene therapy.^{37,38}

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Besides somatic myeloid mutations, other players have been identified as possible determinants of AA and PNH clonal dynamics. We have previously shown that germline HLA configurations imprint different risk of clonal progression (to both secondary PNH and sMN) and survival outcomes.³⁹ In this scenario, immune-privileged lesions or dysregulation of immune pathways may favor the evasion from T cell attack and provide a sustainable balance in the context of CH, cooperating with myeloid genes in intricate ways reported as a “war of clones” “struggling for dominance”.^{18,27,40}

In conclusion, we propose that AA malignant evolution may be the consequence of a relentless autoimmune attack producing a maladaptive response to immunosuppression (**Figure S17**). Our results call for a close monitoring of patients with AA with classical BM smear examination and cytogenetics. Furthermore, information deriving from targeted deep NGS at regular intervals throughout the entire follow-up time must be integrated in the survivorship plan of such patients to allow early interventions. That said, although morphological progression to MDS and occurrence of monosomy 7 are obvious triggers to discuss transplantation, the occurrence (or expansion) of isolated molecular alterations as an indication for transplant is, nowadays, highly debated (except for *TP53* mutations) and must be judiciously interpreted within the context of other variables such as age, donor’s availability and response to IST.

Table 1. Main clinical characteristics of the study cohort.

Variables	Modalities	N=999	AA (N=817)	AA/PNH (N=75)	PNH (N=107)
Gender	Female	502 (50.3)	399 (48.8)	43 (57.3)	60 (56.1)
	Male	497 (49.7)	418 (51.2)	32 (42.7)	47 (43.9)
Age at diagnosis	median [IQR]	34.0 [20.1-54.3]	34 [19.1-56.7]	30.5 [20.5-43.9]	36.1 [27.3-46.9]
PNH clone at diagnosis (N=832)*	no	433 (52)	433 (66.5)	0 (0)	0 (0)
	yes	399 (48)	218 (33.5)	74 (100)	107 (100)
PNH clone (percentage)	median [IQR]	11 [2.4-61.4]	3.3 [1.2-8]	48 [30.8-82.7]	83.5 [61.4-94.5]
Camitta criteria	non-severe		176 (21.5)	48 (64)	
	severe		641 (78.5)	27 (36)	
Karyotype at diagnosis (N=780)*	Normal	725 (93)	598 (91.6)	64 (100)	63 (100)
	Failure	43 (5.5)	43 (6.6)	0 (0)	0 (0)
	Others**	12 (1.5)	12 (1.8)	0 (0)	0 (0)
Cohort	RPH	116 (11.6)	98 (12)	14 (18.7)	4 (3.7)
	CCF	396 (39.6)	336 (41.1)	17 (22.7)	43 (40.2)
	SLS	487 (48.7)	383 (46.9)	44 (58.7)	60 (56.1)

*indicated is the available sample size due to missing data;** including sex chromosome abnormalities and age-related findings not configuring an alternative diagnosis (see also Supplementary Appendix). CCF: Cleveland Clinic Foundation; SLS: Hôpital Saint-Louis; RPH: Ribeirão Preto Hospital; AA: aplastic anemia; PNH: paroxysmal nocturnal hemoglobinuria.

Table 2. Multivariate analysis for severe AA cases (N=504)

Variables		Overall Survival		sMN incidence		sPNH incidence*	
		HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Gender	F	1		1		1	
	M	1.00 (0.69-1.45)	0.99	1.39 (0.82-2.33)	0.22	0.49 (0.27-0.89)	0.02
Response to treatment	CR	1		1		1	
	No CR	4.45 (2.54-7.77)	<0.001	2.60 (1.39-4.84)	0.003	0.76 (0.41-1.41)	0.39
Age (10y effect)		1.29 (1.16-1.43)	<0.001	1.37 (1.20-1.56)	<0.001	0.88 (0.75-1.02)	0.1
Year at transplant (10y effect)		1.35 (0.96-1.88)	0.08	1.14 (0.76-1.72)	0.52	1.47 (0.95-2.28)	0.09
Center effect as frailty			0.015		0.97		0.02

*only SAA. sPNH: secondary paroxysmal nocturnal hemoglobinuria; F: female; M: male; CR: complete response; sMN: secondary myeloid neoplasia; HR: hazard ratio; 95% CI: 95% confidence intervals.

Figure 1. Study design. CONSORT flowchart of patients' inclusion (A) with details of sequenced patients for cross-sectional and longitudinal analysis (B). MN=myeloid neoplasia

Figure 2. Incidence and study of factors influencing malignant progression. Panel A shows the cumulative incidence of malignant progression according to disease phenotypes. At 10 years from diagnosis the risk of progression to a secondary myeloid neoplasms was 12.8% (10.0 - 15.9) for AA, 13.1% (6.0 - 22.9) for AA/PNH overlap syndrome, and 3.4% (0.9 - 9.0) for PNH, respectively ($p=0.13$ globally; $p=0.47$ in AA vs. AA/PNH; $p=0.06$ in AA vs. PNH and AA/PNH vs. PNH by Gray's test). According to disease severity, the 10-year cumulative incidence of malignant progression was 12.4% (9.4 - 15.8) for severe and 13.6% (8.6 - 19.6) for non-severe cases ($p=0.86$ by Gray's test, panel B). Panels C and D show cumulative incidence curves for myeloid progression according to treatment response and age at diagnosis. At 10 years the cumulative incidence of progression was 15.7% (11.5 - 20.6) in patients with partial or no response to treatment vs. 8.5% (4.4 - 14.2) in cases achieving complete response ($p=0.02$). At 10-year the cumulative incidence of progression was 6.6% (3.7 - 10.8) in younger cases (aged ≤ 35) vs. 20.0% (14.3 - 26.2) in older patients ($p<0.001$). Numbers at risk are color-coded and indicated below each curve.

Figure 3. Features of malignant progression. Panel A shows the 5-year overall survival according to bone marrow blast percentage at sMN onset (58.2% [41.1 - 71.9] in patients with $<5\%$ vs. 20.2% [8.6 - 35.3] in those with $\geq 5\%$). Forrest plot in panel B illustrates multivariate analysis for overall survival using a multivariate Cox-cause specific model. In panel C, a waterfall plot depicts the genomic landscape of progressors (N=82 patients with available molecular information). Disease subtype, cytogenetic risk groups according to R-IPSS, R-IPSS risk group (higher >3.5 ; lower ≤ 3.5), and age categories (aged $<60/\geq 60$ years) are color-coded on top while on the right side frequencies of mutations for each gene are shown. Panel D shows the survival curves of main cytogenetic risk groups (numbers at risk for each subgroup are color-coded and provided below the curves). In panel E, a circle plot illustrates the relationship between mutations and cytogenetics. Three cases failed the cytogenetic testing and thereby were removed from the panels including cytogenetics features.

Figure 4. Clonal dynamics in aplastic anemia. In [panel A](#), the pie chart shows the frequency of mutants for *PIGA* and myeloid mutations with a breakdown of cases according to characteristics at onset and treatment response (n=302). Bar charts in [panel B](#) illustrate the difference of molecular spectra between baseline and myeloid progression with frequencies estimated on the total of mutants (n=302 at baseline; n=82 at evolution). [Panel C](#) shows the dynamics of changes in molecular lesions (HLA, *PIGA*, myeloid) in patients sequenced at baseline (D=diagnosis), follow-up (F) and evolution (E, see Figure S13 for details). Frequencies are shown as solid lines on the left, clonal burdens (median VAFs at each time point) are shown as dashed lines on the right with each color representing a different set of mutations. [In panel D](#), a stratified Cox-proportional hazard function adjusting for age was fit to estimate baseline progression hazard rates and generate progression-free survival (PFS) curves of patients with AA and AA/PNH (N=264). Patients were classified based on features (myeloid driver mutations only=Group 1; *PIGA*/PNH clone presence and *BCOR/L1*=Group 2, no mutation=Group 3) previously hypothesized to be associated with myeloid evolution (see also Supplementary Appendix; numbers at risk are indicated below the curves). Particularly, N=7 patients (1 evolved to sMN) with concomitant *PIGA*/PNH clone and myeloid mutations were considered as belonging to Group 2. The bar chart on the lower right illustrates the difference in age at onset in an analysis of patients with pure AA phenotype (n=58).

DATA SHARING STATEMENT

The authors have included all clinical and genetic data in the main text of the article and Supplementary Appendix. Requests for additional information should be sent to the corresponding author.

AUTHOR CONTRIBUTIONS

C.G. supervised the study, participated in patient recruitment, performed molecular analysis, collected, analyzed, interpreted clinical and molecular data and wrote the manuscript; S.P. participated in patient recruitment, collected, analyzed, interpreted clinical and molecular data and edited the manuscript; P.H.P. participated in patient recruitment, collected, and interpreted clinical and molecular data and edited the manuscript; J.E.G. performed statistical analysis on clinical data; L.F.B.C., L.L., M.C.B.I., F.S.deF., B.J.P., M.S., N.D. participated in patient recruitment, collected clinical and molecular data; A.L.P., V.A., L.H., E.C., S. C-Z. performed molecular analyses, A.D. analyzed molecular data; M.T.V. interpreted clinical and molecular data, gave helpful intellectual insights and edited the manuscript; V.V. interpreted molecular data, gave helpful intellectual insights and edited the manuscript; R.PdeL., J.S., R.T.C. and G.S. participated in patient recruitment and in the study design, provided clinical and molecular data, edited the manuscript and gave helpful intellectual insights; J.P.M. designed the study, conceptualized and sponsored the overall project, and wrote the manuscript. All authors read and approved the final version of the manuscript and are accountable for all aspects of the work.

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Figure 1

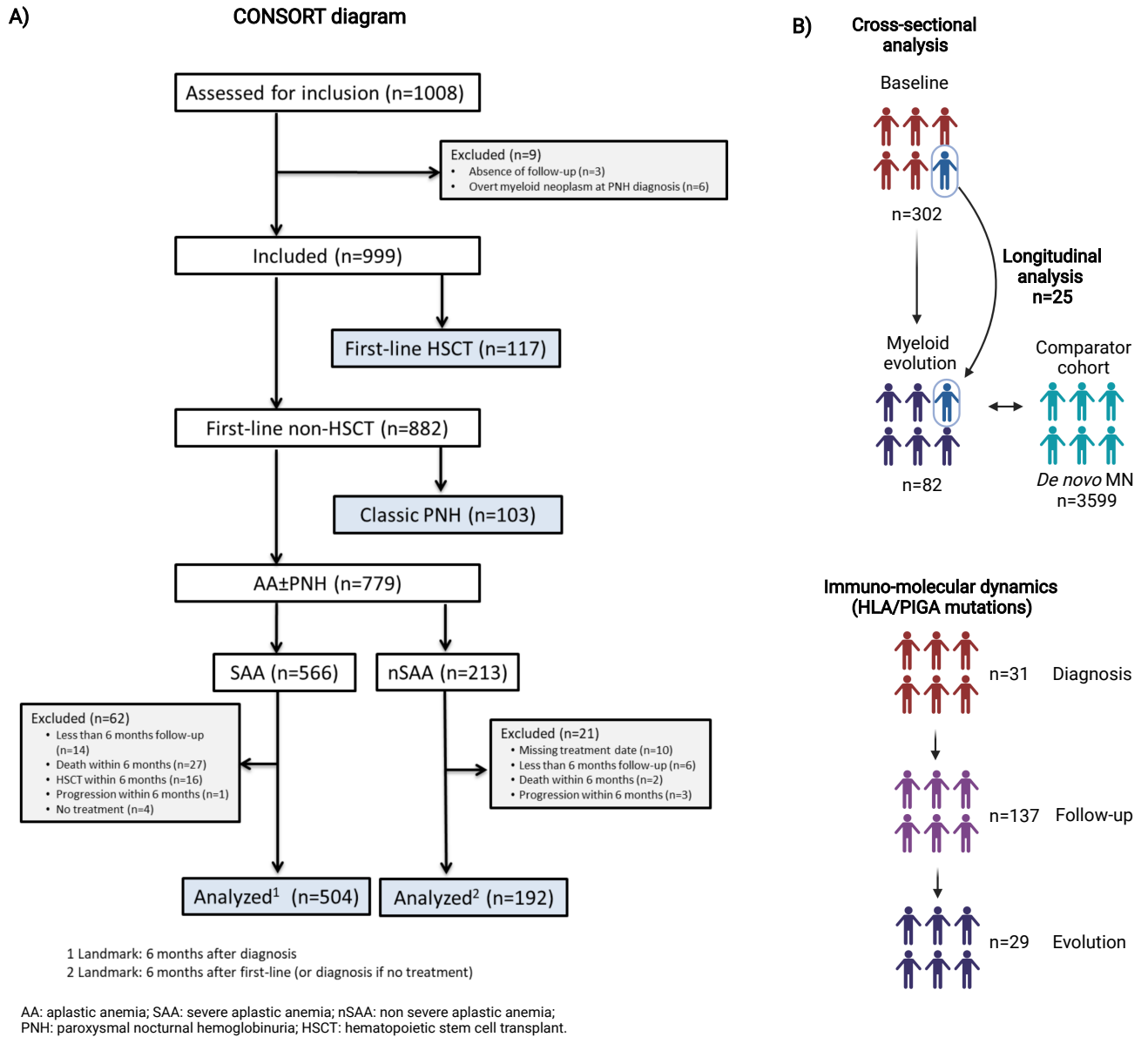
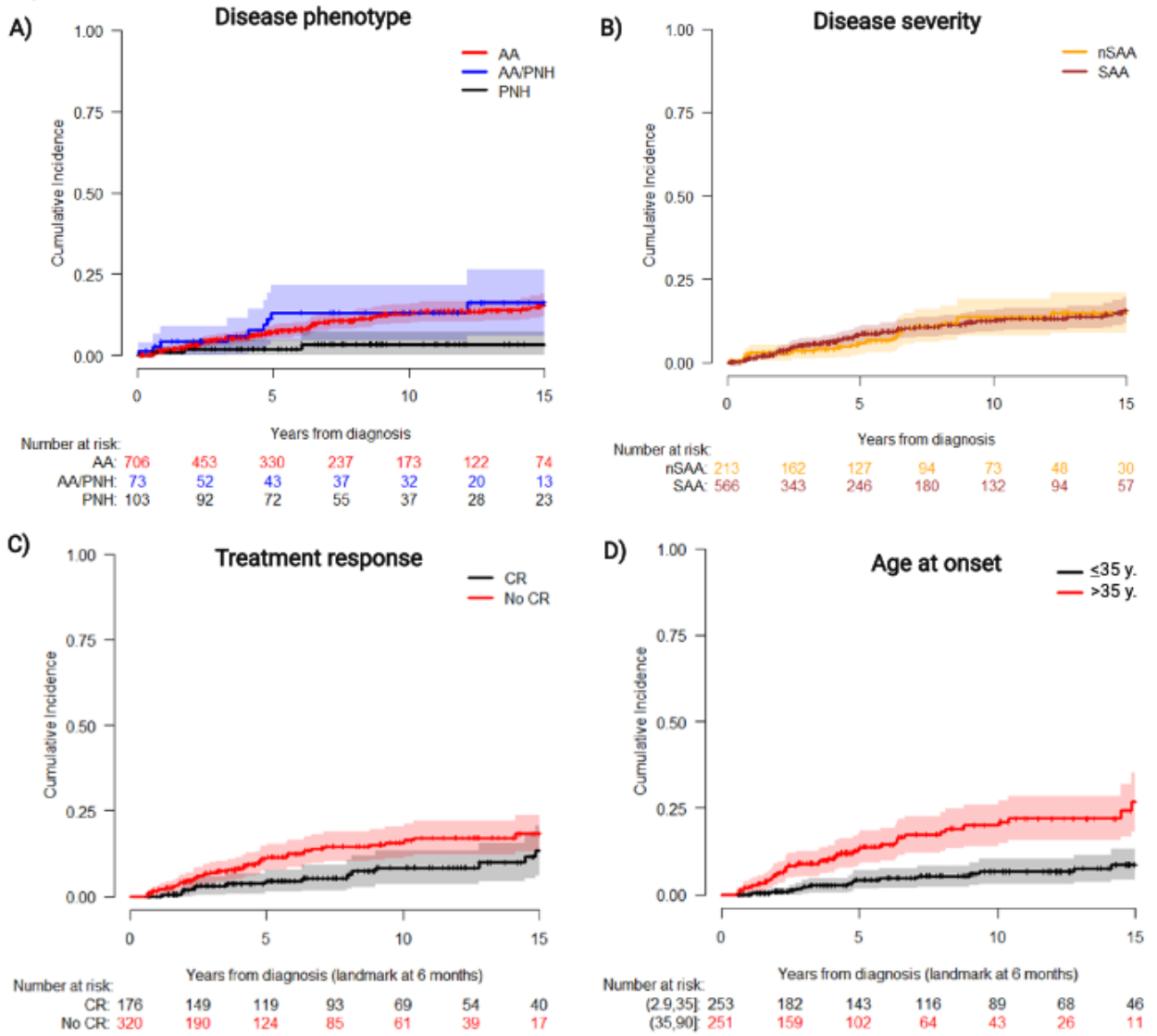


Figure 2



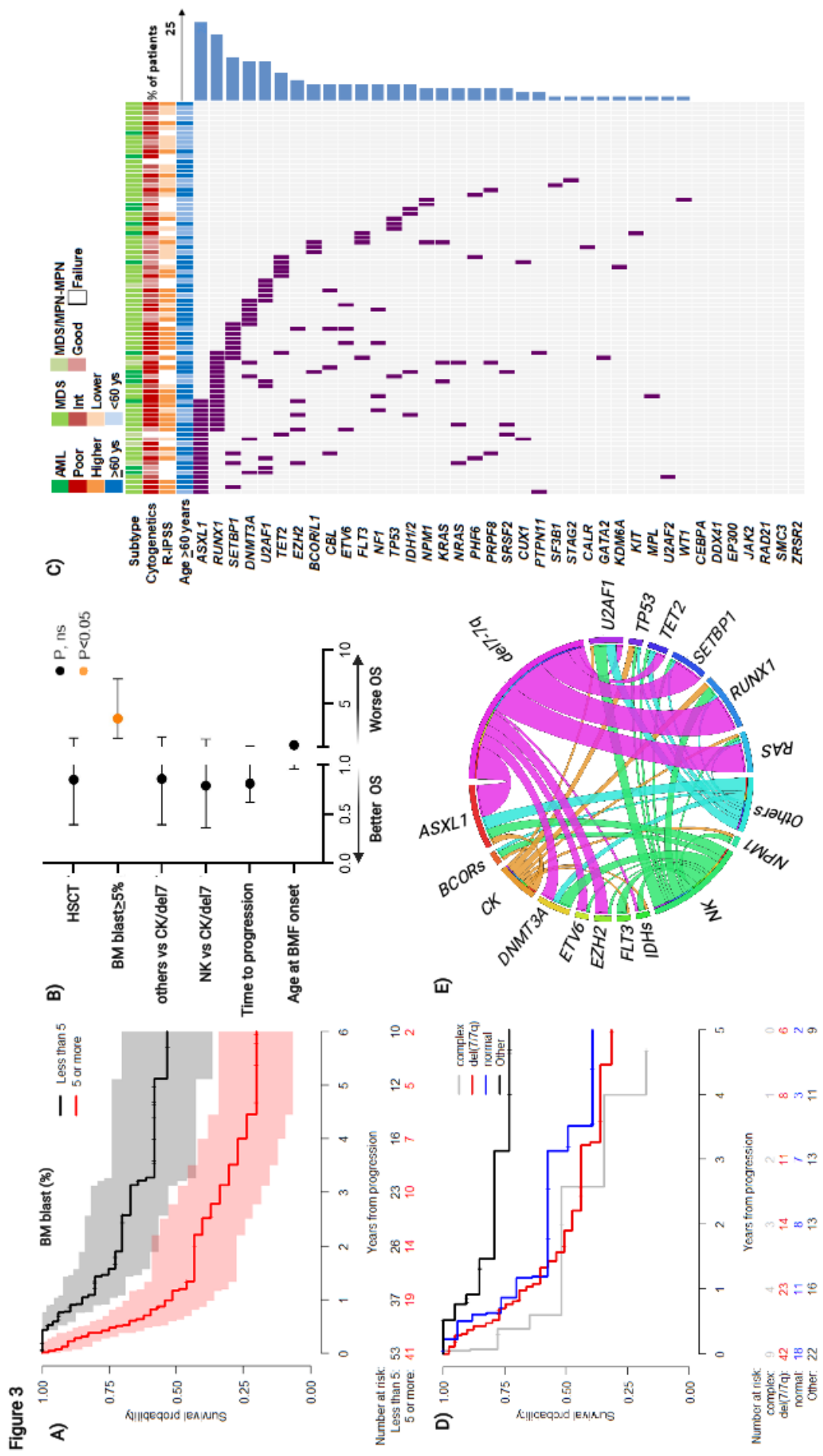
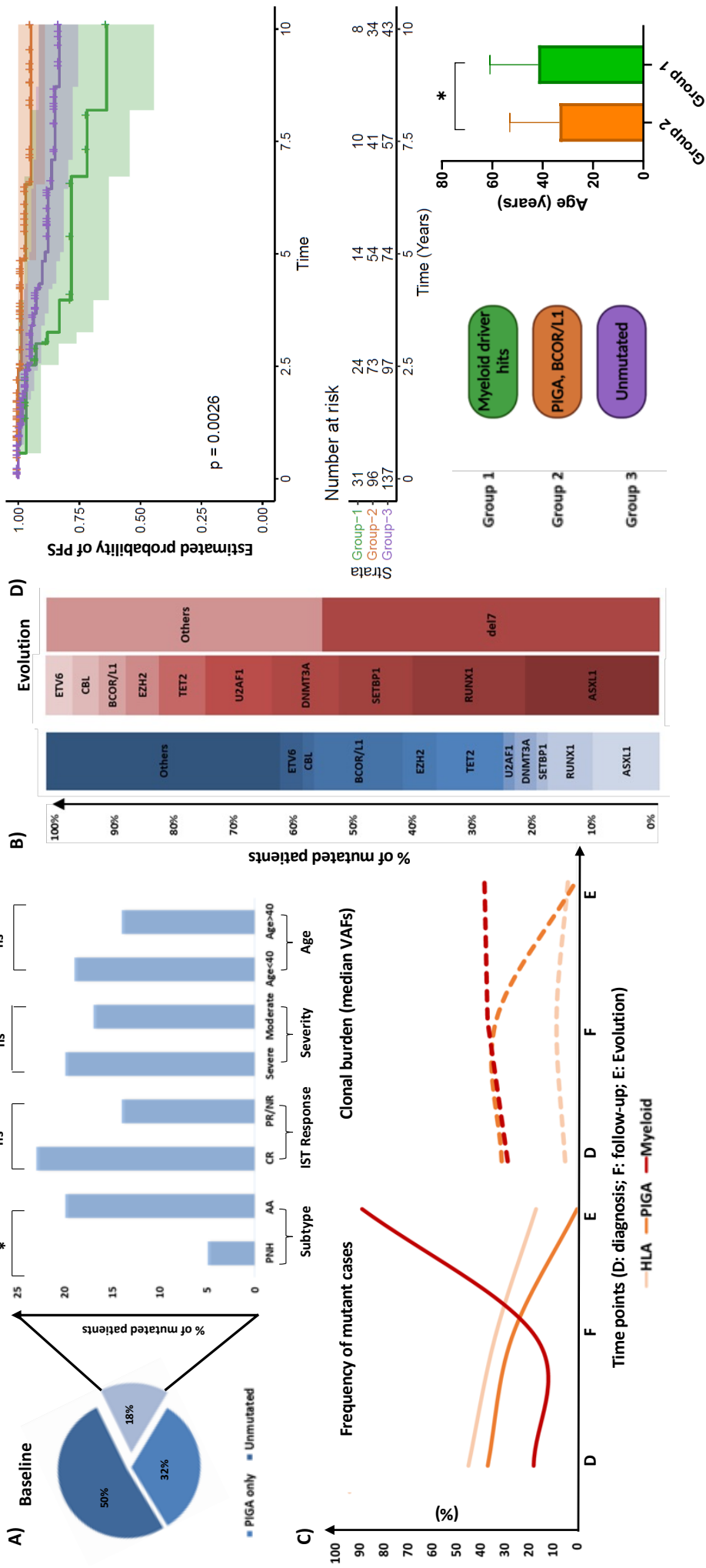


Figure 4



Supplementary Appendix

**Clinical and Molecular Determinants of Clonal Evolution in
Aplastic Anemia and Paroxysmal Nocturnal Hemoglobinuria**

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Study design, objectives, and definitions

1. The research question: PICOT format

In aplastic anemia (AA)/paroxysmal nocturnal hemoglobinuria (PNH) patients, how do baseline clinical, cytogenetics, and molecular characteristics influence the risk of secondary myeloid neoplasm?

P: acquired AA or PNH followed at The Cleveland Clinic Foundation (Cleveland, Ohio, USA), Hospital Saint-Louis (Paris, France), and Ribeirão Preto University Hospital (São Paulo, Brazil) between 1972 and 2020

I: baseline characteristics as risk-factors to secondary myeloid neoplasm and clinico-genomic features of progressors

C: exposed versus non-exposed

O: diagnosis of myeloid neoplasm (WHO 2016) after idiopathic AA or PNH

T: follow-up from landmark to the last visit, defined as six months after AA diagnosis for severe or untreated patients or six months after first-line treatment for non-severe cases (Events: death, hematopoietic stem cell transplant, HSCT)

2. Inclusion/exclusion criteria

- Clinical diagnosis of acquired AA or PNH (Ham Dacie's or flow cytometry PNH clone $\geq 1\%$)
- Follow-up in one of the three participating centers
- No age limits
- Available clinical data

Exclusion criteria:

- Known personal or family history of (decided *a priori*):
 - o Inherited bone marrow failure syndrome.
 - o Myeloid neoplasm.
 - o Idiopathic pulmonary fibrosis.
 - o Cryptogenic hepatic cirrhosis.
 - o Primary immunodeficiency.
- Patients initially diagnosed with acquired AA but who were later re-classified as inherited bone marrow failure syndrome
- Clinical suspicion of inherited bone marrow failure syndrome

3. Objectives

3.1 Primary endpoint and main objective:

Cumulative incidence of secondary myeloid neoplasm and its characteristics (competing events: death and HSCT)

3.2 Secondary objectives:

- Overall survival: time from diagnosis to last follow-up or death
- Secondary PNH incidence: time from AA diagnosis to occurrence of secondary PNH (competing events: death and HSCT)
- Risk-factors for secondary myeloid neoplasm or PNH
- Characterization of the genomic landscape of patients with evolution, focusing on genomic lesions known to have a role in myeloid neoplasms and bone marrow failure syndromes
- Comparison of mutational patterns observed in secondary myeloid neoplasms with the distribution of genetic lesions seen in primary patients with myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML), focusing on chromosome 7 abnormalities

4. Definitions:

AA diagnosis was established according to standard guidelines¹ and severity defined according to Camitta criteria.^{2,3}

Myeloid neoplasm diagnosis was defined according to the 2016 revision to the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemias and, when not already indicated as such, reclassified accordingly after abstraction of data from medical records.⁴

Baseline PNH: positive Ham Dacie's test (historical cases) or flow cytometry PNH clone $\geq 1\%$.

Classical hemolytic PNH: PNH granulocyte clone size $>20\%$ and lactate dehydrogenase >2.5 upper limit of normal.⁵

AA/PNH overlap syndrome: intravascular hemolysis in the presence of a PNH granulocyte clone size $>20\%$ and/or positive Ham Dacie's test and at least two cytopenias.⁶

Secondary PNH: new onset of intravascular hemolysis in the presence of a PNH granulocyte clone size $>20\%$ and/or positive Ham Dacie's test during the follow-up of a patient with AA and no previous clinical evidence of intravascular hemolysis.

Landmark time was defined as six months after AA diagnosis for severe or untreated patients, or six months after first-line treatment for non-severe patients. For the landmark analysis on the cumulative incidence of secondary myeloid neoplasia, all patients with follow-up inferior to 6 months from diagnosis (dead or alive), event (myeloid progression), or competing events (HSCT or death) were excluded.

Complete response was defined as recovery of hemoglobin levels >10 g/dL, absolute neutrophil count $>1,000/$ mm³, and platelet count $>100,000/$ mm³, as previously established.^{7,8}

Partial response was defined as the achievement of transfusion independence with no longer meeting the criteria for severe disease (see above).⁸

No response was defined as patients not meeting criteria for complete nor partial response and requiring transfusion support.

R-IPSS risk groups (higher >3.5; lower \leq 3.5) were defined according to Pfeilstöcker et al.⁹

Patients

We retrospectively analyzed all consecutive patients with a diagnosis of AA and/or PNH at The Cleveland Clinic Foundation (Cleveland, Ohio, USA), Hospital Saint-Louis (Paris, France) and Ribeirão Preto University Hospital (São Paulo, Brazil) between 1972 and 2020. Written informed consent was obtained from each participant. The review of medical records was approved by the internal Institutional Review Boards of each center involved in this study in agreement with the Declaration of Helsinki (IRB 5024 for Cleveland Clinic; IRB 17222419.0.1001.5400 for Ribeirão Preto University Hospital; IRB CD/EB 19-064 for Hospital Saint-Louis). All patients had been regularly followed until November 2020 (or death). Pertinent clinical data including age, gender, disease diagnosis, treatments, cytogenetics, diagnosis at evolution and other clinical complications were collected. PNH clone was defined according to the percentage of GPI-deficient granulocytes detected by flow cytometry as previously described (see below).^{10,11} Specimens were obtained in accordance with ethical committee approvals of participating institutions. In total, our study cohort included 1008 patients with a diagnosis of AA, classical hemolytic PNH and AA/PNH overlap syndrome as per previously published criteria and definitions described above.⁶

Flow Cytometry

Cells were acquired on a XL-MCL (Beckman Coulter) or FC500 cytometer. A 5-color cocktail was used to detect and quantify PNH granulocytes clone size as per established guidelines.^{5,10,12}

Conventional Cytogenetics

G-banded metaphase cytogenetic analysis using standard techniques was performed on bone marrow (BM) aspirates with a median number of metaphases analyzed of 20. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature.¹³

Genomic studies

Myeloid panel. When possible, patients were routinely tested for somatic mutations at diagnosis, when clinically indicated and/or to rule out the possibility of myeloid evolution using the diagnostic panel currently available at the participating institutions. When available, samples from our biorepository were also used to retrospectively study longitudinal clonal dynamics. Briefly, DNA was extracted from peripheral blood or BM specimens. Sequencing libraries were generated according to a paired-end library protocol (Illumina or Agilent Technologies) and sequenced on an Illumina instrument (San Diego, CA) with paired end, and 150x2 cycle reads. A panel of 40 genes (see **Table S1**) was interrogated. Reads were aligned to the reference human genome (Genomic Build GRCh37/hg19). Variants were annotated using Annovar¹⁴ and had a minimum variant allelic frequency (VAF) of 2%, an average of >500X coverage,

and >98% of targeted regions showed over 100X coverage. Variants were classified according to established guidelines¹⁵, and internal bio-analytic pipelines to identify somatic mutations using sequences derived from controls and mutational databases such as dbSNP138, 1000 Genomes or ESP 6500 database, and Exome Aggregation Consortium (ExAC).¹⁶⁻²¹ Furthermore, in silico predictions scores were used to assess potential pathogenicity and clinical relevance according to VarSome classifiers (<https://varsome.com/>). Variants were retained when a strong and/or potential clinical relevance was ascertained after sorting for potential artifacts and according to general population frequencies, previous literature reports, pathogenicity prediction and availability of serial samples. Approximately 4% of the variants detected for which controversial clinical evidence exist (Extended Data) were included based on clinical and genomic criteria, given the rarity of sequencing data in AA/PNH, the inherent differences as compared to classical myeloid neoplasm and the fact that their presence/absence did not affect the results of our analyses.

Deep Targeted PIGA Sequencing. Genomic DNA was amplified with primers designed to cover all exons of the *PIGA* gene and sequenced on an Illumina MiSeq as previously shown for patients enrolled at The Cleveland Clinic.^{5,22}

HLA sequencing and analysis. The TruSight HLA v2 (Illumina, San Diego CA) was utilized to sequence 11 HLA loci (Class I HLA-A, B, and C; Class II HLA-DRB1/3/4/5, HLA-DQA1, HLA-DQB1, HLA-DPA1, and HLA-DPB1) according to the manufacturer's instructions. The DNA libraries were then loaded directly onto a MiSeq system for sequencing. (**Figure S1**) For 10 samples at evolution from the French cohort, the NGSgo®-AmpX (GenDx) was used.

As previously described^{23,24}, after obtaining a full 8-digit typing by means of NovoHLA (Novocraft Technologies, Malaysia), paired-end reads were directly aligned on a per-patient HLA reference with selected alignment options favoring intermediate clipping, high mismatch and gap penalty, multi alignment, and high seed length. After sorting, marking duplicates and indexing procedures, variant calling was performed using VarScan in tumor-only mode. High-quality calls (present in both read mates) with a coverage >20 reads (for the reference base) and 4 reads (for the alternative allele) were prioritized. Non-polymorphic calls were retained after filtering by means of a customized java-based filter, exploiting the multi-alignment files provided by the HLA-IPD/IMGT database.²⁵ Both coding and non-coding variants were topographically and functionally annotated. Only pathogenic or likely pathogenic variants were retained for further analysis.

Unbalanced allelic HLA loss was imputed from sequencing data exploiting read cover ratio as previously described.²⁶ The following formula (where C_i and C_z represent the read coverage for each homologous allele) shows the method used to impute allelic loss:

$$\text{Log}_2 \frac{C_i}{(\sum C_i, C_z)/2}$$

An adjustment for structurally similar alleles was included using the sequence variation defined as "Variant coverage", which is provided by the NovoHLA pipeline. Based on a previous internal validation study on 234 healthy controls, Log_2 ratios <-1.5 were retained as confident allelic loss.

Statistical analysis

Statistical analysis was performed by a third-party statistician (JE) from the European Blood and Marrow Transplant (EBMT) Group, who was the only person able to access and handle de-identified clinical data of the merged cohort, within the respect of the European General Data Protection Regulation (GDPR, 2017/679). As a first step, the three cohorts were tested as to potential center-related differences in terms of rate and timing of secondary myeloid neoplasm (sMN) progression (main outcome), overall survival (OS) and secondary PNH evolution. While no center-related effects were observed for the main outcome (sMN evolution) and secondary PNH evolution, a significant difference was found for OS. In order to take into account the center effect, we introduced a random effect or frailty for each center into the multivariate models (see below).

Quantitative variables were expressed as median, interquartile range, minimum and maximum. Qualitative variables were expressed as numbers and percentages. For all relevant comparisons, comparative analyses between two groups have been performed by two-sided Wilcoxon test for quantitative variables. Fisher test or Chi-square were used for testing independence between groups, for qualitative variables.

OS was defined as the time from diagnosis to last follow-up or death for any cause, and was calculated using Kaplan–Meier estimation.²⁷ Cumulative incidences of sMN and secondary PNH were defined as the time from diagnosis to the acquisition of cytogenetic alterations/evidence of bone marrow dysplasia as per WHO 2016 diagnostic criteria⁴ and new-onset of intravascular hemolysis (see definitions), respectively, and were calculated in a competing risk setting, with death and non-first line HSCT as competing events. Median follow-up was calculated using the reverse Kaplan–Meier estimation and the 95% confidence interval.

Non-adjusted effect of baseline variables was evaluated using Log-Rank and Gray's tests, respectively for OS and cumulative incidence outcome.²⁸ Multivariate impact of baseline variables was evaluated using a multivariate Cox-cause specific model. The risk proportionality of the Cox regression models was tested and the assumption was not violated (i.e., no global test was significant).²⁹ The potential center effect was taken into account in the multivariate Cox models using a random effect term (method also known as frailty).³⁰

The impact of variables was evaluated on AA and separately between non-severe and severe cases. To evaluate the treatment response, landmark analyses were performed at 6 months after the diagnosis and treatment initiation for severe and non-severe AA, respectively. A landmark time of 6 months after diagnosis was used for non-treated cases. Moreover, to account for a possible time period-related effect, we adjusted our analysis based on the year of diagnosis.

Considering all the included patients with available information, a Cox-proportional hazard function adjusting for age was fit to estimate baseline sMN progression hazard rates and generate progression-free survival (PFS) curves. Cases were classified based on features (*PIGA*/PNH clone presence and *BCOR/L1*, no mutation, and myeloid mutations only – see list of our myeloid gene panel in Table 1) previously hypothesized to be associated with sMN evolution.

In a second part, the OS and impact of variables on OS of the patients with sMN were estimated from the time of sMN onset to death or last follow-up. The impact of consecutive HSCT post progression on OS was estimated as time-varying covariate in the Cox multivariate model. Also in this case, a random effect or frailty for each center was introduced.

Finally, a 1:1 matched-pair analysis based on nearest neighbor propensity score matching (PSM) was used to compare post-AA MN cases of this study and matches from a cohort of *de novo* myeloid neoplasm (n=3599) using age, gender, bone marrow blast percentage and disease type as relevant baseline covariates.

All statistical tests were two-sided, and a p-value < 0.05 was considered statistically significant. All analyses and data visualization were generated using the statistical computing environment R (4.0.0 R Core Team, R Foundation for Statistical Computing, Vienna, Austria), Excel Microsoft Office 365 and GraphPad Prism (8.4.0).

Supplementary Figures

Figure S1. Bio-informatic pipeline for HLA mutation detection. Details are provided above.

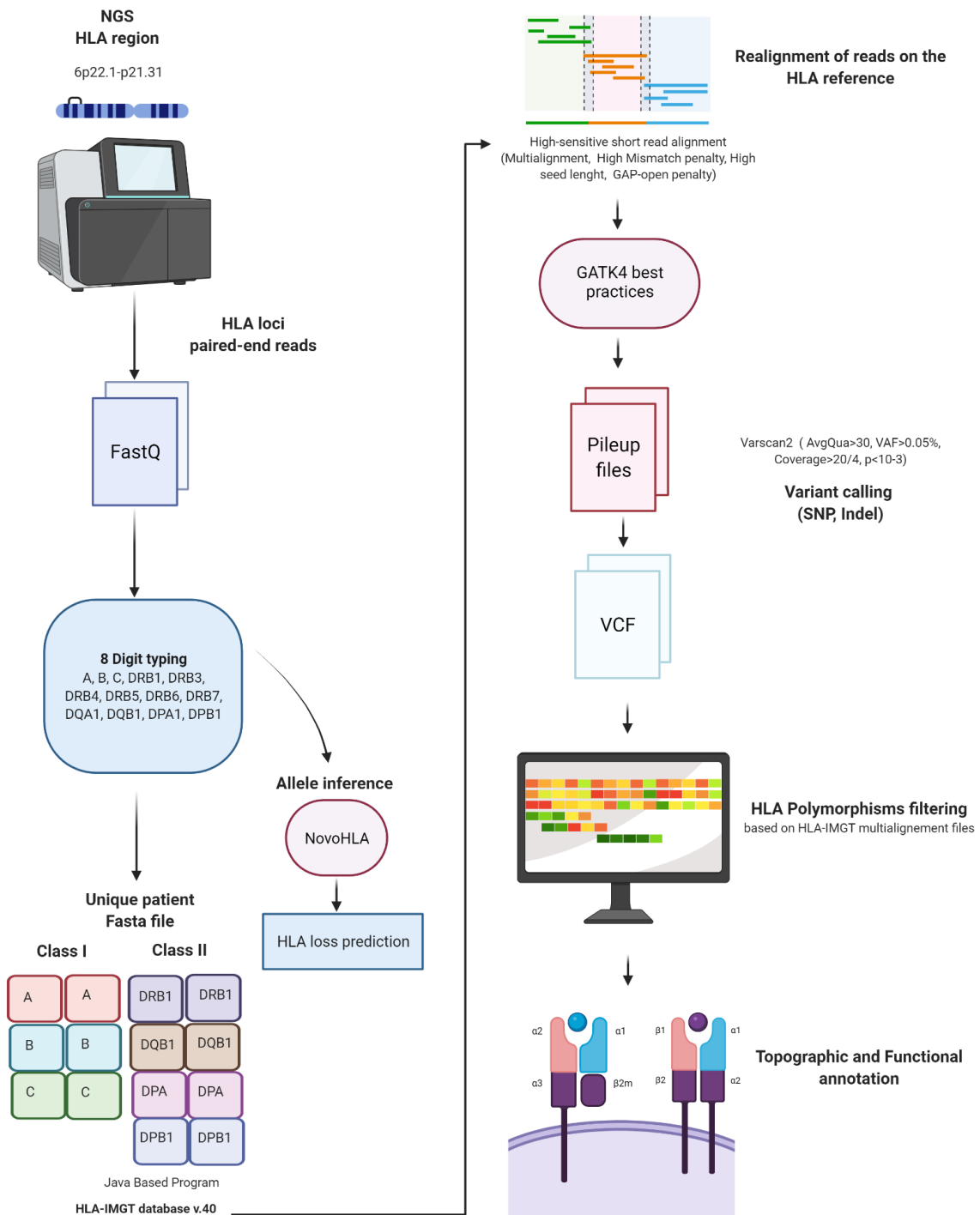
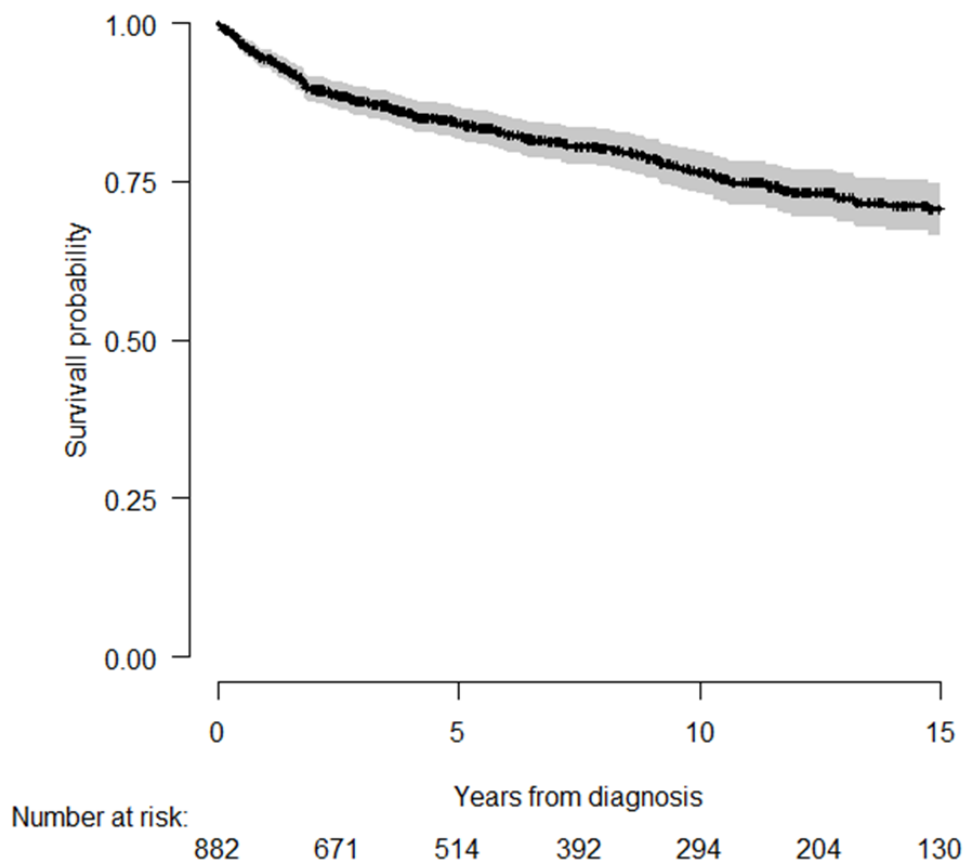
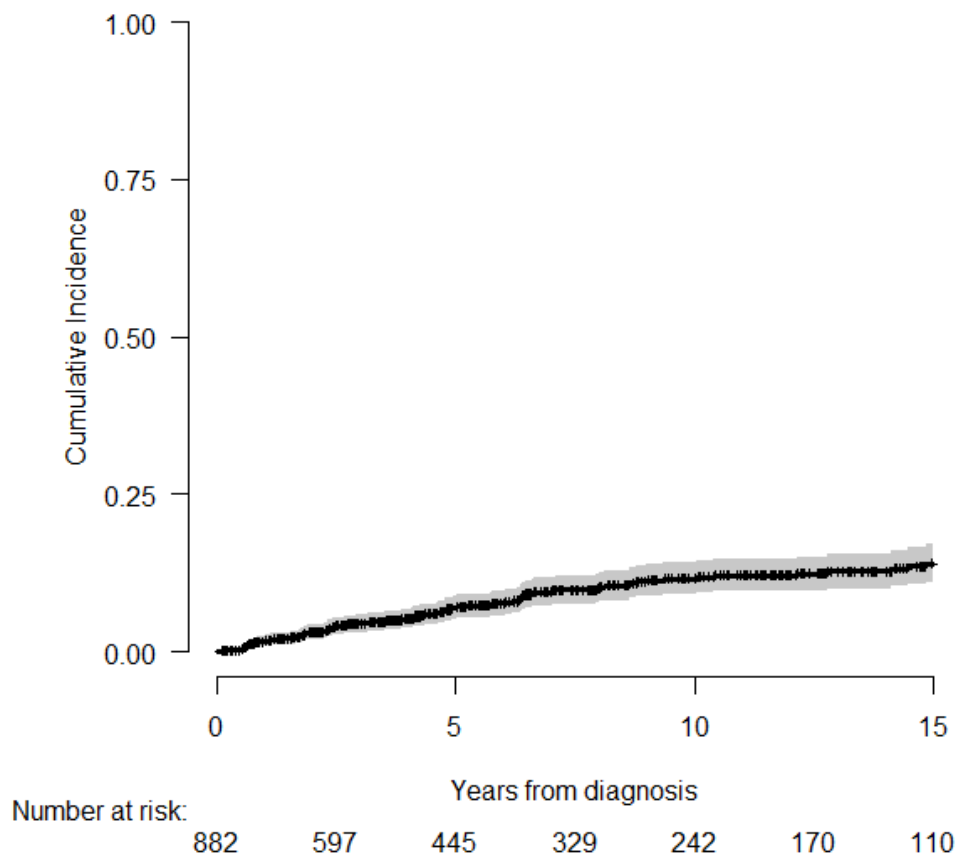


Figure S2. Overall Survival in patients non-transplanted upfront



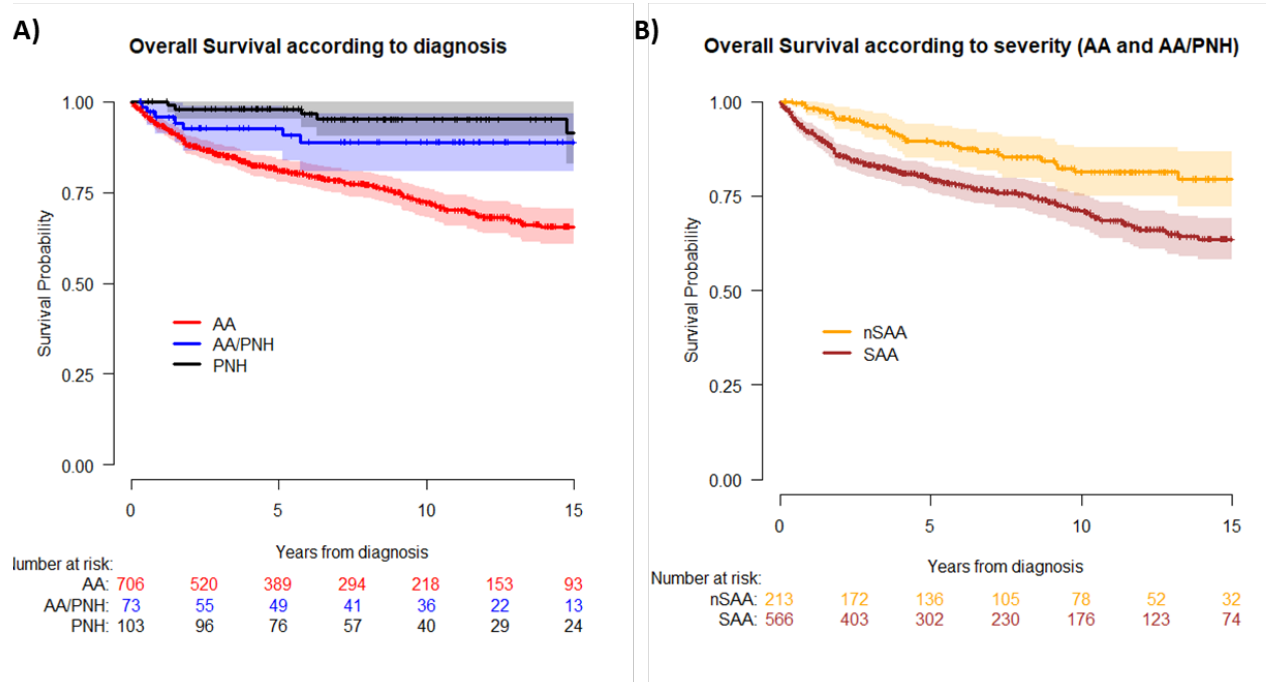
Kaplan-Meier curve shows overall survival (OS) defined as the time from diagnosis of bone marrow failure syndrome (AA, AA/PNH, PNH) to death for any cause. Censoring was applied to cases alive at the last follow-up. With a median follow-up time of 8.6 years (7.8 - 9.2), the 5-year OS was 84.1% (81.3 - 86.5) and 76.4% (72.9 - 79.6) at 10 years. Numbers at risk are indicated below the curve.

Figure S3. Cumulative incidence of malignant evolution in patients non-transplanted upfront



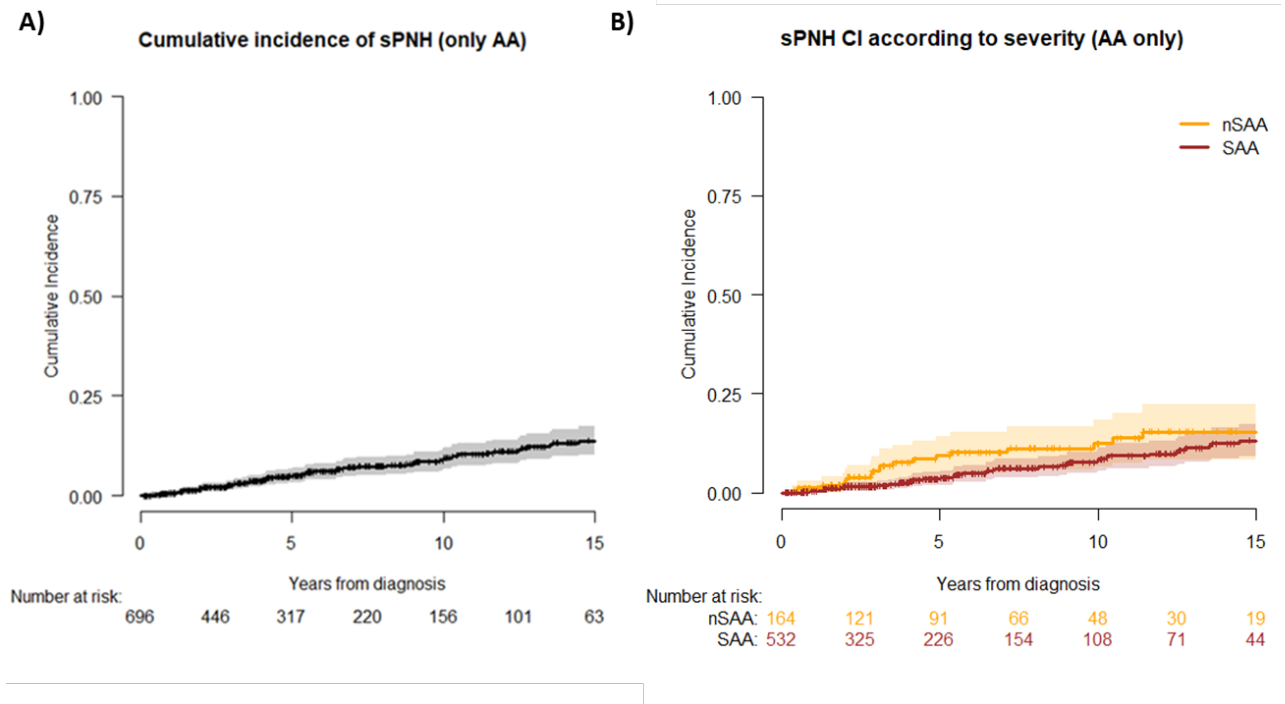
The cumulative incidence of secondary myeloid neoplasm (sMN) evolution was defined as the time from bone marrow failure diagnosis to the acquisition of cytogenetic alterations/evidence of bone marrow dysplasia as per WHO 2016 diagnostic criteria (see above definitions). The 5-year cumulative incidence of sMN was 7.0% (5.4 - 9.0) while at 10 years 11.6% (9.3 - 14.2) of patients progressed to sMN. Numbers at risk are indicated below the curve.

Figure S4. Overall Survival in patients non-transplanted upfront according to disease phenotype (A) and severity (B).



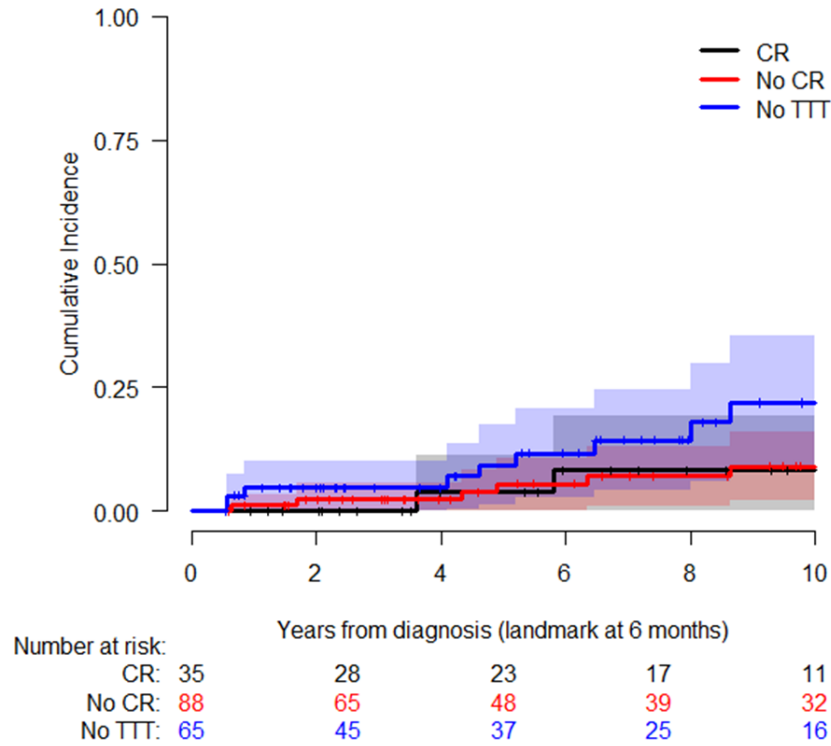
Kaplan-Meier curves show the probability of survival according to disease phenotype ([panel A](#)) and severity in AA and AA/PNH cases ([panel B](#)). Numbers at risk are indicated below the curves and color-coded. In [panel A](#), OS at 10 years was 72.1% (67.9 - 75.9) for AA, 88.8% (77.8 - 94.5) for AA/PNH and 95.2% (87.5 - 98.2) for PNH cases ($p < 0.001$, Log-Rank test). Pairwise comparisons: AA vs. AA/PNH $p = 0.002$; AA/PNH vs. PNH $p = 0.99$; AA vs. PNH $p < 0.001$, Log-Rank test). In [panel B](#), OS at 10 years was 71.0% (62.2 - 75.2) for severe and 81.3% (73.9 - 86.8) for non-severe cases, respectively ($p < 0.001$, Log-Rank test).

Figure S5A-B. Cumulative incidence of secondary PNH evolution in patients non-transplanted upfront



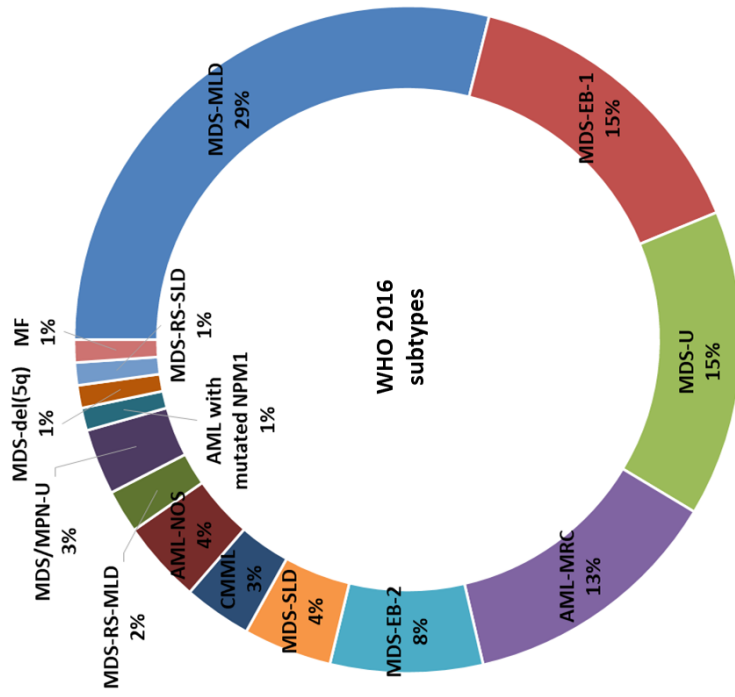
Cumulative incidence of secondary PNH (sPNH) evolution in AA cases overall (panel A) and according to disease severity (panel B). Numbers at risk are indicated below each curve and color-coded when appropriate. The 5-year cumulative incidence of sPNH was 4.9% (3.3 - 6.8) while at 10 years, 9.1% (6.7- 11.9). According to disease severity, the 10-year cumulative incidence of sPNH was 9.0% (6.3 - 12.3) in severe and 15.0% (9.7 - 21.6) in non-severe cases (p=0.12, Gray’s test).

Figure S6. Cumulative incidence of malignant evolution in non-severe AA cases according to treatment and treatment response



Cumulative incidence of secondary myeloid neoplasm (sMN) evolution in non-severe cases according to treatment and response. CR: complete response; no TTT: no treatment received. At 10 years the risk of sMN evolution was 8.3% (1.4 - 23.7) in patients achieving CR, 8.9% (3.5 - 17.4) in non-responders and 21.9% (10 - 36.7) in untreated cases ($p=0.04$; Log-Rank and Gray's tests). Numbers at risk are indicated below the curves and color-coded.

Figure S7. WHO 2016 subtypes of secondary MN cases.

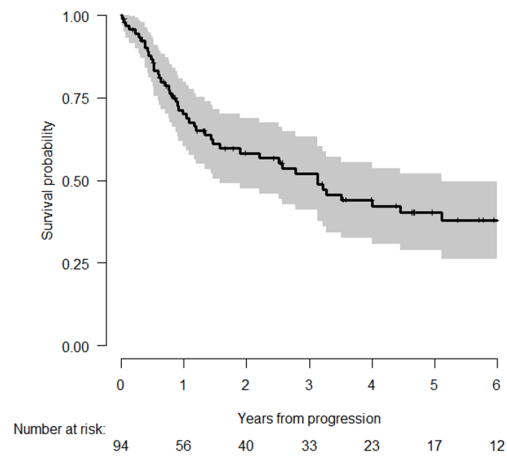


The circle diagram showcases the diagnoses classification⁴. The portions of the circle

of the 94 sMN cases according to the WHO 2016 reflect the frequencies of each entity.

MDS: myelodysplastic syndrome; AML: acute myeloid leukemia; WHO: World Health Organization; MDS-MLD: myelodysplastic syndrome with multilineage dysplasia; MDS-RS-MILD: myelodysplastic syndrome with ringed sideroblasts and multilineage dysplasia; MDS-SLD: myelodysplastic syndrome with single lineage dysplasia; MDS-RS-SLD: myelodysplastic syndrome with ringed sideroblasts and single lineage dysplasia; MDS-EB-1/2: myelodysplastic syndrome with excess blasts-1/2; AML-MRC: acute myeloid leukemia with myelodysplasia-related changes; MDS-U: myelodysplastic syndrome-unclassifiable; AML-NOS: AML not otherwise specified; CMML: chronic myelomonocytic leukemia; MDS/MPN-U: myelodysplastic/myeloproliferative neoplasms-unclassifiable; MF: primary myelofibrosis.

Figure S8. Overall Survival of evolved patients from sMN onset



Kaplan-Meier curve shows overall survival (OS) defined as the time from sMN evolution to death for any cause. Censoring was applied to cases alive at the last follow-up.

With a median follow-up time of 4.7 years (3.6 - 6.3), the 5-year OS was 40.3% (28.8 - 51.6). Numbers at risk are indicated below the curve.

Figure S9. Cytogenetics at evolution and time elapsed from initial bone marrow failure diagnosis

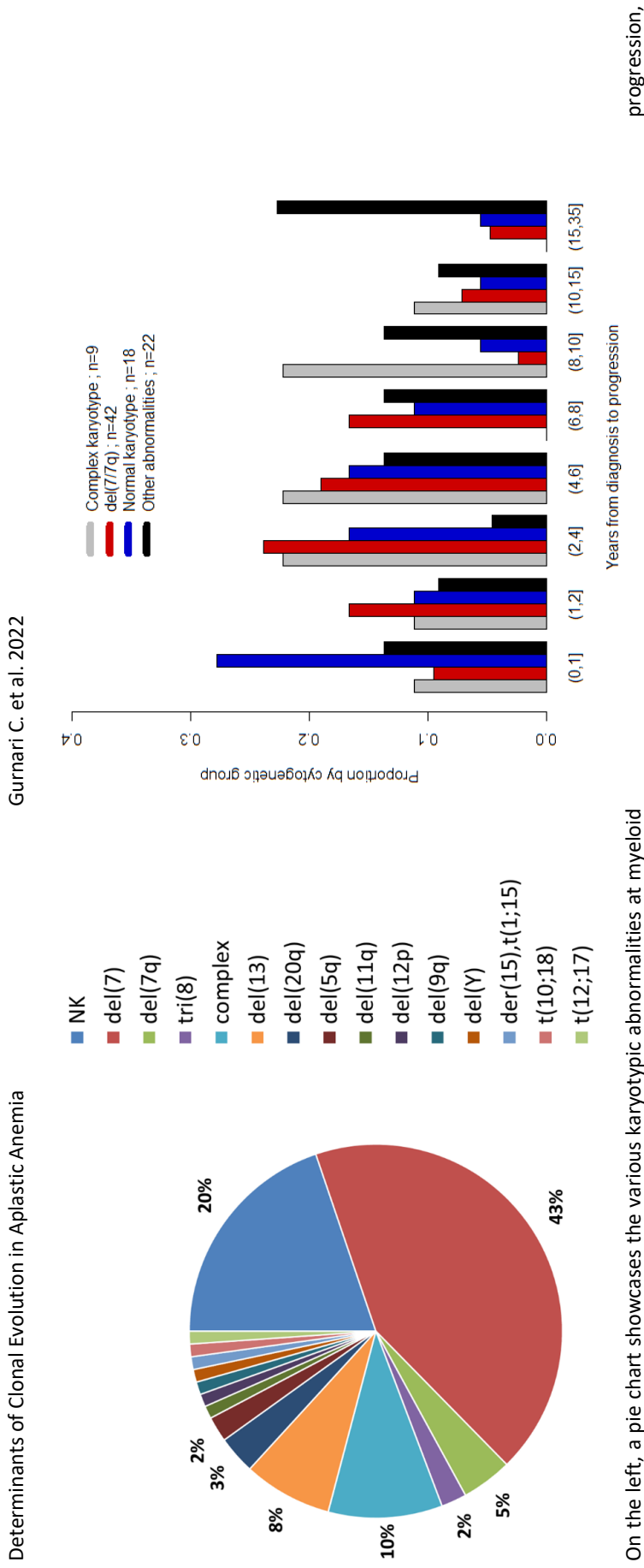
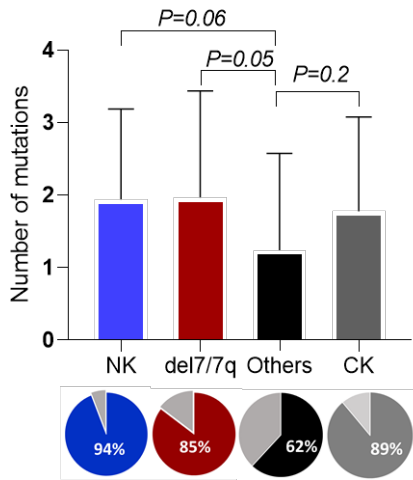
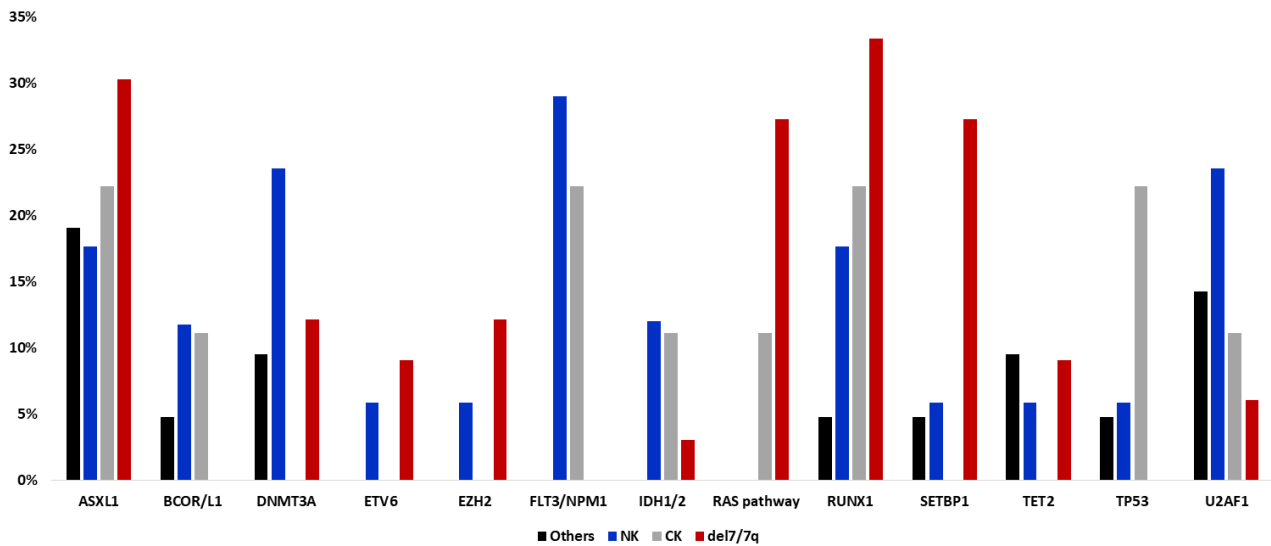


Figure S10 Bar charts illustrating the frequency (A) and type (B) of mutations according to the identified cytogenetic groups in sMN cases.

A)

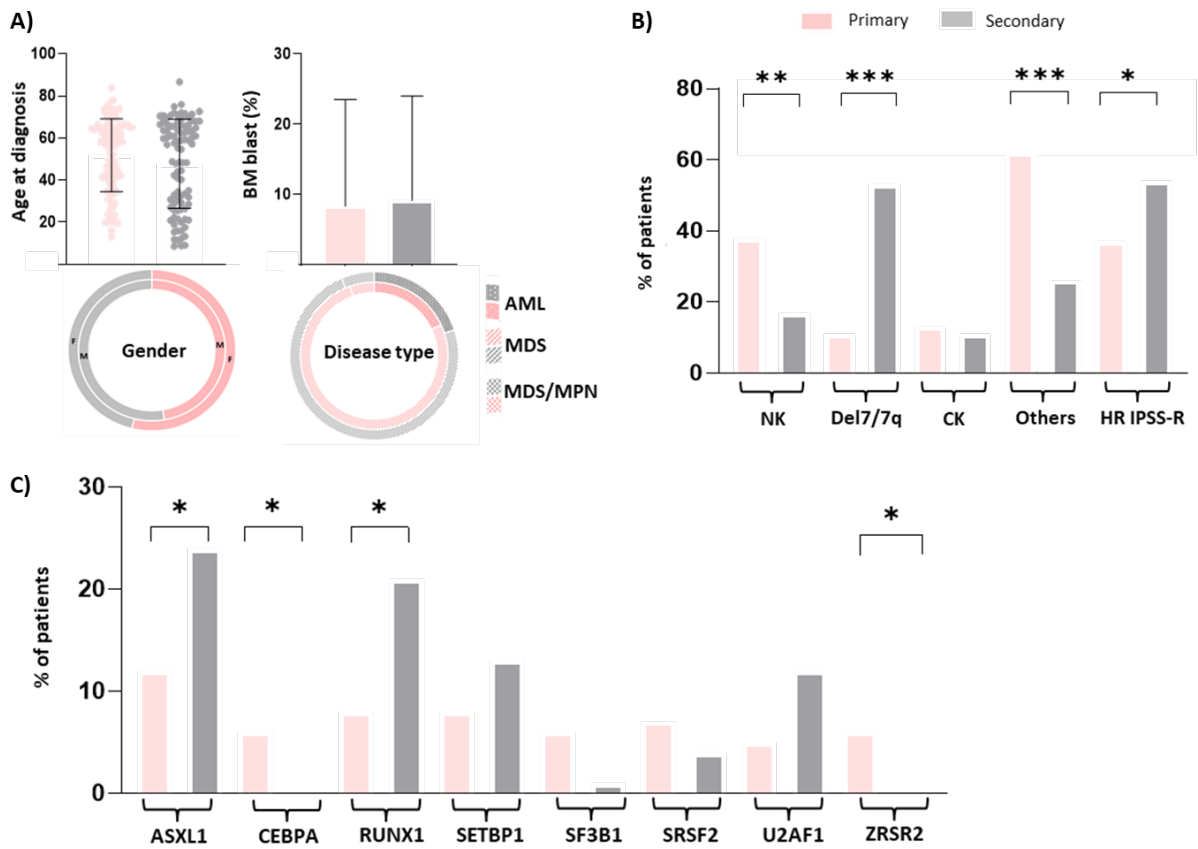


B)



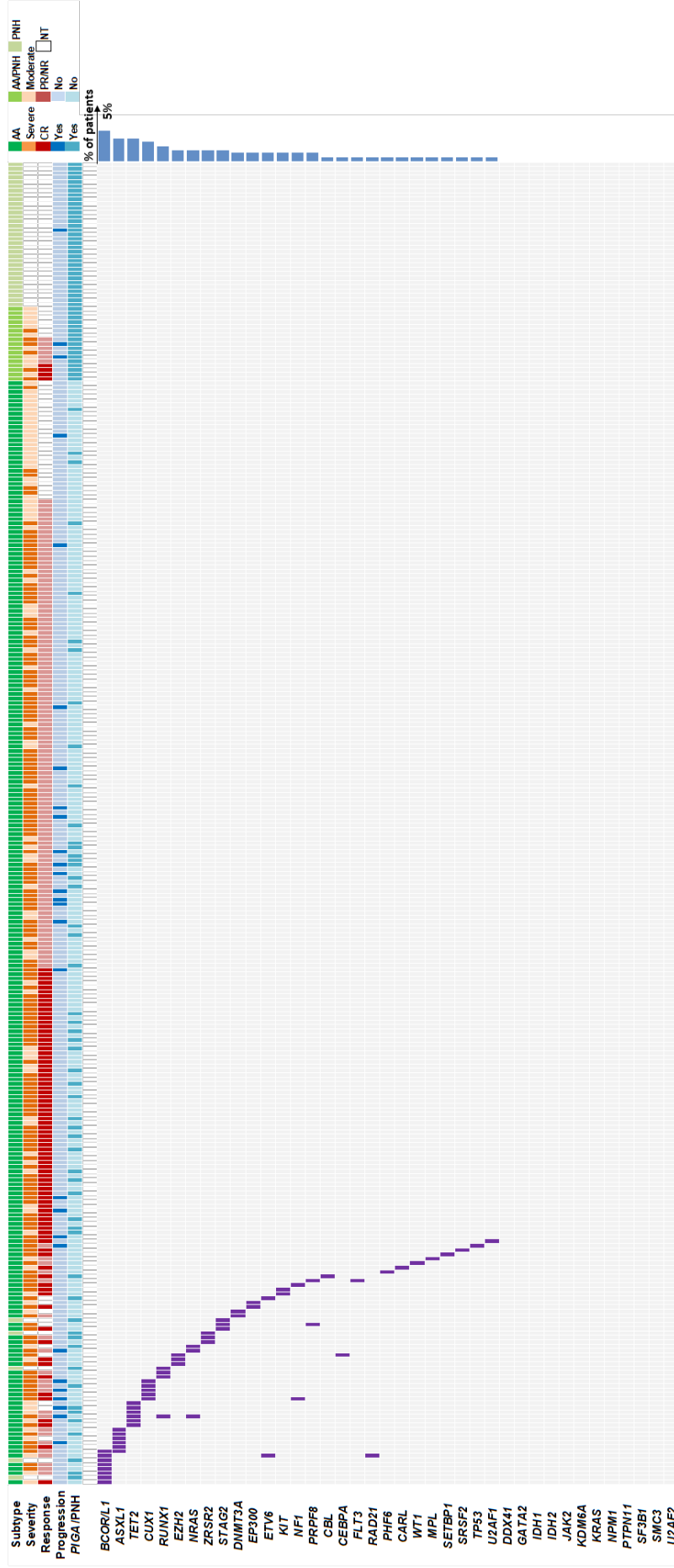
Panel A illustrates the difference in clonal burden according to cytogenetics with pie charts indicating the frequency of mutated cases in each group. In Panel B, the Y-axis shows the frequency of mutated patients. Different colors indicate each cytogenetic group. N=82 cases with available molecular information.

Figure S11. Matched-pair comparison of secondary with primary MN cases.



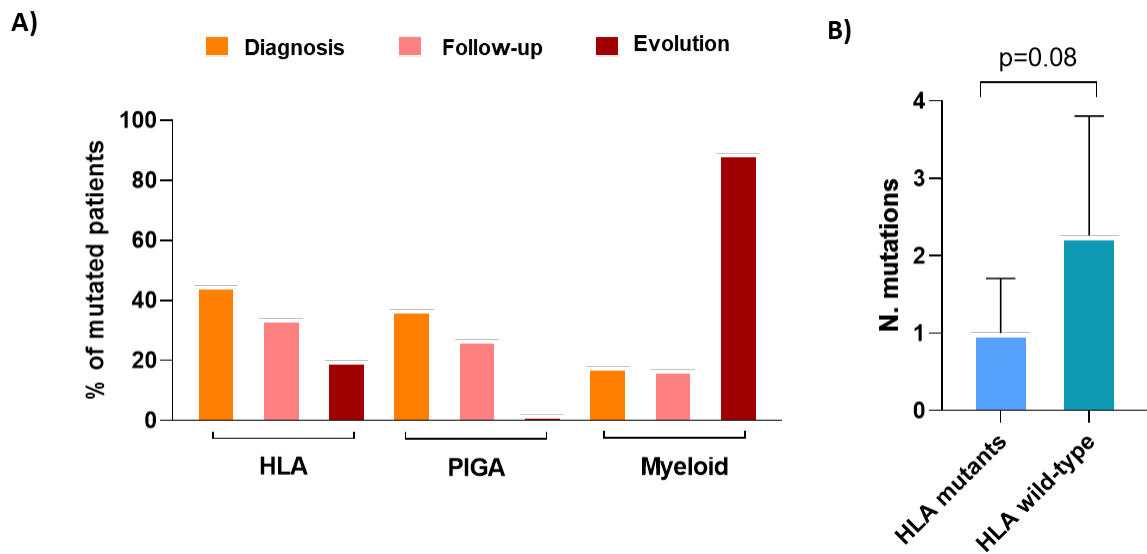
Propensity score matching was applied to reduce any possible confounding effects between sMN (n=94) and our control cohort (n=94 matched patients with a 1:1 ratio from 3599 *de novo* cases) by using age, gender, bone marrow blast percentage and disease type as relevant baseline covariates (confounders). Panel A shows equal (=not statistically significant) distribution of possible confounders between the 2 groups. Panel B and C illustrate differences in cytogenetic and mutational landscape, respectively. *P<.05, **P<.01, ***P<.0001. The pink color indicates primary, while the grey color secondary to AA/PNH cases. R-IPSS risk groups (higher>3.5; lower<3.5; see above definitions).

Figure S12. Waterfall plot showcasing the mutational landscape at baseline of patients with available information.



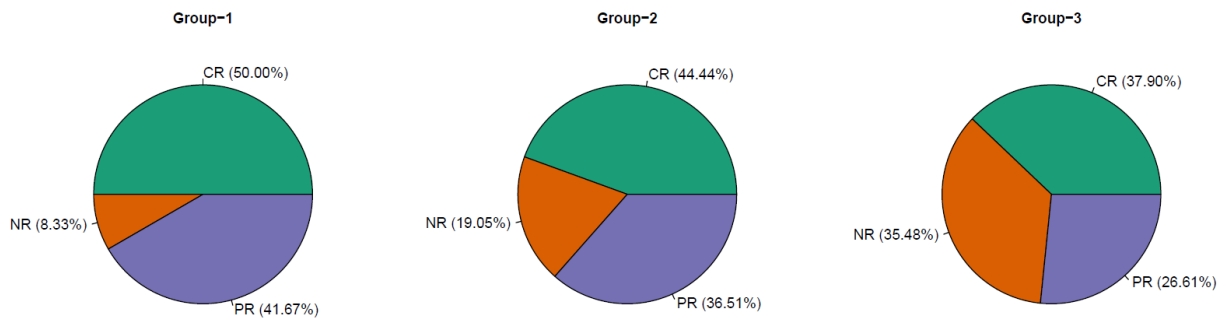
Waterfall plot summarizes the frequency of gene mutations in a sub-cohort of 302 patients with available information at baseline. Disease phenotypes, AA severity, treatment response, progression to myeloid neoplasia, and presence of *PIGA*/PNH clone(s) are color-coded on top of the waterfall plot. Frequencies of mutations for each gene are shown on the right. Median variant allelic frequency for myeloid mutations was 31% (IQR, 15-46).

Figure S13. Details on the cross-sectional analysis of immune-molecular dynamics.



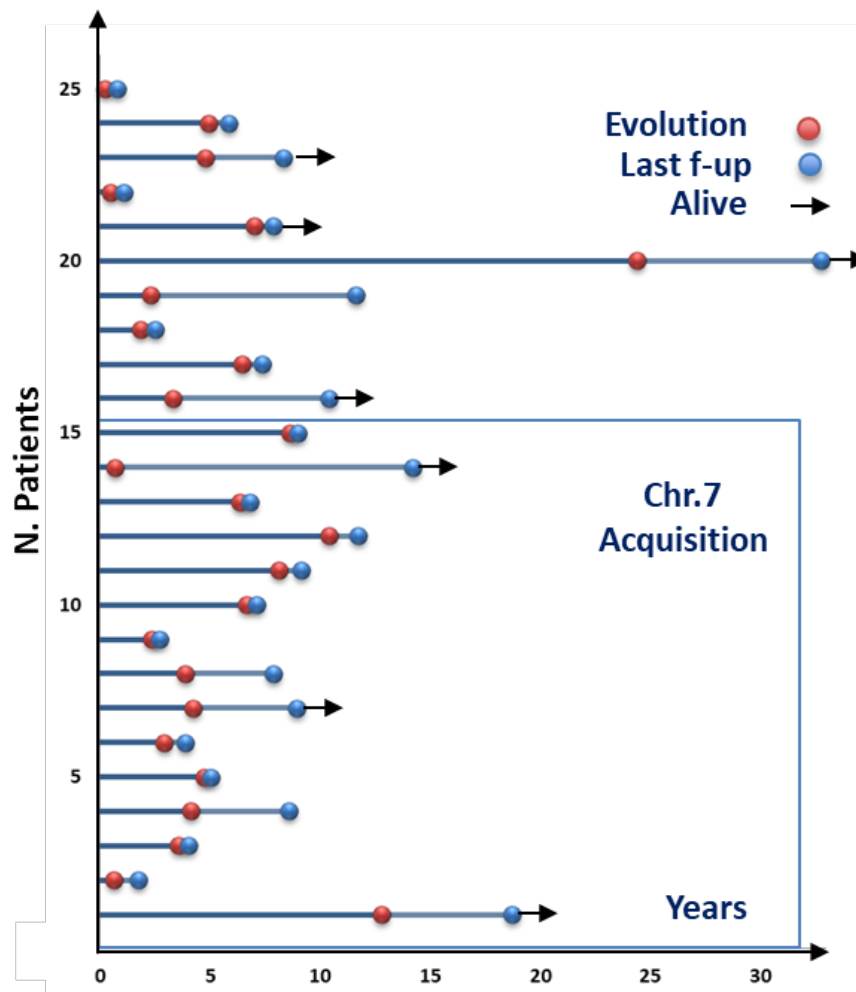
For this analysis, only patients with concomitant information on the 3 panels at each time point were used: diagnosis (n=31), follow-up (n=137), and myeloid evolution (n=29). Panel A shows details on the frequency of cases mutant for HLA, *PIGA*, and myeloid mutations at each time point. Panel B shows a trend for lower myeloid burden (number of myeloid hits) at evolution in carriers of HLA mutations (n=5) vs. wild-type patients (n=23).

Figure S14. Details on treatment response according to mutational status



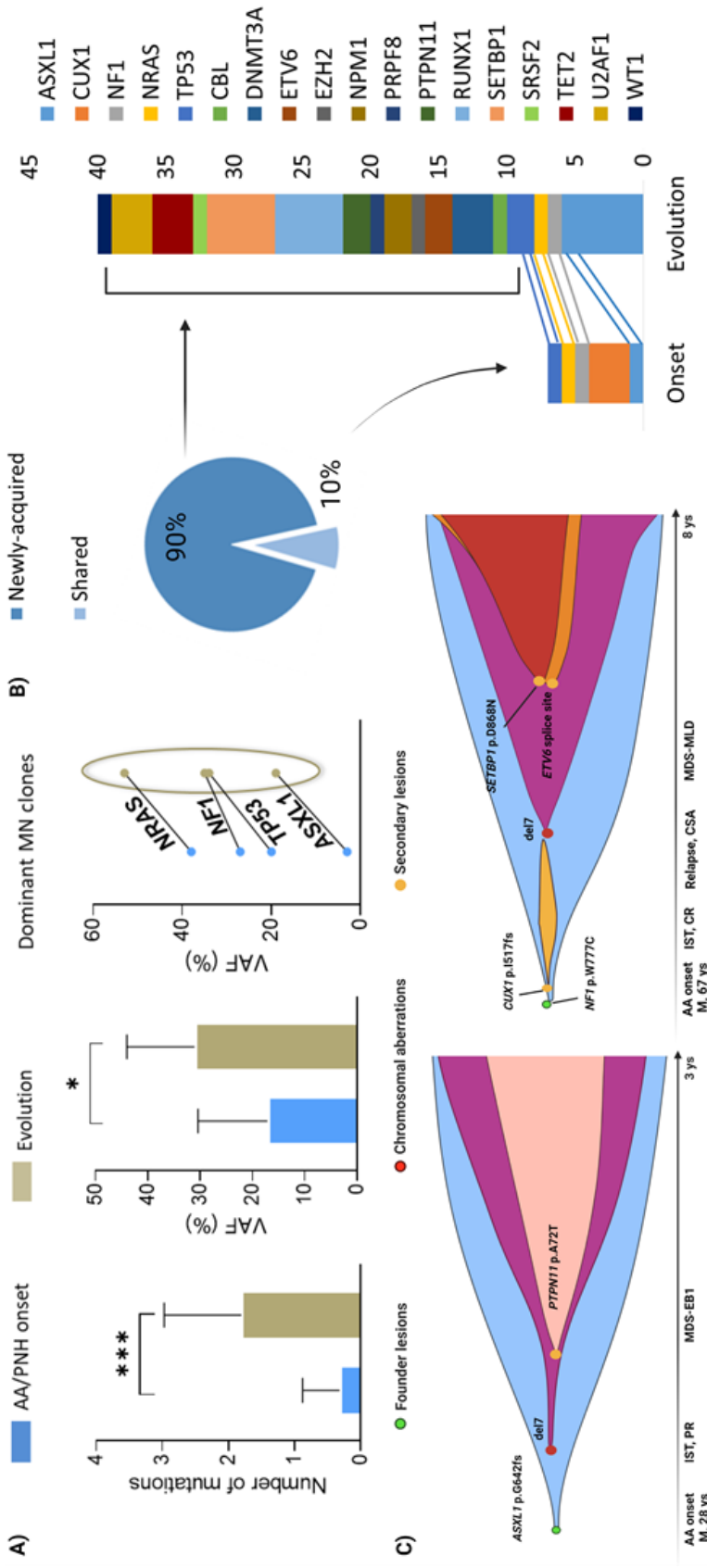
Pie charts showcase the response to initial IST for patients in each group according to mutational status and progression risk (Group 2: *PIGA*/PNH clone presence and *BCOR/L1*, Group 3: No Mutation; Group1: all other myeloid mutations; see also Figure 4 and above for details). No statistically significant differences were found when considering both CR vs. PR/NR ($p=0.54$) and CR/NR/PR separately ($p=0.08$). $N=302$. CR=complete response; PR=partial response; NR=no response.

Figure S15. Swimming plot of the 25 patients studied longitudinally



Swimmer plot illustrates the disease course of the 25 longitudinal patients. Patients on the bottom acquired chromosome 7 aberrancies at evolution.

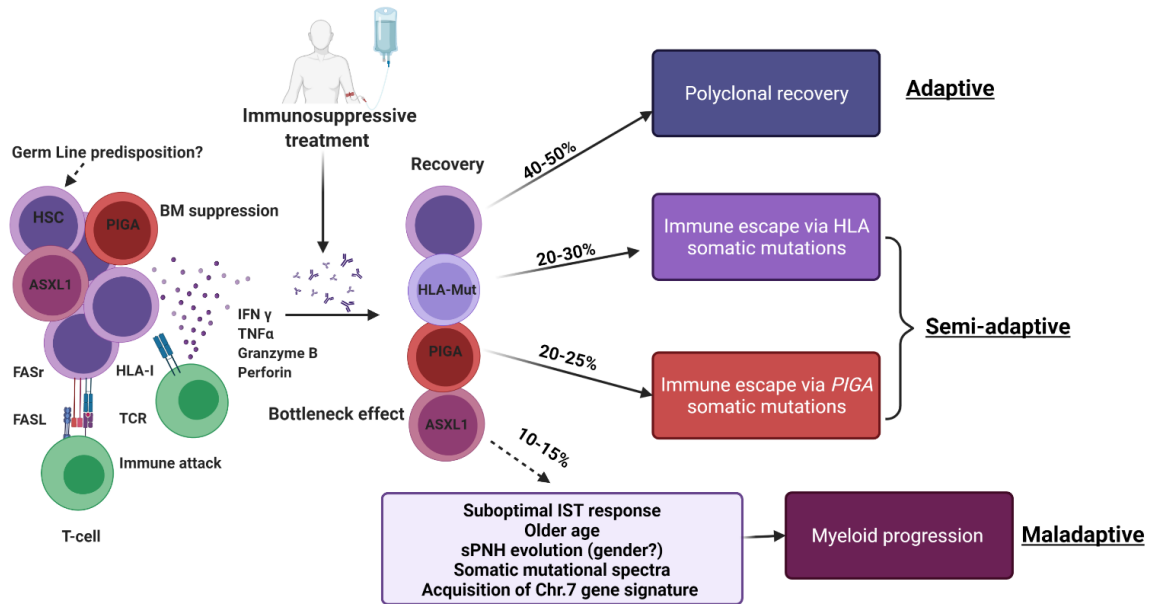
Figure S16. Longitudinal molecular studies of 25 serial cases.



In panel A, bar charts demonstrate difference in number and clonal burden of mutations in serial patients studied at onset and myeloid progression with exemplificative patients whose mutations were present already at BM failure onset on the right. Panel B shows the molecular architecture of cases at onset and disease progression with the

pie chart illustrating the percentage of mutations shared at both time points. Lines between the two bar charts associate shared mutations. In [panel C](#), two exemplificative patients are shown. On the left, the fish plot showcases a 28 year-old male with severe AA treated with conventional immunosuppression (anti-thymocyte globulin and cyclosporine A) achieving a partial response and subsequently evolving after 3 years to a myelodysplastic syndrome (MDS) with excess blasts type 1. The *ASXL1* mutation present at AA onset (p.G642fs, variant allelic frequency, VAF 3%) constituted the bulk of disease at MDS evolution (VAF 19%) with monosomy 7 (45,XX,-7[20]) and an additional *PTPN11* (p.A72T, VAF 13%) mutation acquired at evolution. On the right, a case of a 67 year-old man with severe AA is illustrated. The patient had an initial complete response to standard immunosuppressive treatment, subsequently relapsed and eventually progressed to a MDS with multilineage dysplasia after 8 years from initial diagnosis. At AA onset, the patient harbored *CUX1* (p.I517fs, VAF 5%) and *MF1* mutations (p.W777C, VAF 26%) while at the time of progression he lost the *CUX1* lesion and the *MF1* mutation increased in VAF (35%). Moreover, the patient acquired monosomy 7 (46,XX,-7,+mar[20]) and two additional alterations in *SETBP1* (p.D868N, VAF 35%) and *ETV6* (c.163+1G>A splice site variant, VAF 36%) genes.

Figure S17. Modes of evolution in AA



The cartoon shows the theorized modes of clonal evolution in AA. Following the immune attack and standard immunosuppression, the hematopoietic stem cell (HSC) pool is reduced (bottleneck effect), and few remaining stem cells repopulate the bone marrow. Patient-specific variables (age), the effects of immunological forces (*PIGA*, HLA) and the presence of specific myeloid mutations at onset may play a role in shaping the disease trajectories of individual patients towards adaptive (i.e., normal), semi-adaptive (i.e., HLA/ *PIGA*-mutant hematopoiesis) or maladaptive (i.e., malignant) escape mechanisms.

Supplementary Tables

Table S1. List of common genes studied on the targeted sequencing panels

<i>ASXL1</i>	<i>CEBPA</i>	<i>ETV6</i>	<i>IDH2</i>	<i>MPL</i>	<i>PRPF8</i>	<i>SF3B1</i>	<i>TP53</i>
<i>BCOR</i>	<i>CUX1</i>	<i>EZH2</i>	<i>JAK2</i>	<i>NF1</i>	<i>PTPN11</i>	<i>SMC3</i>	<i>U2AF1</i>
<i>BCORL1</i>	<i>DDX41</i>	<i>FLT3</i>	<i>KDM6A</i>	<i>NPM1</i>	<i>RAD21</i>	<i>SRSF2</i>	<i>U2AF2</i>
<i>CALR</i>	<i>DNMT3A</i>	<i>GATA2</i>	<i>KIT</i>	<i>NRAS</i>	<i>RUNX1</i>	<i>STAG2</i>	<i>WT1</i>
<i>CBL</i>	<i>EP300</i>	<i>IDH1</i>	<i>KRAS</i>	<i>PHF6</i>	<i>SETBP1</i>	<i>TET2</i>	<i>ZRSR2</i>

Table S2. Main baseline characteristics of patients undergone HSCT upfront (N=117).

Variables		HSCT (N=117)	AA (N=111)	AA/PNH (N=2)	PNH (N=4)
Follow-up	median [IQR]	8.2y (5.7 - 10.6)			
Age	median [IQR] (range)	23.3 [17-34] (3.1-52.1)	23.3 [17-34] (3.1-52.1)	23 [19.3-26.8] (15.5-30.5)	35.4 [30.5-39.1] (24.7-41.4)
Gender	F M	52 (44.4) 65 (55.6)	49 (44.1) 62 (55.9)	1 (50) 1 (50)	2 (50) 2 (50)
Severity	non-severe severe	11 (9.7) 102 (90.3)	9 (8.1) 102 (91.9)	2 (100) 0 (0)	
PNH clone (N=83)*	no yes	51 (61.4) 32 (38.6)	51 (66.2) 26 (33.8)	0 (0) 2 (100)	0 (0) 4 (100)
PNH percentage (N=83)*	median [IQR] (range)	3.2 [0.9-11.5] (0.1-99)	3.0 [0.9-10] (0.1-45)	56/99	NA
Karyotype (N=76)*	normal failure others	62 (81.6) 12 (15.8) 2 (1.3)	60 (81.1) 12 (16.2) 2 (1.4)	1 (100) 0 (0) 0 (0)	1 (100) 0 (0) 0 (0)

*indicated is the available sample size due to missing data. AA: aplastic anemia; PNH: paroxysmal nocturnal hemoglobinuria; HSCT: hematopoietic stem cell transplant; F: female; M: male; y:years. For categorical variables, numbers in parentheses indicate percentages.

Table S3. Characteristics of patients not undergone upfront HSCT (N=882).

Variables	Modalities	N=882	AA (N=706)	AA/PNH (N=73)	PNH (N=103)
Age	median [IQR]	36.6 [20.6-57.1]	37.2 [19.9-59.3]	30.8 [20.5-44]	36.1 [27.3-47.4]
	(range)	(3-89.4)	(3-89.4)	(5.8-86.7)	(7.7-84.8)
Gender	F	450 (51)	350 (49.6)	42 (57.5)	58 (56.3)
	M	432 (49)	356 (50.4)	31 (42.5)	45 (43.7)
Severity	non-severe		167 (23.7)	46 (63)	
	severe		539 (76.3)	27 (37)	
PNH clone (N=749)*	no	382 (51)	382 (66.6)	0 (0)	0 (0)
	yes	367 (49)	192 (33.4)	72 (100)	103 (100)
PNH percentage (N=749)*	median [IQR]	13 [2.7-65.5]	3.5 [1.3-8]	44.5 [30.2-81.4]	83.5 [61.4-94.5]
	(range)	(0.1-99.9)	(0.1-84)	(0.4-99)	(12-99.9)
Karyotype (N=712)*	normal	663 (93.1)	538 (91.8)	63 (98.4)	62 (100)
	failure	31 (4.4)	31 (5.3)	0 (0)	0 (0)
	others	18 (2.5)	17 (2.9)	1 (1.6)	0 (0)
N. of myeloid mutations (N=302) *	0	246 (81.2)	196 (79.3)	17 (100)	33 (86.8)
	1	48 (15.8)	43 (17.4)	0 (0)	5 (13.2)
	2	6 (2)	6 (2.4)	0 (0)	0 (0)
	3	2 (1)	2 (0.9)	0 (0)	0 (0)
PIGA mutant (N=232)*	No	154 (66.4)	142 (78)	5 (33.3)	7 (20)
	Yes	78 (33.6)	40 (22)	10 (66.7)	28 (80)
First line treatment (N=869)*	None	171 (19.7)	54 (7.8)	22 (31)	95 (93.1)
	ATG/CSA based	526 (60.5)	494 (71)	32 (45.1)	0 (0)
	CSA based	115 (13.2)	102 (14.7)	12 (16.9)	1 (1)
	Eltrombopag	18 (2.1)	17 (2.4)	1 (1.4)	0 (0)
	Other	39 (4.5)	29 (4.2)	4 (5.6)	6 (5.9)

*indicated is the available sample size due to missing data. AA: aplastic anemia; PNH: paroxysmal nocturnal hemoglobinuria; F: female; M: male; ATG: anti-thymocyte globulin; CSA: cyclosporine A. For categorical variables, numbers in parentheses indicate percentages.

Table S4. Main baseline, treatment, and response characteristics of patients with severe AA (N=504)

Variables	Modalities	All (N=504)	SAA (N=481)	SAA/PNH (N=23)
PNH clone (N=415)*	no	265 (63.9)	265 (67.4)	0 (0)
	yes	150 (36.1)	128 (32.6)	22 (100)
PNH percentage (N=415)*	median [IQR]	4 [1.3-11]	3 [1.2-6.5]	37.4 [27-90]
	(range)	(0.1-99)	(0.1-84)	(0.4-99)
Age	median [IQR]	34.9 [19.1-58.1]	35.2 [19.1-58.5]	29.4 [20.4-40.9]
	(range)	(3-85.4)	(3-85.4)	(9.6-78.6)
Gender	F	240 (47.6)	225 (46.8)	15 (65.2)
	M	264 (52.4)	256 (53.2)	8 (34.8)
Karyotype (N=431)*	normal	394 (91.4)	371 (90.9)	23 (100)
	failure	25 (5.8)	25 (6.1)	0 (0)
	other	12 (2.8)	12 (3.0)	0 (0)
N. of myeloid mutations (N=155)*	0	124 (80)	118 (79.2)	6 (100)
	1	25 (16.1)	25 (16.8)	0 (0)
	2	5 (3.2)	5 (3.4)	0 (0)
	3	1 (0.6)	1 (0.7)	0 (0)
PIGA mutant (N=122)*	No	97 (79.5)	94 (80.3)	3 (60)
	Yes	25 (20.5)	23 (19.7)	2 (40)
First line treatment (N=500)*	ATG/CSA based	453 (90.6)	430 (90.1)	23 (100)
	CSA based	34 (6.8)	34 (7.1)	0 (0)
	Eltrombopag	7 (1.4)	7 (1.5)	0 (0)
	Other	6 (1.2)	6 (1.3)	0 (0)
Response to treatment (N=496)*	CR	176 (35.5)	169 (35.7)	7 (31.8)
	no CR	320 (64.5)	305 (64.3)	15 (68.2)
Pts. receiving >1 line of treatment (N=498)*	no	250 (50.2)	236 (49.7)	14 (60.9)
	yes	248 (49.8)	239 (50.3)	9 (39.1)
N. of lines received (N=497)*	1	250 (50.3)	236 (49.8)	14 (60.9)
	2	155 (31.2)	151 (31.9)	4 (17.4)
	3	61 (12.3)	58 (12.2)	3 (13)
	4	20 (4)	20 (4.2)	0 (0)
	≥5	11 (2.2)	9 (1.9)	2 (8.7)
Eltrombopag (N=501)*/&	no	382 (76.2)	363 (75.9)	19 (82.6)
	yes	119 (23.8)	115 (24.1)	4 (17.4)
Eculizumab (N=501)*/&	no	444 (88.6)	438 (91.6)	6 (26.1)
	yes	57 (11.4)	40 (8.4)	17 (73.9)
Subsequent transplant	no	380 (75.4)	362 (75.3)	18 (78.3)
	yes	124 (24.6)	119 (24.7)	5 (21.7)

*indicated is the available sample size due to missing data; [§]ever received. AA: aplastic anemia; PNH: paroxysmal nocturnal hemoglobinuria; F: female; M: male; ATG: anti-thymocyte globulin; CSA: cyclosporine A; SAA: severe AA; CR: complete response. For categorical variables, numbers in parentheses indicate percentages.

Table S5. Univariate analysis for clonal progression and survival outcomes in severe AA cases (N=504)

Variables	Modalities	10-year Overall Survival	10-year Cumulative Incidence of sMN	10-year Cumulative Incidence of sPNH
Disease phenotype	SAA	73.7 [68.5-78.1]	13.3 [10-17.1]	
	SAA/PNH	95.7 [72.9-99.4]	5.4 [0.3-22.4]	
	p-value	0.02	0.19	
Gender	F	75.2 [67.9-81.0]	12.9 [8.4-18.6]	12.1 [7.4-18.0]
	M	74.5 [67.5-80.2]	13 [8.8-18.0]	6.0 [3.1-10.2]
	p-value	0.85	0.26	0.01
Response to treatment (N=496)*	CR	89.6 [82.1-94.0]	8.5 [4.4-14.2]	15.8 [9.8-23.1]
	No CR	67.3 [60.6-73.1]	15.7 [11.5-20.6]	5.0 [2.6-8.6]
	p-value	<0.001	0.02	<0.001
N. of myeloid mutations (N=155)*	0	71.4 [60.4-79.9]	15.6 [9.1-23.6]	14.8 [8.2-23.4]
	>=1	55.4 [33-73.1]	16.9 [5.0-34.8]	4.6 [0.3-19.8]
	p-value	0.42	0.67	0.24
PIGA mutations (N=155)*	No			5.5 (1.7 - 12.7)
	Yes			41.3 (20.4 - 61.1)
	p-value			<0.001
PNH clone (N=415)*	no	70.7 [63.5-76.8]	12.7 [8.5-17.9]	4.7 [2.1-8.7]
	yes	83.9 [75.8-89.4]	11.7 [6.6-18.4]	19.8 [11.2-30.3]
	p-value	0.02	0.99	<0.001
Age	(≤35]	88.0 [82.6-91.8]	6.6 [3.7-10.8]	9.9 [6.0-14.8]
	(>35]	58.9 [50.4-66.5]	20.0 [14.3-26.2]	7.8 [4.1-13.0]
	p-value	<0.001	<0.001	0.09

*indicated is the available sample size due to missing data. AA: aplastic anemia; PNH: paroxysmal nocturnal hemoglobinuria; F: female; M: male; SAA: severe AA; CR: complete response; sPNH: secondary PNH; sMN: secondary myeloid neoplasia. Non-adjusted effect of baseline variables was evaluated using Log-Rank and Gray's tests, respectively for overall survival and cumulative incidence outcomes (sMN and sPNH).

Table S6. Main baseline, treatment and response characteristics of patients with non-severe AA (N=192)

Variables	Modalities	nSAA (N=192)	nSAA (N=151)	nSAA/PNH (N=41)
PNH clone (N=169)*	no	78 (46.2)	78 (60.9)	0 (0)
	yes	91 (53.8)	50 (39.1)	41 (100)
PNH percentage (N=169)*	median [IQR]	14 [4.2-44.2]	5.4 [2-12]	63 [37-84]
	(range)	(0.1-99)	(0.1-27)	(14-99)
Age	median [IQR]	37.2 [22-58.8]	38.7 [22.1-60.2]	33.1 [21-44.6]
	(range)	(5.1-89.4)	(5.1-89.4)	(5.8-86.7)
Gender	F	113 (58.9)	90 (59.6)	23 (56.1)
	M	79 (41.1)	61 (40.4)	18 (43.9)
Karyotype (N=169)*	normal	163 (96.4)	127 (96.2)	36 (97.3)
	failure	3 (1.8)	3 (2.3)	0 (0)
	other	3 (1.8)	2 (1.6)	1 (2.7)
N. of myeloid mutations (N=83)*	0	68 (81.9)	59 (79.7)	9 (100)
	1	14 (16.9)	14 (18.9)	0 (0)
	2	1 (1.2)	1 (1.4)	0 (0)
PIGA mutant (N=64)*	no	42 (65.6)	40 (71.4)	2 (25)
	yes	22 (34.4)	16 (28.6)	6 (75)
First line treatment	None	65 (33.9)	45 (29.8)	20 (48.8)
	ATG/CSA based	28 (14.6)	21 (13.9)	7 (17.1)
	CSA based	69 (35.9)	58 (38.4)	11 (26.8)
	Eltrombopag	7 (3.6)	7 (4.6)	0 (0)
	Other	23 (12)	20 (13.2)	3 (7.3)
Median time to Treatment (years)	median [IQR]	0.37 [0-1]	0.4 [0-1]	0.5 [0.1-0.9]
	(range)	(0-30.6)	(0-17)	(0-30.6)
Response to treatment (N=188)*	CR	35 (18.6)	31 (20.8)	4 (10.3)
	No CR	88 (46.8)	73 (49)	15 (38.5)
	No TTT	65 (34.6)	45 (30.2)	20 (51.3)
Pts. receiving >1 line of treatment (N=189)*	no	142 (75.1)	110 (73.3)	32 (82.1)
	yes	47 (24.9)	40 (26.7)	7 (17.9)
N. of lines received* (N=189)	0	65 (34.4)	45 (30)	20 (51.3)
	1	77 (40.7)	65 (43.3)	12 (30.8)
	2	16 (8.5)	14 (9.3)	2 (5.1)
	3	17 (9)	14 (9.3)	3 (7.7)
	4	5 (2.6)	5 (3.3)	0 (0)
	≥5	9 (4.7)	7 (4.7)	2 (5.1)
Eltrombopag (N=191)*/&	no	150 (78.5)	112 (74.7)	38 (92.7)
	yes	41 (21.5)	38 (25.3)	3 (7.3)
Eculizumab (N=190)*/&	no	137 (72.1)	133 (88.7)	4 (10)
	yes	53 (27.9)	17 (11.3)	36 (90)
	no	171 (89.1)	135 (89.4)	36 (87.8)

Subsequent transplant	yes	21 (10.9)	16 (10.6)	5 (12.2)
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*indicated is the available sample size due to missing data; [§]ever received; No TTT: no treatment; AA: aplastic anemia; PNH: paroxysmal nocturnal hemoglobinuria; F: female; M: male; ATG: anti-thymocyte globulin; CSA: cyclosporine A; SAA: severe AA; nSAA: non-severe AA; CR: complete response. For categorical variables, numbers in parentheses indicate percentages.

Table S7. Univariate analysis for clonal progression and survival outcomes in non-severe AA cases (N=192)

Variables	Modalities	10-year Overall Survival	10-year Cumulative Incidence of sMN	10-year Cumulative Incidence of sPNH **
Disease phenotype	nSAA	80 [70.5-86.8]	11.5 [5.9-19.1]	
	nSAA/PNH	86.2 [69.7-94.1]	16.4 [6.5-30.4]	
	p-value	0.43	0.03	
Gender	F	88.9 [80.1-93.9]	7.2 [2.9-14.3]	8.1 [2.8-17.1]
	M	74.7 [61.7-83.8]	17.8 [9.3-28.4]	11.6 [4.6-22.2]
	p-value	0.15	0.1	0.73
Response to treatment (N=188)*	CR	88.2 [60.6-96.9]	8.3 [1.4-23.7]	12.4 [2.9-29.1]
	No CR	79.7 [68.5-87.3]	8.9 [3.5-17.4]	11.5 [5.0-21.1]
	No TTT	81.6 [64-91.2]	21.9 [10-36.7]	4.7 [0.3-20.1]
	p-value	0.31	0.04	0.19
N. of myeloid mutations (N=83)*	0	77.4 [62.7-86.9]	6.7 [1.6-17]	13.3 [5.2-25.4]
	>=1	50.6 [14-79]	10.0 [0.4-37.6]	16.9 [2.3-43.3]
	P value	0.66	0.68	0.85
PIGA mutated (N=64) *	No			10.5 [2.4-25.5]
	Yes			38.1 [12.6-63.9]
	p-value			0.002
Age	(2,9,35]	87.6 [76.4-93.7]	12.3 [5.6-21.8]	12.6 [5.0-23.9]
	(35,90]	76.2 [64-84.7]	12.6 [5.9-22]	6.9 [2.5-14.3]
	p-value	0.04	0.96	0.85

*indicated is the available sample size due to missing data; **exclusion of 3 patients with sPNH before 6 months; No TTT: no treatment. No TTT: no treatment; AA: aplastic anemia; PNH: paroxysmal nocturnal hemoglobinuria; F: female; M: male; SAA: severe AA; nSAA: non-severe AA; CR: complete response; sPNH: secondary PNH; sMN: secondary myeloid neoplasia. Non-adjusted effect of baseline variables was evaluated using Log-Rank and Gray's tests, respectively for overall survival and cumulative incidence outcomes (sMN and sPNH).

Table S8. Main characteristics at secondary myeloid progression (N=94)

Variables	Modalities	N=94
Gender	F	36 (38.3)
	M	58 (61.7)
Age at progression	median [IQR] (range)	61.0 [34.4-69.3] (11-87.5)
Time from diagnosis to progression (years)	median [IQR] (range)	4.5 [1.8-7.7] (0.7-33.1y)
BM blasts	median [IQR] (range)	3.5 [1-8.8] (0-66)
MN diagnosis	AML	17 (18.1)
	MDS	71 (75.5)
	MDS/MPN	5 (5.3)
	MPN	1 (1.1)
	AML-MRC	12 (12.8)
	AML-NOS	3 (3.2)
	AML with mutated <i>NPM1</i>	1 (1.1)
	CMML	3 (3.3)
	MDS-del(5q)	1 (1.1)
	MDS-EB-1	14 (14.9)
	MDS-EB-2	7 (7.4)
	MDS-MLD	28 (29.8)
	MDS-RS-MLD	2 (2.1)
	MDS-RS-SLD	1 (1.1)
	MDS-SLD	4 (4.3)
	WHO 2016	MDS-U
MDS/MPN-U		2 (2.1)
MF		1 (1.1)
MPN-U		1 (1.1)
Cytogenetics	complex	9 (9.8)
	del(7/7q)	42 (44.6)
	normal	18 (19.1)
	other	22 (23.4)
	failure	3 (3.1)
R-IPSS (MDS cases)	higher	37 (57.8)
	lower	27 (42.2)
N. of myeloid mutations (N=82)*	0	16 (19.5)
	1	26 (31.7)
	2	15 (18.3)
	>2	25 (30.5)
Treatment (N=90)*	no	40 (44.4)
	CHT	11 (12.2)
	HMA	17 (18.9)

	HSCT	19 (21.1)
	other	3 (3.3)
HSCT all lines	no	60 (63.8)
	yes	34 (36.2)
	progression/primary disease	16 (35.5)
	relapse	3 (6.7)
	brain hemorrhage	1 (2.2)
Cause of death (N=45)*	GvHD	3 (6.7)
	infection	15 (33.3)
	others	3 (6.6)
	suicide	1 (2.2)
	TRM	3 (6.7)

*indicated is the available sample size due to missing data. WHO 2016 diagnoses are according to Arber et al⁴. R-IPSS (higher>3.5; lower \leq 3.5) were defined according to Pfeilstöcker et al.⁹ F: female; M: male; CHT: chemotherapy; HMA: hypomethylating agents; HSCT: hematopoietic stem cell transplant; GvHD: graft versus host disease; TRM: transplant-related mortality defined as death due to any transplant-related complication other than GvHD. For categorical variables, numbers in parentheses indicate percentages.

Table S9. Baseline characteristics of patients subsequently progressed to sMN (N=94)

Variables	Modalities	N=94
Diagnosis of BMF	AA	77 (81.9)
	AA/PNH	11 (11.7)
	PNH	6 (6.4)
PNH clones (N=74)*	no	37 (50)
	yes	37 (50)
PNH percentage at diagnosis (N=74)*	median [IQR]	7 [3.2-47]
	(range)	(0.3-99.5)
Age at diagnosis	median [IQR]	56.5 [28.4-65.5]
	(range)	(7.6-86.7)
Gender	F	36 (38.3)
	M	58 (61.7)
Severity	non-severe	25 (28.4)
	severe	63 (71.6)
N. of myeloid mutations (N=25)*	0	18 (72)
	1	6 (24)
	2	1 (4)
PIGA mutations (N=18)*	No	16 (88.9)
	Yes	2 (11.1)
First-line treatment	None	18 (19.1)
	ATG/CSA based	55 (58.5)
	CSA based	13 (13.8)
	Eltrombopag	2 (2.1)
	Other	6 (6.4)
Response to first-line treatment (N=93)*	CR	16 (17.2)
	No CR	59 (63.4)
	No TTT	18 (19.4)
Number of lines received (N=93)*	0	18 (19.4)
	1	39 (41.9)
	2	18 (19.4)
	>3	18 (15.5)
Second-line treatment received (N=93)*	no	38 (50.7)
	yes	37 (49.3)
Second-line treatment (N=31)*	ATG+CSA	13 (41.9)
	ATG+CSA+Epag	1 (3.2)
	CSA	6 (19.4)
	CSA+Epag	1 (3.2)
	Other	9 (29)
	Other+Epag	1 (3.2)
Response to second line (N=26)*	CR	1 (3.8)
	NR	14 (53.8)

 PR 11 (42.3)

*indicated is the available sample size due to missing data; no TTT: no treatment; CR: complete response; PR: partial response; NR: no response; ATG: anti-thymocyte globulin; CSA: cyclosporine A; No TTT: no treatment; AA: aplastic anemia; PNH: paroxysmal nocturnal hemoglobinuria; F: female; M: male; SAA: severe AA; nSAA: non-severe AA; Epag: eltrombopag. For categorical variables, numbers in parentheses indicate percentages.

Table S10. Univariate analysis for overall survival from MN diagnosis in progressed cases (N=94)

Variables	Modalities	5-year Overall Survival
Gender	F	28.2 [11.8-47.3]
	M	46.8 [32.2-60.2]
	p-value	0.47
Bone marrow blast %	< 5	58.2 [41.1-71.9]
	>=5	20.2 [8.6-35.3]
	p-value	<0.001
Diagnosis at sMN evolution	AML	20.0 [4.0-44.7]
	Not AML	44.7 [31.4-57.1]
	P value	0.001
N. of myeloid mutations (N=82)*	0-1	43.6 [26.6-59.5]
	>=2	41.2 [23.2-58.3]
	p-value	0.44
Cytogenetics	Normal	39.3 [14.5-63.7]
	Complex or del(7/7q)	28.7 [14.9-44.2]
	Other abnormality	61.9 [37.7-79]
	p-value	0.08
Age at BMF onset	(7.5,35]	52.2 [32.4-68.8]
	(35,87]	32.3 [18.5-46.9]
	p-value	0.06
Age at progression	< 60	49.4 [32.6-64.2]
	>=60	30.4 [15.6-46.8]
	p-value	0.17
Time to progression	Early	38.5 [17.7-59]
	Late	41.5 [27.8-54.6]
	p-value	0.6

*indicated is the available sample size due to missing data. F: female; M: male; AML: acute myeloid leukemia; BMF: bone marrow failure; sMN: secondary to AA/PNH myeloid neoplasm. Non-adjusted effect of baseline variables on overall survival was evaluated using Log-Rank test.

Table S11. Univariate analysis for overall survival according to mutational status (most frequent mutations) in progressed cases (N=82 patients with molecular information)

Overall survival from MN progression			
Marker	N=82	Univariate HR (95% CI)	p-value (Wald)
<i>ASXL1</i>	20	1.49 (0.74-3.00)	0.26
<i>RUNX1</i>	17	1.35 (0.66-2.73)	0.4
<i>SETBP1</i>	11	1.51 (0.67-3.42)	0.32
<i>DNMT3A</i>	10	Nonproportional risk	0.02 (Log-Rank)
<i>U2AF1</i>	10	0.78 (0.30-1.99)	0.6
<i>TET2</i>	7	1.83 (0.71-4.70)	0.21
<i>EZH2</i>	5	0.27 (0.04-1.98)	0.2
<i>CBL</i>	4	0.29 (0.04-2.13)	0.23
<i>ETV6</i>	4	1.12 (0.27-4.67)	0.87
<i>FLT3</i>	4	0.77 (0.11-5.66)	0.8
<i>TP53</i>	4	12.03 (3.67-39.44)	<0.001

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2.1.2 Discussion and limitations

With a median follow-up of more than 8 years, no transplanted patient (n=117) progressed to MDS/AML, suggesting that donor hematopoiesis outcompetes recipient premalignant clones that might exist at the moment of transplantation [median age 23.3 (3.1 – 52.1)] years. Of note, conditioning regimens for AA are rather immunosuppressive than myeloablative.^{45,154,155} In other words, conditioning itself does not kill LT and ST-HSCs.

Among severe AA (SAA), we have confirmed that suboptimal response to immunosuppression and age at BMF onset were independent prognostic factors for secondary myeloid malignancy and worst survival.¹⁵⁶ Likewise, the prevalence of CHIP increases with age. Older patients might already carry premalignant clones that are positively selected under stress.

Among non-severe cases (nSAA), we observed that untreated patients had the higher cumulative incidence of secondary myeloid malignancy. However, we could not adjust for confounding variables. As for CCUS and SAA with poor response to immunosuppression, stress haematopoiesis might help select premalignant clones.

We observed that classic PNH had a lower cumulative incidence of MDS/AML than AA/PNH and AA alone, although we were underpowered to show a significant difference. Arguably, a less stressed haematopoiesis might play a protective role in clonal selection. Classic PNH patients had fewer myeloid mutations at diagnosis. The presence of PNH clone in aplastic patient did not affect the risk of secondary myeloid malignancy.

The most frequently found mutations at AA diagnosis were in consonance with the literature and overlapped with CHIP: *BCOR*, *BCORL1*, *TET2*, and *ASXL1*.^{19,96,97} The presence of any mutation at AA onset did not affect the risk of MDS/AML evolution. However, myeloid driver mutations predicted higher risk of progression when compared to absence of mutations; isolated *PIGA/HLA* mutations predicted the lowest risk. These findings also confirm previously published data.^{96,98,156} Patients bearing myeloid mutations need close monitoring.

We could compare the molecular profile of patients sequenced at AA baseline and evolution in a cross-sectional analysis: *PIGA* or *HLA^{mut}* VAFs tended to increase among non-progressors and decrease in progressors, followed by an increase myeloid genes. The inverted clonal dynamics supports that *PIGA* or *HLA^{mut}* are not drivers of transformation. Among progressors, we could backtrack the driver mutation at AA for 4/25.

Expectedly, the prognosis of progressors was poor and comparable to published results.¹⁵⁷ Blast count at MDS/AML diagnosis and older age predicted independently worst survival.

MDS secondary to AA differed cytogenetically from primary MDS, presenting worse karyotypes. Most post-AA MDS patients had at least one detectable myeloid mutation and nearly two thirds, ≥ 2 .

Chromosome 7 abnormalities and complex karyotypes conferred poor prognosis. Diversely from R-IPSS, normal karyotype at MDS/AML diagnosis did not predict good prognosis. The interval between AA and MDS/AML was shorter and normal karyotype was associated with classic leukemic drivers such as *NPM1*, *FLT3*, *IDH1/2*, and *U2AF1*.

According to the 2016 WHO revision for myeloid malignancies, isolated del(13q) is sufficient for MDS diagnosis. Historically, in the context of AA, del(13q) is considered

to confer a good prognosis.^{105,158} From 44 published del(13q) post-AA, five progressed to high-risk disease (monosomy 7 or excess of blasts)^{158–160} We did not have enough cases of isolated del(13q) to draw conclusions. Such patients might need close monitoring.

Despite having published the largest cohort on AA/PNH so far, we were underpowered to assess the contribution of individual mutations to the risk of progression. Besides, we observed a higher risk of myeloid malignancy among treatment-naïve nSAA in univariate analysis, but again we were underpowered to adjust for confounding factors.

The availability of NGS (the first patient included in the model was diagnosed in 1972), the indications for sequencing, and the evolution of panels used throughout the years made that we did not have complete molecular data in over half of the cohort, precluding some analyses. We must be cautious interpreting molecular observations. On the other hand, we had excellent completeness of clinical data.

2.1.3 Lessons learnt from this study

I gained experience in bibliographical research, literature review, project and grant writing, grant management, IRB approval (Brazil and France), statistical analysis plan co-writing with a statistician, establishment of international collaborations, and teamwork. Collaborative research is an excellent learning tool for combining different points of view towards a common interest.

Working with co-PIs and a statistician, I have learnt how to create tidy databases and improve preexisting ones. We had the chance to discuss the strengths and weaknesses of our statistical plan, data analyses and visualisation, and draw data-driven conclusions.

In parallel, I have learnt how to identify the cases fulfilling the inclusion criteria, collect clinical data, retrieve biological samples and extract DNA (blood and marrow frozen samples, FFPE marrow biopsies, blood and marrow smears). I did not perform the NGS myself but worked closely with the molecular biology, histocompatibility, and bioinformatics teams.

2.1.4 Perspectives

Molecular profiling using next-generation sequencing is currently performed using heterogeneous panels at non-standardised time points during AA natural history. However, clinicians still have no tool to stratify such patients before first-line treatment by the risk of secondary myeloid neoplasm (evolution. The effect of specific myeloid gene mutations at diagnosis (treatment-naïve patients) and its associations with other factors (e.g., age at onset, conventional cytogenetics, PNH clone, and disease severity) is currently unknown.

We aim to combine clinical, cytogenetic, and molecular characteristics at baseline (treatment-naïve) to develop the first tool for sMN risk-stratification in immune AA.

We propose the development of a novel tool for stratifying AA patients. AA is a rare disease, and the studies published so far did not have enough patients and power to develop a risk score. Studies have described that somatic mutations are frequent events in the natural history of aplastic anaemia and can be already seen in patients' first evaluation^{96,98,161–166}. The study is relevant because the predictive value of such mutations in AA is unknown. A score will help stratify the risk of malignant transformation and allow clinicians to adapt the follow-up strategy for high-risk patients (closer monitoring to assess the best moment for treatment modification).

Because of the rarity of the disease, the most effective strategy to fill this gap is establishing a sizeable multicentric consortium. Based on available data (please see the table below) and considering database updates, we estimate this study is feasible.

Studies reporting molecular data on aplastic anemia non transplanted upfront													
	Gurnari	Groarke	Negoro**	Park	Babushok	Huang	Yoshizato*	Kulasekararaj	Heuser	Lane	Total		
Journal	JCO	Leukemia	Blood	BJH	Cancer Genetics	Haematologica	NEJM	Blood	Haematologica	Leukemia			
Year	2022	2022	2017	2017	2015	2015	2015	2014	2014	2013			
Technique	NGS	NGS	WES / NGS	NGS	WES	NGS	WES / NGS	NGS	NGS	Sanger			
Centers	Paris, Cleveland, Brazil	NIH	Cleveland, Basel	Seoul	U. Penn	Peking	Cleveland	NIH	Japan	King's	Hannover	Dana Farber	
n (AA + AA/PNH)	779	642	199	210	22	138	24	256	159	150	38	33	2650
Age at BMF onset	37.2	31.9	52	42	14.5	30.7	46.9	29	60	41.6	30	34.8	
Male (%)	383 (49.2)	362 (56.4)	71 (53.4)	105 (50)	9 (41)	81 (58.7)	15 (62.5)	157 (61.3)	72 (45.3)	71 (54.6)	18 (47)	22/39 (46)	
PNH (positive/total, %)	264/646 (40.9)	140/366 (38.2)	-	15/105 (14.3)	10 (45.5)	20 (14.5)	16 (66.7)	146 (57)	95 (59.7)	61/150 (40.6)	17/35 (48.6)	4/31 (12.9)	
Severe (%)	566 (70.8)	-	48/133 (36.1)	57 (27.1)	21 (95.4)	81 (58.7)	21 (87.5)	256 (100)	67 (42.1)	74 (52.8)	27 (71)	20 (74.3)	
Cytogenetics (%)	650 (83.4)	-	-	210 (100)	22 (100)	138 (100)	-	-	-	143 (95)	25 (65.8)	33 (100)	
Baseline molecular data (%)	256 (32.8)	-	59	-	13 (59.1)	-	5	0	35	63 (18.2)	-	-	431
sMN events	88	93	35	18	0	9/100 (9)	5/24 (20.8)	36/256 (14.1)	42.6	17	1 (2.6)	-	308
Median FU (years)	8.6	4.7	-	-	1.2	-	5.9	4.9	6.3	3.8	7	-	

* Risk of overlapping patients with Gurnari and Groarke

**Risk of overlapping patients with Gurnari and Yoshizato

We propose a multicentric cohort study. We plan to study a discovery cohort with sufficient clinical and biological data to develop the so-called International Consortium on Aplastic Anemia Risk Score (ICAARIS) model; we will validate the model internally using the bootstrap method and externally on an independent validation cohort.

2.2 Developing a mouse model of somatic mosaicism in Fanconi Anaemia

2.2.1 Aim of the work

Today, FA patients with BMF undergo HSCT if eligible. The 15-year OS reaches 80%,⁷² and transplantation reduces the risk of progression towards MDS/AML.⁷⁵ Revertant patients seem not to progress to MDS/AML and gene therapy mimics this functional reversion. Gene therapy has the advantages of not needing any conditioning regimen and not causing GvHD. However, it is unknown if gene therapy modifies the risk of progression. Considering that HSCT provides excellent results for the haematologic complications of FA and neither reduces the risk of solid cancers, we asked ourselves if somatic functional reversion reduces the risk of progression to MDS/AML.

2.2.2 The scientific question

We would need long follow-up with many patients measure the impact of gene therapy on leukemogenesis. Therefore, we worked on developing a model of genetic reversion in *Fancg*^{-/-} mice. We aimed to test the fitness of gene-corrected or preleukemic subclones in the Fanconi context. Our main objective is to demonstrate that functionally corrected *Fancg*^{-/-} HSPCs have an adaptative advantage over non-corrected premalignant *Fancg*^{-/-} HSPCs *in vivo*.

2.2.3 Methods

2.2.3.1 Mice

C57BL/6J *Fancg* (**Figures 8 and 9**) were kept in specific opportunist pathogen free (SOPF) environment and mating scheme was *het x het*. All animals used for experiences were older than 7 weeks. All experiments were carried according to European regulations (APAFIS#28841-2021010510188985v2 – Modélisation du mosaïcisme somatique et selection clonale dans un modèle murin de l'Anémie de Fanconi).

2.2.3.2 Cell harvesting, lineage-negative and LSK purification

C57BL/6J *Fancg* with the appropriate genotype were euthanized by cervical dislocation, and had their lower limbs dissected for femur, tibia, and, later during my thesis, hip retrieval. Under laminar flow, individual mononuclear cell suspensions were prepared after bones were crushed, filtered, and purified from red blood cells (Red Blood Cell Lysing Buffer Hybri-Max™, Sigma Life Sciences, Saint-Louis, MO, #R7757-100mL). Lin⁻ were obtained using a lineage cell depletion kit (Miltenyi, Bergisch Gladbach, Germany, #130-090-858) and magnetically sorted (Miltenyi, #130-090-976, #130-042-401) according to the manufacturer's instructions. LSK (Lin⁻, Sca1⁺, c-kit⁺) were purified from the magnetically sorted Lin⁻ by flow cytometry (Aria III, Becton and Dickinson, Franklin Lakes, NJ).

2.2.3.3 Murine embryonic fibroblasts

Ten cages containing each one adult C57BL/6J *Fancg*^{+/-} male and two adult C57BL/6J *Fancg*^{-/-} females were set up for mating at 5PM. The next morning, all females were checked for a copulation plug and weighted. On the day E12.5, females gaining at least two grams were euthanized by cervical dislocation and dissected for uterus retrieval. Each embryo was individually genotyped, dissected under a microscope, and had its head and red organs removed. Under laminar flow, embryos of the same genotype were grouped by three. Cells suspensions were obtained by repeated pipetting back and forth using 3mL/embryo ice-cold 0.05% Trypsin-EDTA. Then, fragments were further dissociated using scalpels in a 10cm cell-treated Petri

dish. Twenty-seven (9mL per 1mL of trypsin) millilitres of DMEM supplemented with de-complemented 10% foetal bovine serum, and 1% penicillin/streptomycin were added and kept at 37°C and hypoxia for 48h. Then, cells were retrieved using 0.25% Trypsin-EDTA (Gibco, #25200-056), washed with PBS and re-seeded or frozen with FBS + 10% DMSO in liquid nitrogen for further use.

2.0×10^6 cells were seeded in cell-treated T75 flasks in DMEM (Gibco, #41966-029) supplemented with de-complemented 10% foetal bovine serum, and 1% penicillin/streptomycin and kept at 37°C and hypoxia until confluency.

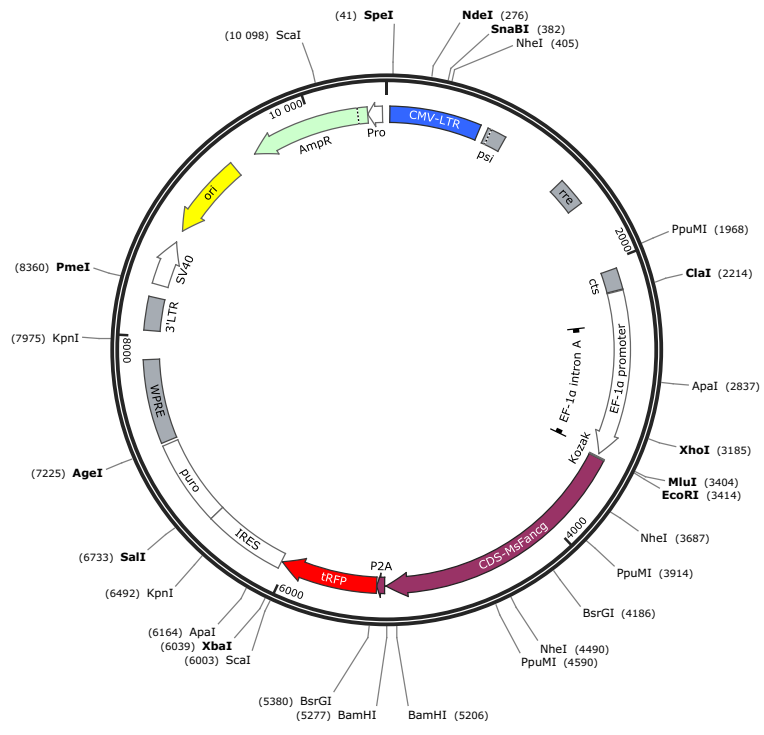
2.2.3.4 Lentiviral production and titration

I did not carry out all the steps described in this paragraph myself (I was not habilitated to access the biosafety level 3 facility). Lentiviral virions were produced using jetPRIME reagents (Polyplus-transfection, France) by co-transfection of viral production plasmids (VSV-G, PAX, and transfer plasmid) in T150 cm² tissue culture flasks containing 2×10^6 HEK-293T cells by flask in Advanced DMEM + 1% penicillin/streptomycin + 1% L-glutamine + 10 foetal bovine serum, according to the manufacturer's protocol. Viral supernatants were filtered through a 0.45µm HV Durapore membrane (Merck Millipore) and concentrated by ultra-centrifugation at 24,000 rpm for two hours at 4 °C, and stored at -80 °C.

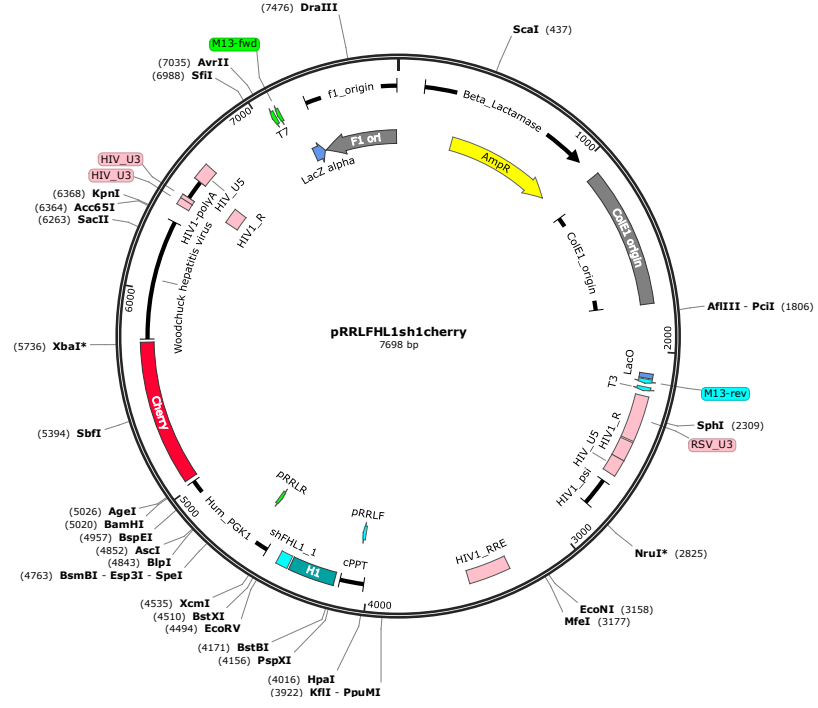
2.2.3.5 Transfer plasmids and lentiviral transduction

All the plasmids I used in this project were already available in the lab and I profited from an already existing transduction protocol. pLenti-hEF1a-*mFancg*-tRFP, pLenti-hEF1a-*mFancg*-eGFP, pLVX-hEF1a-*mEvi1*-ZsGreen, pLVX-hEF1a-*mock*-ZsGreen, pRRL-hPGK-shFHL1-*mCherry* (**Figure 18**) were transduced overnight for 16 hours with the lentiviral supernatants and polybrene 4µg/L with different multiplicities of infection (range 0- >1000). For *in vivo* experiments, treated cells were harvested and washed at day 1. Transduction efficacy was measured by flow cytometry (FACS Aria III cell sorter, Beckton Dickinson) or Fortessa Analyzer, Beckton Dickinson). For *in vitro* experiments, cells were re-seeded for another 24h. Gene overexpression was checked using specific immunoblot, RT-qPCR, and flow cytometry

We chose *Evi-1* because it is located in the long arm of chromosome 3. EVI-1 is frequently amplified among de novo and Fanconi AML (because of +3q). Using *Evi1*, the team from Hildeberg had 100%-penetrant AML in *Fanca*^{-/-} B6 model (data presented in the 1st Translational Research Conference: from the cell to the cure of the disease, 2020).



TLO2020_Lenti-hEF1a-msFancg-P2A-trFP-IRES-puro mock
10 540 bp



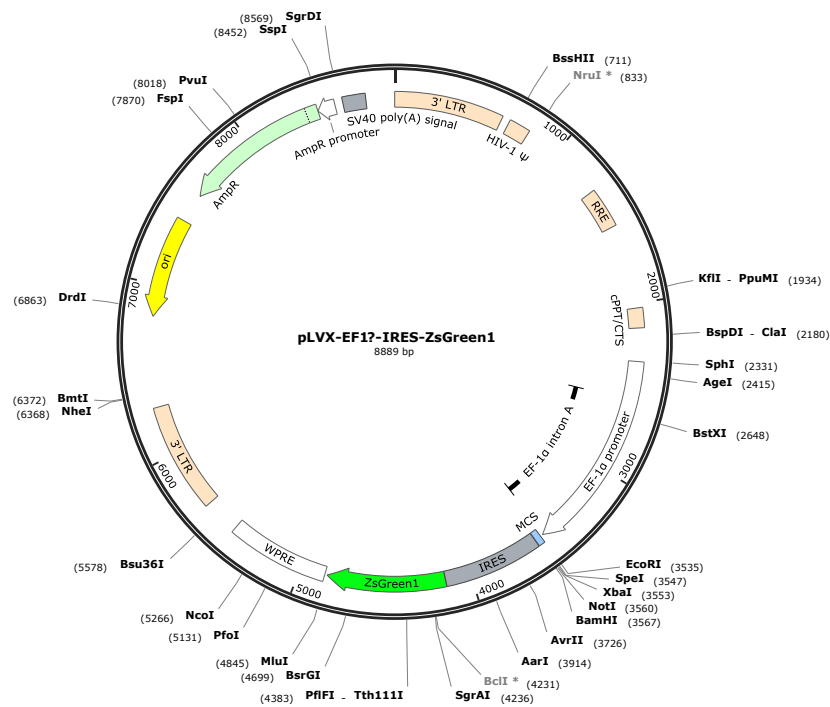


Figure 18: Examples of plasmid backbones used in this work.

2.2.3.6 Cell culture

Haematopoietic cells

About 1×10^6 Lin⁻ cells were seeded in round-bottom 96-well plates in StemSpan™ (Stem Cell Technologies, Vancouver, BC, #09600) supplemented with 1% penicillin/streptomycin for lentiviral transduction, 100μL/well.

MEFs

About 2.0×10^6 cells were seeded in cell-treated T75 flasks in DMEM (Gibco, #41966-029) supplemented with decomplexed 10% foetal bovine serum, and 1% penicillin/streptomycin and kept at 37°C and hypoxia until confluency. Then, cells were retrieved using 0.25% Trypsin-EDTA (Gibco, #25200-056), washed with PBS and re-seeded for experiments, or frozen as cell pellets for Western Blot or RT-qPCR.

NIH 3T3 cell lines and HEK 293T cell lines

About 0.9×10^6 mycofree cells were seeded in cell-treated T150 flasks in DMEM (Gibco, #41966-029) supplemented with decomplexed 10% foetal bovine serum, and 1% penicillin/streptomycin and kept at 37°C and normoxia until confluency. Then, cells were retrieved using 0.25% Trypsin-EDTA (Gibco, #25200-056), washed with PBS and re-seeded or frozen as cell pellets for Western Blot or RT-qPCR.

2.2.3.7 RNA extraction and RT-qPCR

DNA was extracted and purified from frozen cell pellets using the RNeasy Plus Micro kit (Qiagen, Hilden, Germany, #74034) following the manufacturer's instructions. RNA was quantified using the NanoDrop One (Thermo Fischer, Waltham, Massachusetts, MA).

cDNA was synthesized from purified RNA using the SuperScript Vilo Master Mix (Thermo Fischer, Carlsbad, CA, #11755-050) following the manufacturer's instructions. The protocol was 10min of annealing at 25°C, 60min of retrotranscription

at 42°C, and 5min of enzymatic inactivation at 85°C (Veriti Thermal Cycler, Applied Biosciences, Waltham, Massachusetts, MA).

Quantitative PCR was performed using the PowerSYBR Green PCR Master Mix (Applied Biosciences, Waltham, Massachusetts, MA, #4367659) following the manufacturer's instructions in the StepOne Plus PCR System (Applied Biosciences, Waltham, Massachusetts, MA). Except for *Fancg*, the protocol used was 10min holding stage 95°C, 50 cycles 15s 95°C and 50s 60°C, 15s 95°C, 1min 60°C and 15s 95°C melting curve. For *Fancg*, the second step of the cycling stage was at 61.5°C. Gene expression was normalized with the expression of *Gapdh*.

The primers used for PCR reactions were:

Evi-1 forward: ATTTTCATCGGGAACAGCAAC
Evi-1 reverse: ACGAGTCACCCCTAGCTCCT
Fancg forward: TGCTAGCAAGTCGATGCCTG
Fancg reverse: GGGAGAACCTTGTCTCTGAGC
Gapdh forward: CATCACTGCCACCCAGAAGACTG
Gapdh reverse: ATGCCAGTGAGCTTCCCGTTTCAG

2.2.3.8 Protein extraction and Western Blot

Protein extracts were prepared from frozen cell pellets with a RIPA lysis and extraction buffer (Thermo Fisher). Samples were dosed using the QuantiPro™ BCA Assay Kit (Sigma Life Sciences, ref: 1002803688) according to manufacturer's instructions. Absorbance was measured by CLARIOstar Plate Reader (BMG Lab Tech, France).

Western Blots were performed using a standard protocol. The antibodies used were against murine *Fancg* (sc-393382, Santa Cruz Biotechnology; PA5-27117, Thermo Fisher; ab-151516, Abcam), human EVI1 C50E12, Cell Signaling Technology), actin (Santa Cruz) and vinculin (Santa Cruz).

2.2.3.9 Competitive transplantation experiments

C57BL/6J Ly5.1 (CD45.1) wild-type mice were lethally irradiated (9Gy single-dose) one day before transplantation. A total of 5×10^5 lineage-negative cells suspended in 200µL of phosphate buffered saline (PBS) were injected in the tail vein. The number of effectively transduced cells varied from 2×10^4 to 7×10^4 for each condition and were kept at a 1:1 ratio between groups. For some experiments, 5×10^5 to 1×10^6 whole bone marrow *Fancg*^{-/-} cells were co-injected to support haematopoiesis during the first weeks after lethal irradiation. For one experiment, transduced cells were sorted (Aria III, BD) prior to injection. Engraftment was followed monthly by marrow or blood sampling (CD45.1-PE, CD45.2-APC from BD Biosciences, eGFP, and RFP). Mice were euthanized if signs of discomfort or after 6 months of follow-up. Flow cytometry analyses were performed on Aria III or Fortessa Analyzer.

2.2.3.10 Colony-forming unit assays

3000 lineage-negative or 100-300 LSK cells were plated and cultured in MethoCult 3434 medium (Stem Cell Technologies) and colonies were counted using standard criteria on the 7th day. Cells were treated or not with different doses of mytomicin (MMC)(Roche).

2.2.4 Results

2.2.4.1 Mouse model validation

First of all, we have confirmed our model was suitable for experiments. Lineage negative- or LSK cells harvested from C57BL/6J are sensitive to low doses of mytomicin, whereas paired wild-type cells were resistant (**Figure 19**). *Fancg* mRNA was absent from different tissues from *Fancg*^{-/-} mice. Western blot experiments were not specific enough to compare *Fancg* protein between genotypes (data not shown). I tested three different antibodies, including one targeting amino-acids 1-160 (deleted 1-163).

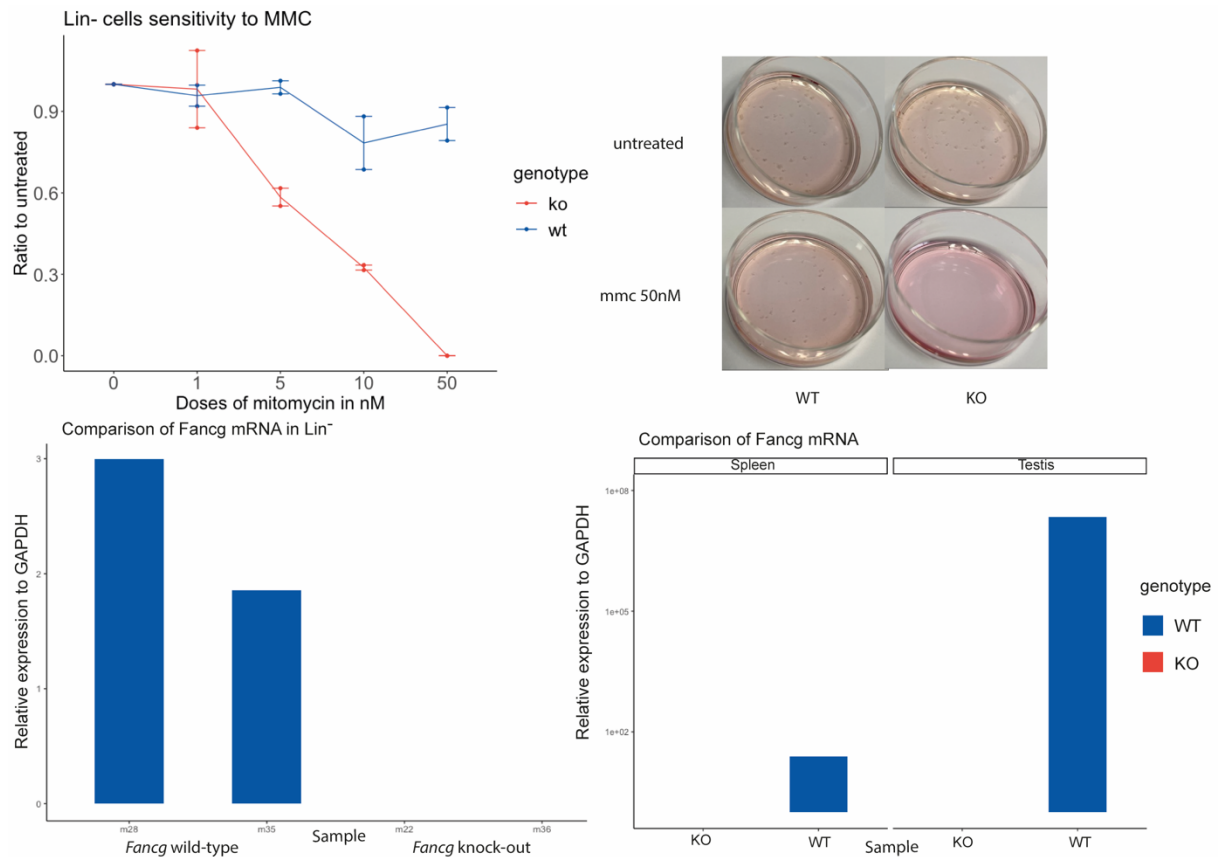


Figure 19: Validating the mouse model

Upper left: CFU assay of KO vs WT lineage-negative cells treated with MMC. Upper right: CFU-assay of KO vs WT LSK cells treated with MMC. Absence of colonies in KO at 50nM. Bottom left and right: Absence of *Fancg* mRNA in different tissues of *Fancg* KO mice.

Fancg^{-/-} mice have high embryonic lethality and the birth rate of the KO genotype was inferior about 10%. Therefore, the number of KO animals was a limitation for my experiments. To mitigate sample availability, I tested if collecting the hip bone would increase Lin⁻ retrieval (**Figure 20**). Collecting femurs, tibias, and hips did not increase the dissection time and increased significantly the number of viable Lin⁻.

Comparison between two strategies for collecting KO Lin⁻

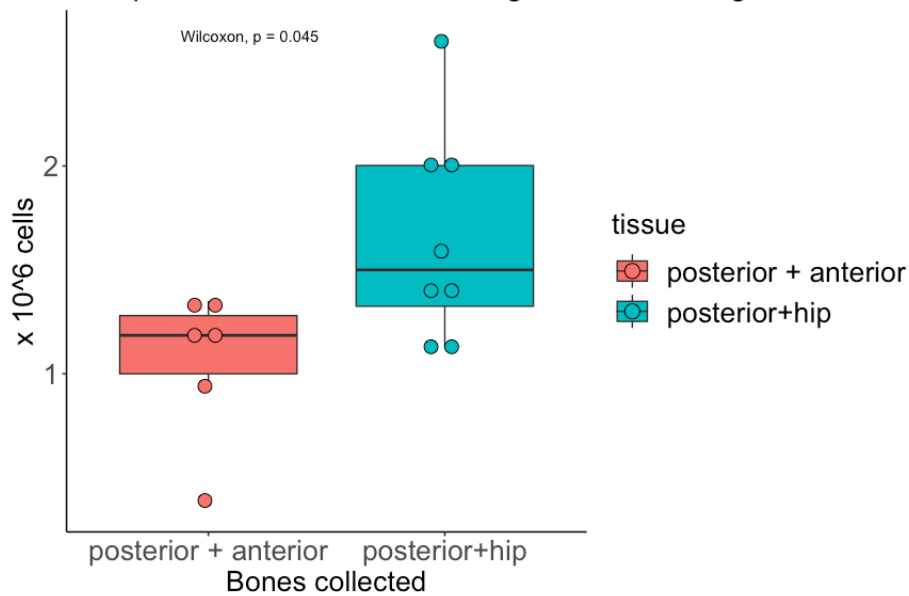


Figure 20: Harvesting Lin⁻

Comparison of the number of viable lineage-negative cells retrieved from femurs, tibias, and anterior limbs (orange) against femurs, tibias and, hips (green).

2.2.4.2 Transduction and plasmid expression

Next, I tested the transduction protocol in Lin⁻ cells. In this early stage of my thesis, I had not controlled the multiplicity of infection (I used 10 μ L of the lentiviral supernatant per 10⁵ cells) and, therefore, had significant variability from one experiment to another. The mean transduction efficiency in Lin⁻ for Lenti-RFP plasmids was 55% of single cells; for pLVX-ZsGreen, 21% at day one.

Despite variability, transduction increased many hundred-fold the expression of *Fancg* in KO Lin⁻ cells normalized to untreated wild-type (**Figure 21**).

The increase in *Fancg* expression was also detectable at the protein level (**Figure 21**). For *Fancg* Western blot, I used KO MEF because of the number of non-specific bands present when using Lin⁻.

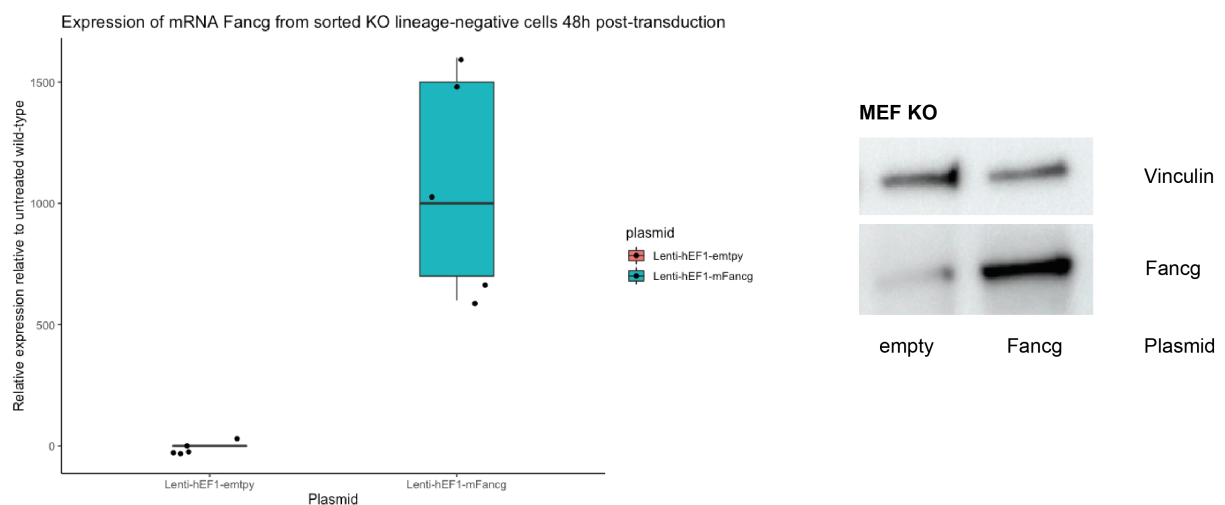


Figure 21: *Fancg* overexpression.

Left panel: RT-qPCR comparing *Fancg* expression at 48h in RFP-sorted KO cells by plasmid. Right panel: Western blot of KO MEF transduced with *Fancg* plasmid or control.

The mRNA expression for Evi-1 was increased over 50-fold in MEF compared to control. This increase was seen in the protein level (**Figure 22**).

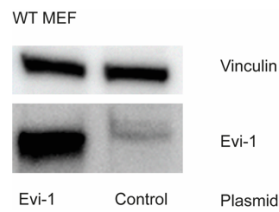


Figure 22: Evi1 protein expression after transduction

2.2.4.3 Competitive experiments

Following the validation of cell harvest and transduction, I have tried to mimic gene therapy. I have transduced KO Lin⁻ CD45.2 either with Lenti-EF1a-*Fancg*-RFP ou Lenti-EF1a-*control*-eGFP and re-injected in competition (1:1 ratio) into 5 lethally irradiated CD45.1 wt. In the first experiment, all recipient mice developed skin ulcers from D+40 and were euthanized. The median CD45.2 engraftment was 98.2% (range: 96.9 – 99.2). There were no detectable RFP or eGFP cells. I repeated the experiment, however, had to sacrifice mice because of primary graft failure.

Because of the negative results, I have re-assessed the Lin⁻ culture and transduction steps. After exchanging with colleagues that performed similar experiments, we have noticed a 40% differentiation of Lin⁻ at 24h in culture. A possible cause was the change in the culture medium lot. My colleagues tested different culture conditions and, from this moment on, I have not added cytokines to the culture medium (lower differentiation rate).

Then, I focused on developing a model of clonal dominance between KO Lin⁻ transduced with Lenti-EF1a-*Fancg*-RFP (gene-corrected) or pLVX-EF1a-*Evi1*-ZsGreen (pre-malignant). Figure represents schematically the next three experiments.

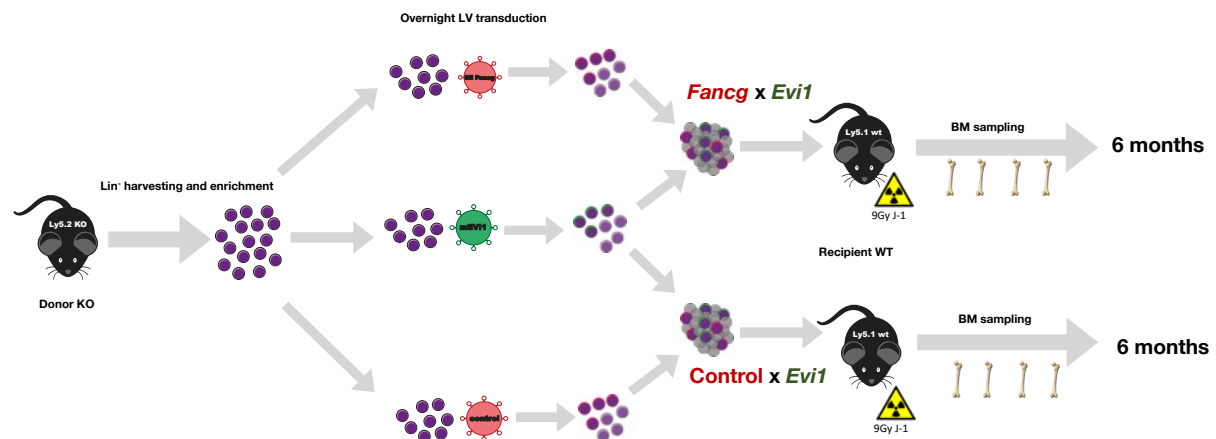


Figure 23: Schematic representation of competitive transplantation experiments.

Following Lin⁻ harvest, purification, and transduction, a 1:1 cell mix was injected in the tail vein of lethally irradiated wt CD45.1 mice. Transplanted mice were monthly followed up to 6 months.

In the first attempt to carry out the experience represented in **Figure 23** (n=10), four mice died or were euthanized within 15 days from irradiation because of graft failure. In the second (n=6) and third (n=6) attempts, I added KO whole bone marrow supporting cells. This strategy allowed me to overcome graft failure; however, one mouse was euthanized in each experiment because of skin ulcers. Ten other mice were received only *Evi1*--transduced cells.

In total, I have transplanted 42 mice and 21 reached 6 months follow-up (9 primary graft failure, 7 euthanized because of skin ulcers, 5 of unknown causes. Transduced cells were not detectable in blood or marrow in any analysed mice. On the other hand, donor engraftment was excellent (**Figure 24**).

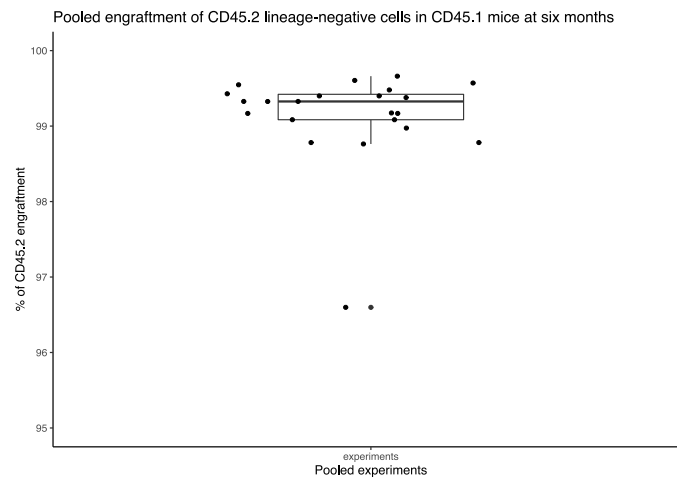


Figure 24: Engraftment

Nearly all of recipients' mononuclear marrow haematopoietic cells were of donor origin (CD45.2) at 6 months.

Therefore, the transplantation steps were functional (9Gy irradiation and KO support whole bone marrow). I have discussed with the animal facility team about my results and, specially, the skin ulcer clinical picture. Serial sampling assessments for infectious organisms in the animal facility were negative and the experimental plan is not in favour of graft-versus-host disease (inbred strain and co-isogenic backgrounds). Thus far, the aetiology is skin ulcers is not clear.

Notwithstanding, the absence of transduced cells in all analysed mice intrigued me. So, I investigated why it did not work.

2.2.4.4 Colony-forming unit assay

The first question I asked myself was how the transduced cells behaved in culture. So, I transduced Lin⁻ with pLVX-EF1a-*Evi1*-ZsGreen or pLVX-EF1a-control-ZsGreen and put in methylcellulose culture, bulk and sorted. Each week I measured the proportion of ZsGreen-positive cells in flow cytometry. Astonishingly, the proportion dropped below 5% in all conditions by day 14 (**Figure 25**). My hypothesis was of a viral failure or a negative selection of transduced cells.

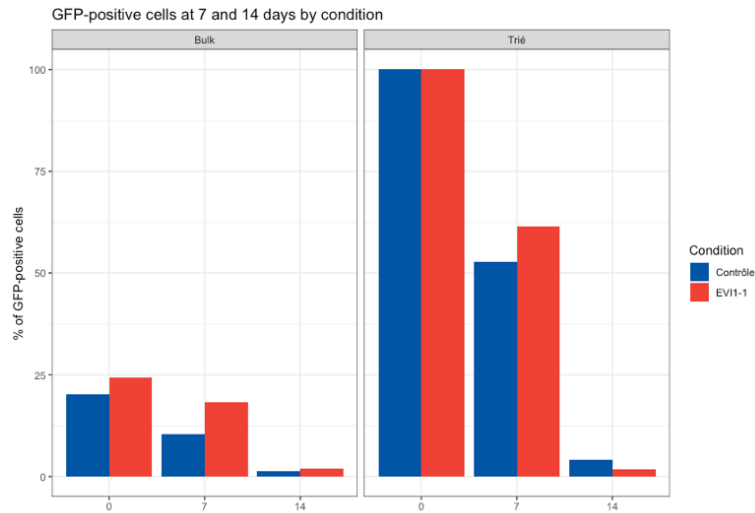


Figure 25: CFU-assay of transduced KO Lin- cells

2.2.4.5 Transduced MEF

To assess if the loss of RFP or GFP/ZsGreen signal was tissue specific, I transduced wt MEF with different constructions and kept them in culture for 4 passages (**Figure 26**). Contrary to what I observed for Lin⁻ cells, the signal remained stable. These results strongly supported that viral integration worked. Therefore, negative selection was the main possibility.

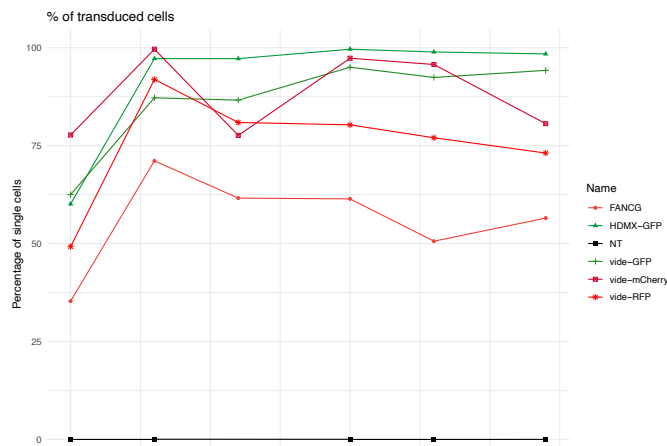


Figure 26: Proportion of transduced MEF through time

2.2.4.6 Multiplicity of infection, efficacy, and viability

The first uncontrolled variable I had in mind was the viral supernatant. We had an important batch-to-batch lentiviral titration variability, reaching 4 log between some batches (**Figure 27**). In other words, the number of virions per cell was unknown.

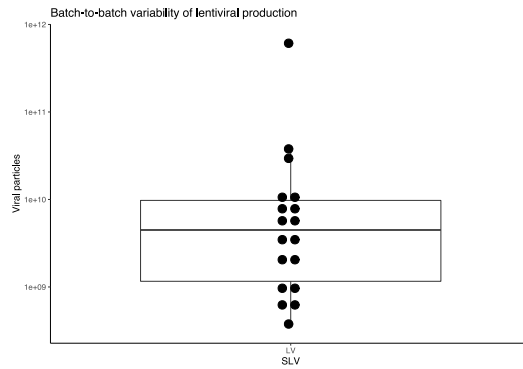


Figure 27: qPCR of viral titration, pooled constructions.

So, to choose the best ratio, I transduced KO and WT Lin⁻ with different multiplicities of infection and measured the efficiency by flow cytometry. At MOI<50, the increase in efficiency was evident with the increase in viral concentration (**Figure 28**). However, it seems of no benefit to go beyond 50.

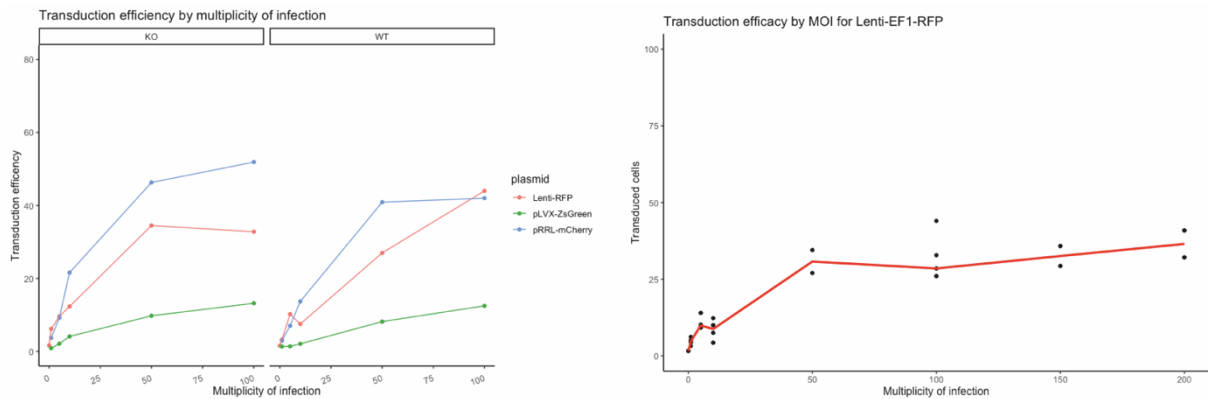


Figure 28: Efficiency x multiplicity of infection

Left panel: Transduction efficiency with different constructions at increasing MOIs. Right panel: Transduction efficiency by multiplicity of infection using the Lenti-EF1a-RFP construction.

Next, I measured apoptosis in transduced cells using annexin V. Surprisingly, nearly 80% of cells transduced with pLenti-RFP constructions were apoptotic from MOI 1 and higher MOIs had little or no effect, whereas more than 70% of nontransduced cells from a same sample were viable (internal control, **Figure 29**). Therefore, the high MOI could not explain the negative selection, at least not alone.

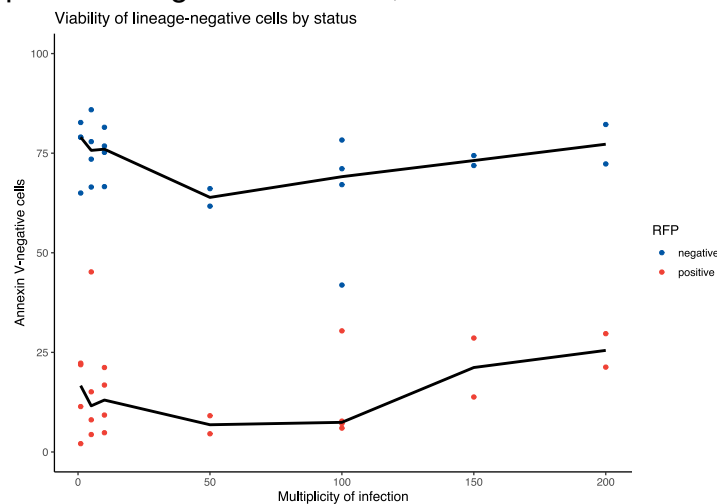


Figure 29: Viability of lineage-negative cells by transduction status.

Within a same sample, the viability of transduced cells (red dots) was much lower than nontransduced cells (blue dots).

Intrigued by such observations, I tested apoptosis in KO and WT cells. Viral integration causes double strand breaks and might be too toxic to Fanconi cells. Furthermore, I tested different constructions to assess if results were associated with a viral factor. Surprisingly, nearly all transduced cells with pLVX or pLenti were apoptotic, regardless of the genotype (**Figure 30**).

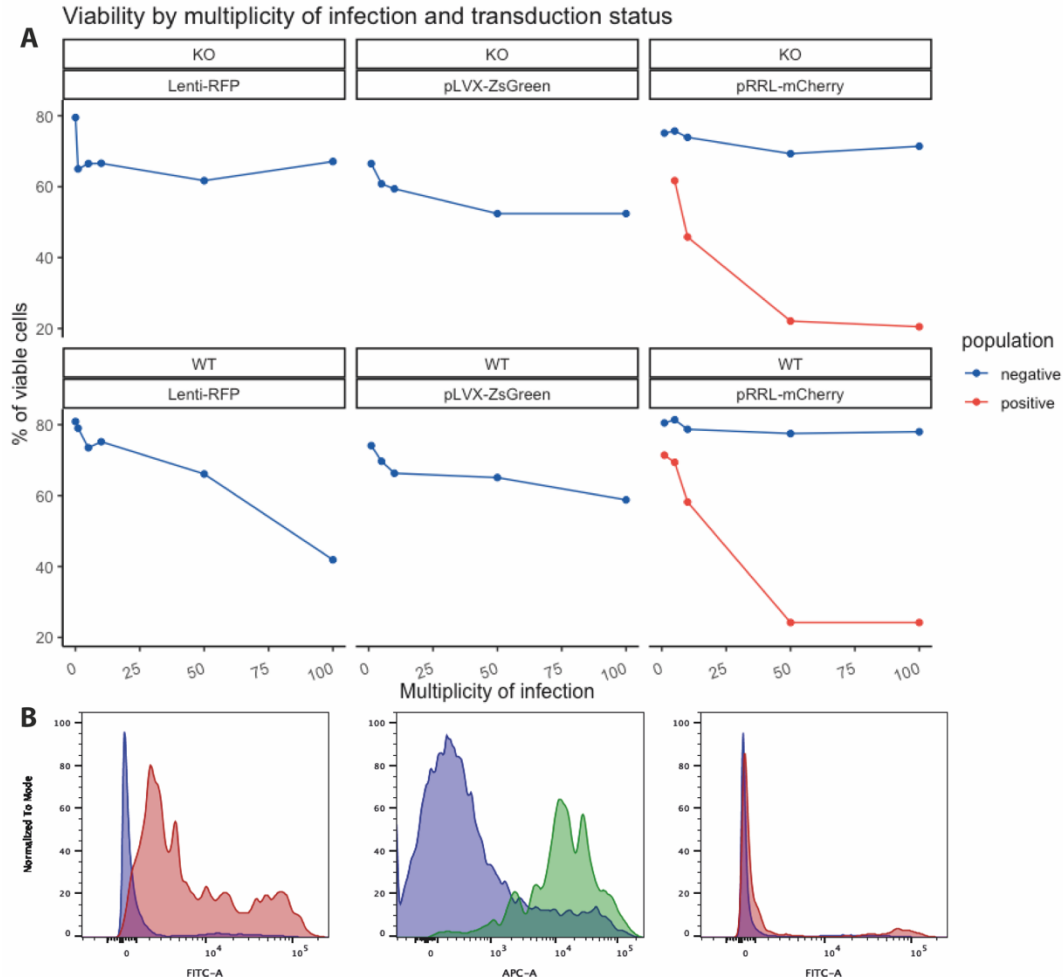


Figure 30: Viability x MOI x plasmid x genotype

A) Viability by multiplicity of infection, transduction status, construction, and genotype. B) Representative flow cytometry histogram of transduced and non-transduced cells at MOI of 5.

Based on these observations, the construct seems to play a major role on cellular toxicity.

2.2.5 Discussion and lessons learnt from this study

My main objective was to investigate if the functional correction of HSPCs was protective against the evolution into AML (**Figure 17**). We have observed that, among revertant patients, none presented overt MDS/AML. Our hypothesis was that functionally revertant HSPCs would outcompete FA-deficient cells in the haematopoietic niche. Revertant cells would, therefore, halt the process of premalignant clonal dominance seen in FA patients (1q+, 3q+, MDS/AML).

The validation of our hypothesis can have therapeutic consequences: in transplanted FA patients, the FA haematopoietic tissue is replaced by a healthy one. Besides reducing the burden of oligoclonality by debulking possible pre-existing premalignant clones, the new allogeneic immune system's surveillance detects and

eliminates residual cells. However, this benefit comes with a cost: FA patients are very sensitive to genotoxic stress and transplant conditioning regimens highly depend on alkylating agents (irradiation is rarely used for Fanconi patients). Besides conditioning short-term toxicity, transplanted patients are at risk of graft-versus-host disease. GvHD might further increase the risk of solid cancers among FA patients. Notwithstanding, HSCT clinical outcomes for FA patients improved greatly in the last decades and survival is excellent for young patients transplanted before transformation.

Gene therapy offers an alternative: *ex vivo* corrected autologous cells overcame the risk of GvHD and engrafted without any prior conditioning regimen, reflecting an ecologic advantage over Fanconi cells *in vivo*.

To test our hypothesis, I worked on a preestablished C57BL/6J *Fancg*^{-/-} Ly5.2 available at IRSL animal facility. The team was knowledgeable on the model and on the style of experiences I would perform. I am grateful for the competence transfer. I could also profit from an already validated transduction protocol and viral constructs that were already available at the lab.

After validating the pre-transplantation steps, I have started *in vivo* experiences one after another. When I noticed the absence of ZsGreen or RFP cells in the follow-up, I had concluded that it was too early to detect transduced cells. It was after reaching 6 months FU for the first experiment that I concluded the others would not work as well.

I have encountered some experimental difficulties along the process. First of all, *Fancg*^{-/-} are born at sub-mendelian rates and we had to increase the number of het x het couples (KO mice are infertile). Besides, I changed the strategy of cellular harvesting to increase yield.

Another problem was early mortality following irradiation. Undoubtedly, one factor that progressed through time was my skill of injecting, and I am confident that it has affected my first results. However, I still observed mortality within 15 days following irradiation after having mastered the technique. Co-injecting untreated whole bone marrow cells surmounted early mortality.

Some mice presented skin ulcers and the aetiology remains unclear. Initially, I thought it would be GvHD, but after discussing with many other researchers, this hypothesis is unlikely. Infection does not seem to be the case either. C57BL/6J can spontaneously develop ulcerative dermatitis.¹⁶⁷ It is my diagnosis of exclusion.

Contrary to competitive transplantation against WT cells,⁹¹ *Fancg*^{-/-} Lin⁻ result in excellent engraftment when injected alone (**Figure 24**). Yet, I could not understand the absence of transduced cells following donor engraftment with 6 months follow-up. I controlled all the other variables I have identified (Lin⁻ differentiation, integration, gene expression, transduction efficacy, multiplicity of infection).

Surprisingly, I found out that nearly all the cells transduced with the constructions I used in this project died within 48h (**Figure 30**), and, likely, the cells that were still alive at that moment died soon after. Cells had good viability with a third construct that a colleague uses in her project. The mortality following Lenti-hEF1-tRFP and pLVX-EF1-ZsGreen explained my results, both *in vivo* and *in vitro*.

What both plasmids have in common that is different from the third plasmid is the promoter: hEF1, human elongation factor 1 is a strong promoter used for gene-overexpression. Indeed, I noticed that *Fancg* or *Evi1* were many hundred-fold overexpressed in transduced cells. Such promoters have the advantage of robust expression and are less frequently silenced in hematopoietic cells than other promoters. However, strong promoters can also increase the expression of *cis*-

neighbouring genes to the viral insertion site. Perhaps unwanted genetic overexpression was toxic for the Lin⁻.

2.2.6 Perspectives

I will test different constructs with different promoters with *Fancg / Evi1*. I will validate the transduction and gene expression as I did for the first couple of constructions. I will also test if the addition of retronectin allows for higher transduction efficacy at lower MOIs and, therefore, lower viral toxicity. After choosing the most adequate construction and MOI, I will perform CFU assays to test the persistence of transduced cells after passages. I will test the functional reversion of the cellular FA phenotype by demonstrating that gene-corrected *Fancg*^{-/-} became resistant to MMC (CFU).

Another difference from the original protocol is that I will use LSK instead do Lin⁻ because they are more enriched in true stem cells and have lower differentiation (data not shown).

Once these steps work (a viral construction with a less powerful promoter and LSK cells), I will perform another *in vivo* experiment.

Chapter 3: Concluding remarks

CH is part of physiological ageing. Surveillance is important to adapt / modify the therapeutic strategy before progression to AML/MDS regardless the BMF status.

However, CH is more frequent among BMF patients than in the general population. In the presence of unfavourable intrinsic (i.e., DNA-repair deficiency and consequent p53/p21 response) or extrinsic (i.e, immune aplastic anaemia or radio-chemotherapy) haematopoiesis can be sustained by more fit clones. Such clones can have built-in proliferative advantages (arguably pre-malignant, as in CHIP), or simply be more adapted to the environmental (protective, as in reversion) and mediate homeostasis. Somatic genetic rescue is not restricted to the haematopoietic tissue: in inflammatory bowel disease, clones resistant to IL-17 help maintain intestines' function not taking part in tumorigenesis; on the contrary, tumour formation was reduced in a mice model.^{168,169}

BMF patients with CH seem to present two very different natural histories: lower risk of MDS/AML as reversion in IBMF; higher risk to MDS/AML as specific myeloid driver mutations in BMF (**Figure 31**). Although GPI⁻ or HLA⁻ seem to confer a protection at the cellular level, it seems not to confer a competitive advantage over the remaining GPI⁺ or HLA⁺ on the long run. Indeed, MDS/AML can stem from such cells in patients with PNH or HLA loss of function mutations or 6p-LOH. On the other hand, reversion or compensation are seen across IBMF (besides FA, GATA2,¹⁷⁰ TBD,^{134,151,171} and SDS¹³⁶), and transformation to MDS/AML seem exceptional.

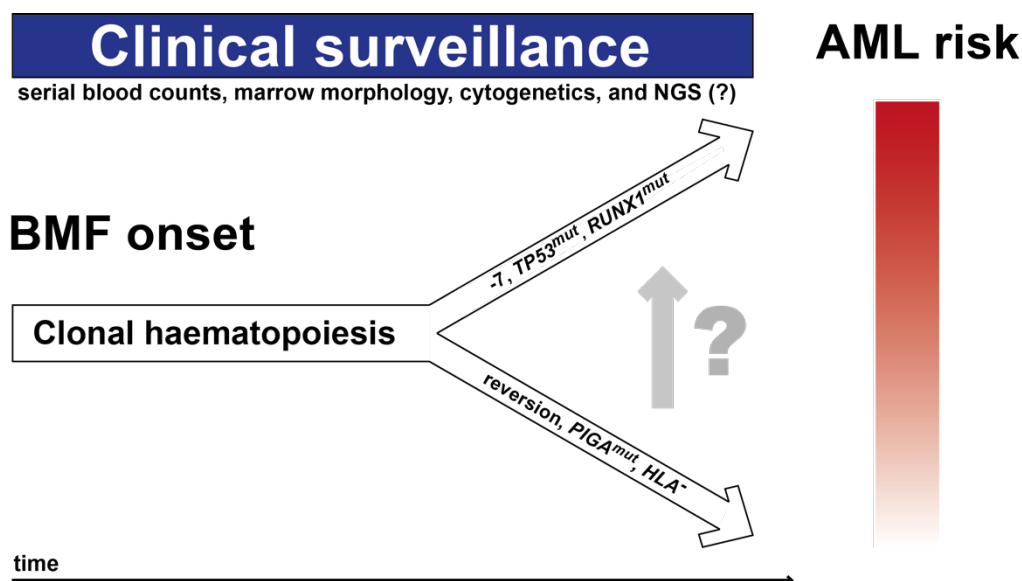


Figure 31: Different natural histories for BMF patients

Close surveillance including at least complete blood counts, marrow morphology and conventional cytogenetics at regular intervals is mandatory for all BMF patients. We still have insufficient evidence to recommend indiscriminate NGS – an isolated molecular abnormality will hardly change therapeutics. NGS and functional studies (as FANCD2 Western Blot) be considered on an individual basis.

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Annexes

Annexe I: Rare Germline *Complement Factor H* Variants in Patients with Paroxysmal Nocturnal Hemoglobinuria

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Key point

- Rare germline *CFH* variants are overrepresented among PNH patients

Abstract

The response to eculizumab among paroxysmal nocturnal hemoglobinuria patients is variable. In this cohort study, we used next-generation sequencing to screen 84 PNH patients treated with eculizumab for complement regulatory gene variants, compared the allele frequencies with populational data and a geographically-matched control group, and looked for an association between the presence of variants and response to treatment. Transfusion independence was evaluated at six months. Sixteen patients presented rare variants, 9 in the *CFH* gene. Germline *CFH* variants were more frequent among PNH patients than in healthy controls ($p=0.02$) or public data ($p<0.001$) and were more likely to be transfusion dependent at six months after eculizumab initiation ($p=0.015$). With a median follow-up of 5.8 years, all but one *CFH*-variant patient received transfusions, and two developed a thrombosis. No *CFH*-variant patient fulfilled the criteria for severe aplastic anemia from the initiation of eculizumab. We show for the first time that *CFH* variants are overrepresented among PNH patients and that they were associated with suboptimal response to eculizumab. Patients' molecular stratification to tailored treatments, e.g., proximal complement inhibition, could reduce costs and morbidity.

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare disease that manifests classically as intravascular hemolysis, bone marrow failure, and thrombosis. It is caused by somatic mutations in the *PIGA* gene, resulting in impaired glycosylphosphatidylinositol (GPI) biosynthesis.¹ GPI anchors many proteins on cells' surfaces, including the decay-accelerating factor (CD55) and the membrane inhibitor of reactive lysis (CD59) – which are modulators of the alternative and terminal complement pathway. Impaired GPI synthesis leads to complement-mediated hemolysis, the hallmark of PNH.²

The most widely available treatments for PNH act as terminal-complement inhibitors, binding to C5, halting hemolysis, and reducing the occurrence of thrombosis.³⁻⁵ The clinical response varies from complete to none.^{6,7} C5 polymorphisms, red blood cell C3d opsonization, and genetic variants in complement regulatory genes might partly explain this variability.⁸⁻¹⁰

Complement factor H (CFH) is a crucial alternative pathway regulator acting on the host-cell surface or in plasma, protecting them from complement damage. Impaired regulatory functions of factor H, caused by genetic abnormality or autoantibodies, have been linked to kidney diseases such as atypical hemolytic uremic syndrome and C3 glomerulopathy.¹¹ Both membrane-bound regulators and soluble complement inhibitors, particularly factor H and factor I, provide efficient protection from unwanted complement-mediated damage, especially under conditions with strong complement activation.

Therefore, we aimed to screen PNH patients for constitutional variants in complement regulatory genes, compare variant frequencies from PNH patients with population data and a geographically-matched healthy control group, and correlate the presence of such variants with response to anti-C5 treatment. We searched if these variants were associated with transfusion dependence six months after eculizumab initiation.

Study design

Data collection

We studied patients diagnosed with PNH by flow cytometry or Ham Dacie's test, who received treatment with eculizumab, were enrolled in the , and had available blood samples for molecular profiling with next-generation sequencing (NGS). Patients were excluded if follow-up was inferior to 6 months, insufficient clinical data for transfusion independence, inclusion in a clinical trial within six months from the start of eculizumab therapy, or upfront clinical trial. All participants signed informed consent. This research was carried on by the Helsinki Declaration. The research, registry, and biobank were approved by the *Comité de protection des personnes Ouest VI*.

Gene sequencing

Genomic DNA was extracted from blood, buccal swabs, and fibroblasts samples. All coding and flanking intronic sequences of *CFH*, *CFI*, *MCP* and *C3* genes were analyzed by NGS as reported.^{11,12} We defined rare when a variant minor allele frequency was below 0.1% in the general population. We considered pathogenic those in which the genetic change affected the protein function (by *in vitro* functional studies supporting a damaging effect on the gene product), found in a disease-related functional domain, or affected protein expression (nonsense, frameshift, canonical ± 1 or two splice sites variants, well-demonstrated lack of *in vitro* synthesis, or quantitative

deficiency in the patient's plasma as previously described for complement regulatory genes).¹³ Other variants were classified as variants of uncertain significance (VUS).^{13,14} We confirmed variants' constitutional nature by sequencing DNA extracted from buccal swab samples or from cultured skin fibroblasts.

Statistical analyses

Qualitative variables are presented as numbers and percentages and compared with Fisher's exact test. Quantitative variables are presented as medians with ranges and interquartile ranges and compared with the Wilcoxon test. The distribution of rare variants in this cohort was compared to a control group of 80 healthy individuals and public data (1000 Genomes).

Transfusion dependence was the primary endpoint, defined as more than two red blood cell transfusion episodes during six months as a binary outcome. The secondary outcome was the event-free survival (EFS), defined as the first event of red blood cell transfusion after 28 days from eculizumab initiation (loading dose), thrombosis, hematopoietic stem cell transplantation (HSCT), evolution to a WHO 2016-defined myeloid neoplasia or death. Patients free of events were censored at the last follow-up. The EFS was estimated using the Kaplan-Meier estimator. The impact of baseline variables on the primary and secondary outcome was tested using Fisher or Wilcoxon tests and log-rank tests, respectively. We used the reverse Kaplan-Meier method to estimate the median follow. All tests were bilateral with a 5% level. We present EFS with 95% confidence intervals. We used the R software (R Core Team 2021. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>) for statistical analyses and figures.

All clinical and genetic data are presented in the main text or supplementary material. Requests for additional information (deidentified data) should be sent to the corresponding author.

Results and discussion

We found that rare germline *CFH* variants were overrepresented among PNH patients and associated with suboptimal response to anti-C5 treatment (based on transfusion requirements).

Eighty-four patients were eligible for the genetic variant screening, and 83 were evaluable for the primary and secondary objectives. **Supplemental Figure 1** shows the patients' inclusion flow chart.

Patients' characteristics are described in **Supplemental Table 1**. PNH diagnoses ranged from 1978 to 2019. Two patients started eculizumab after relapsed for PNH following HSCT for aplastic anemia, and with autologous reconstitution. Patients bearing a *CFH* variant were older at PNH diagnosis. Other baseline characteristics and PNH clinical picture at diagnosis were not different between variant *CFH* and wild-type (WT) *CFH*. During follow-up, seven patients presented a myeloid neoplasm: six myelodysplastic syndromes, one acute myeloid leukemia, and one chronic myelomonocytic leukemia. Three patients died: two progressions of myeloid malignancy and one of hemorrhage. None of these patients had a rare variant in the studied genes.

Twenty-eight patients had a thrombosis before eculizumab initiation: nine Budd-Chiari syndromes, six calf/femoral deep vein thrombosis, three strokes, two pulmonary

embolisms, two cerebral sinus thrombosis, one mesenteric artery, one splenic, one cochlear thrombosis, and three unspecified sites.

We identified 16 patients with a rare variant in complement regulatory genes: *CFH* (n=9), *C3* (n=4), and *CFI* (n=3) (**Supplemental Table 2**). Among these rare variants, four were deemed pathogenic. We confirmed the germline nature of variants in 10 patients using DNA from buccal swab samples (n=2) or cultured skin fibroblasts (n=8). *CFH* variants were significantly overrepresented among PNH patients in our study when compared to healthy controls (p=0.02) or public data (p<0.001) (**Table 1**).

CFH variants or previous transfusions affected transfusion independence at six months after eculizumab initiation in univariate analysis (33.3% vs. 75.7% for *CFH*-variant and *CFH*-WT, respectively, p=0.015, **Supplemental Table 3**); no other baseline characteristics impacted transfusion dependence.

The median follow-up was 5.8 years [95%CI: 5.2 - 7.3]. The 6-year EFS was 39.5 [95%CI: 27.7-51.0] and 0% for the *CFH*-standard and *CFH*-variant groups, respectively (**Figure 1** and **Supplemental Table 4**). All but one *CFH*-variant patient received transfusions after eculizumab loading dose (**Supplemental Table 4**). One patient remained transfusion independent (never transfused, eculizumab started after a thrombotic event). Two patients had thrombosis under eculizumab therapy. No *CFH*-variant patient fulfilled the criteria for severe aplastic anemia from the initiation of eculizumab. Overall, patients younger at PNH diagnosis, without a *CFH* variant, and transfusion independent before eculizumab treatment had a better 6-year EFS (**Supplemental Table 4**).

Our data support that FH protection is necessary for the survival of PNH cells even during the C5 cleavage inhibition. Factor H is a negative regulator of the alternative pathway, binding to C3b.¹⁵ Among *CFH*-variant patients, we found that the LDH (n=6) was mildly higher, whereas hemoglobin levels (n=7) were lower (**Supplemental Figure 2**) from day 28 to 6 months. These observations are compatible with extravascular hemolysis caused by C3d opsonization (**Supplemental Figure 3**).

Specific *CFH* variants may downsize factor H activity, upregulating the alternative pathway and regulating the C3 and C5 convertases. In patients under anti-C5 treatment, the upstream C3b accumulation might aggravate hemolysis and contribute to poor response to eculizumab. Not all identified *CFH* variants are pathogenic using stringent criteria of functional deficiency. The role of FH on the PNH patient RBC surface is unclear. However, few studies suggested that FH plays an important RBC-protective role and that variation of FH levels may impact the erythrocytes' lifespan.¹⁶

A limitation of this study was the heterogeneity of biological data during follow-up (reticulocyte count and CH50 activity), precluding analyses. We acknowledge the possibility of referral bias (national reference center). We were underpowered to perform subgroup or multivariate analyses because of the rarity of the disease.

Besides anti-C5, the FDA recently approved an anti-C3.¹⁷ Moreover, a factor B inhibitor presented promising results in a phase 2 study.¹⁸ Patients bearing these *CFH* variants might respond differently to proximal complement inhibitors.

The functional consequences of *CFH* variants and their possible role in GPI-deficient clones' positive selection are incompletely known to date. Our findings may help better understand the heterogeneity of PNH clinical course and response to anti-C5 treatment. NGS is becoming progressively accessible in clinical care, and patients' molecular stratification in choosing the treatment is standard for many diseases. PNH

drugs are expensive. Tailored treatments could reduce costs and morbidity, ultimately improving patients' quality of life. Our results need validation and justify a collaborative study to confirm the impact of *CFH* variants on response to PNH treatment.

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Authorship contributions: PHP helped elaborate the project, participated in patient recruitment, collected, analyzed, interpreted clinical and molecular data, and wrote the manuscript; JEG performed statistical analysis on clinical data; FSF participated in patient recruitment and collected samples; PED participated in patient recruitment and collected clinical data. PVM and SR performed molecular analyses; ACL collected clinical data; LL and JS prepared and biobanked samples and gave helpful intellectual insights. GS participated in patient recruitment, gave helpful intellectual insights, and edited the manuscript. VFB performed molecular analyses, interpreted molecular data, helped elaborate and sponsor the project, and edited the manuscript. RPL helped elaborate and sponsored the project, participated in patient recruitment, analyzed, interpreted clinical and molecular data, and edited the manuscript. All authors read and approved the final version of the manuscript and are accountable for all aspects of the work.

Conflicts of Interest Disclosures: The authors declare no competing financial interests.

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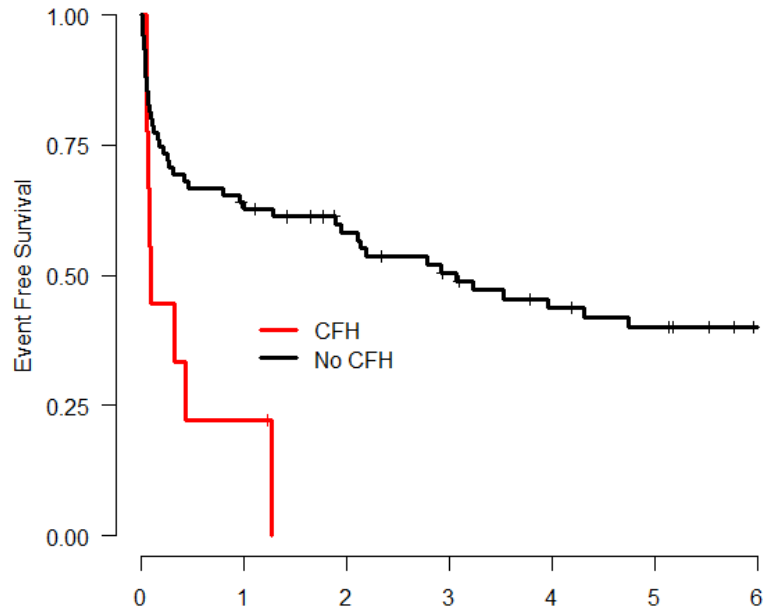
Legends:

Table 1. Prevalence of rare variants (MAF<0.1%) among PNH patients and controls

n: number of individuals carrying rare variant. None of the tested patients carry rare variants in MCP and CFB genes.

^a <https://gnomad.broadinstitute.org>

Figure 1: Event-free survival of PNH patients bearing a *CFH* variant (red) versus without a *CFH* variant (black). Events were first red blood cell transfusion after the eculizumab loading dose, thrombosis, HSCT, evolution to myeloid neoplasia, or death. The 6-year EFS for the no *CFH* group was 39.5 months [95%CI: 27.7-51.0].

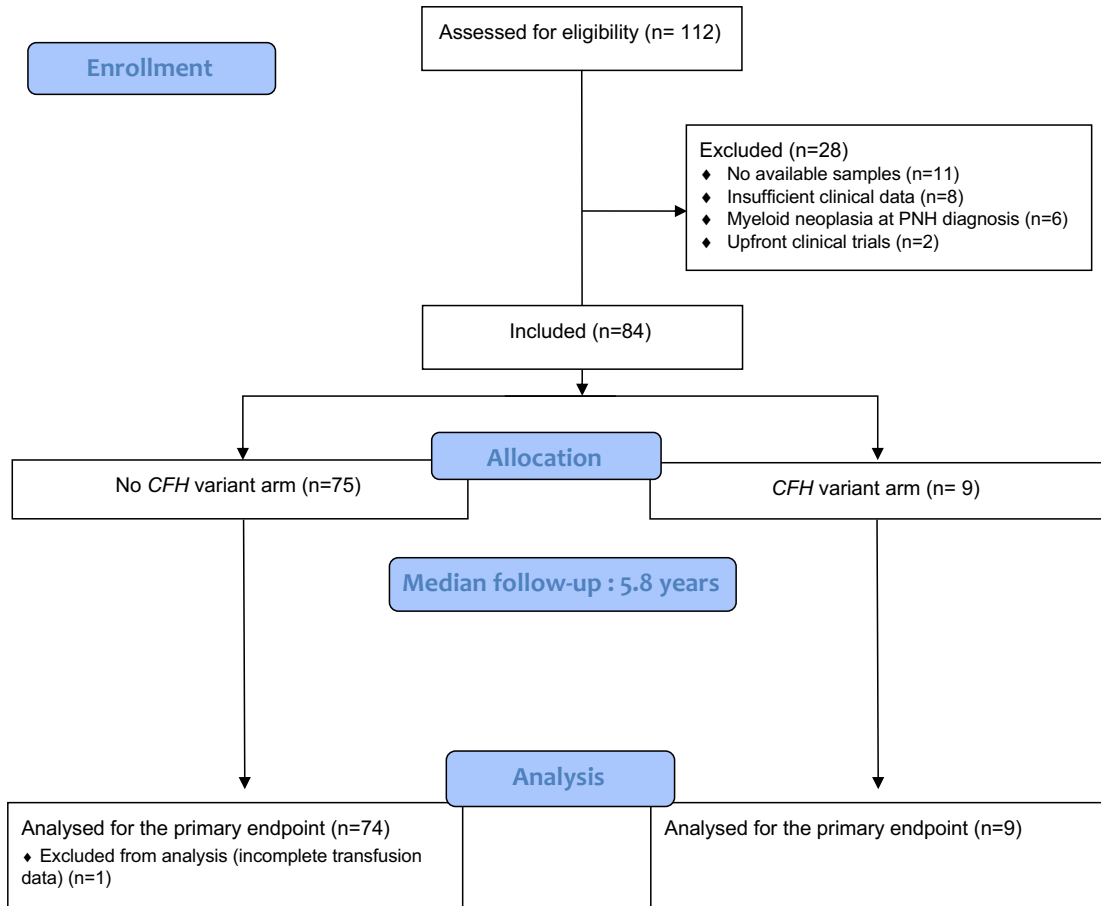


	From 28 days after Eculizumab initiation (years)						
Number at risk:	0	1	2	3	4	5	6
CFH:	9	2	0	0	0	0	0
No CFH:	75	46	38	31	25	22	17

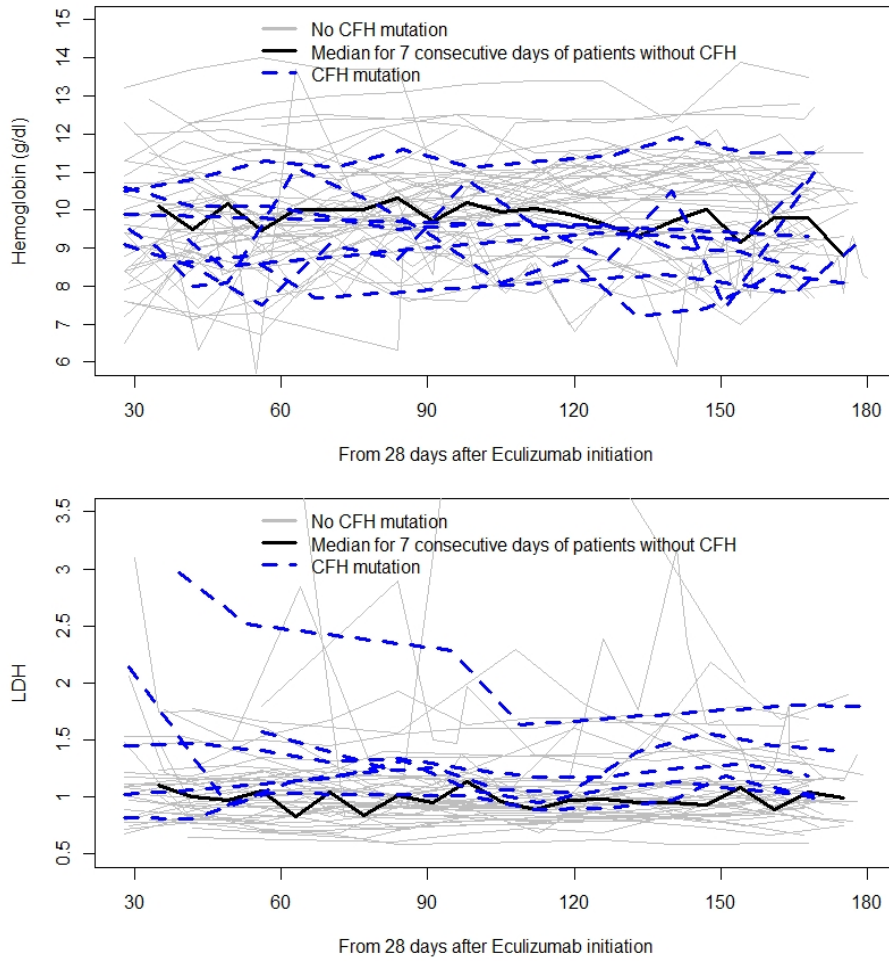
	PNH (n=84)	French controls (n=80)	1000 Genomes controls (n=503) ^a	p (PNH vs French controls)	p (PNH vs 1000 genomes)
Individuals with rare variants	16 (19)	5 (6.3)	35 (7.0)	0.02	0.001
Individuals with at least one pathogenic variant	4 (4.8)	0 (0)	2 (0.4)	0,12	0,005
<i>CFH</i> rare variant, n (%)	9 (10.7)	1 (1.3)	8 (1.6)	0.02	<0.001
Pathogenic variant, n (% of <i>CFH</i> rare variant)	3 (3.6)	0 (0)	0 (0)	0.25	0.003
<i>CFI</i> rare variant, n (%)	3 (3.6)	0 (0)	7 (1.4)	0.25	0.16
Pathogenic variant, n (% of <i>CFI</i> rare variant)	1 (1.9)	0 (0)	2 (0.4)	1	0.37
<i>C3</i> rare variant, n (%)	4 (4.8)	4 (5)	13 (2.6)	1	0.29
Pathogenic variant, n (% of <i>C3</i> rare variant)	0 (0)	0 (0)	0 (0)	Not tested	Not tested

Supplemental appendix

Supplemental Figure 1: Patients' study inclusion flow diagram

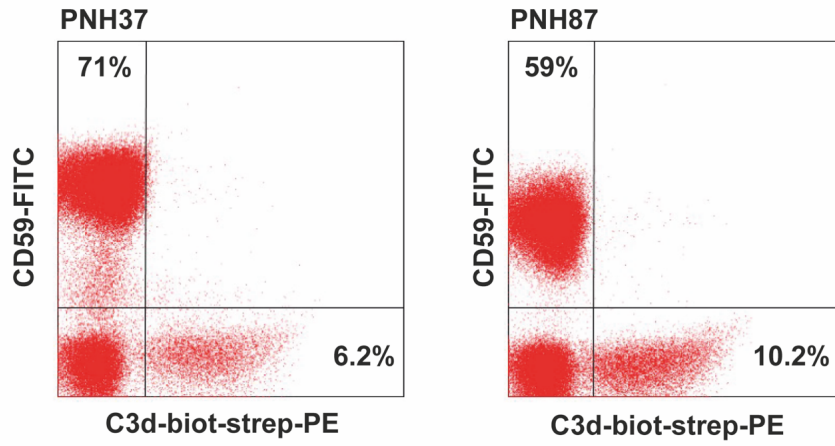


Supplemental figure 2: 7-day moving medians for LDH and hemoglobin



Supplemental figure 2 shows the evolution of biological parameters. Black lines indicate a 7-day moving median for patients without rare *CFH* variants. LDH is indicated as the upper limit of normal ratio. Blue lines indicate *CFH*-variant patients, and background gray lines indicate patients without rare *CFH* variants.

Supplemental Figure 3: Flow cytometry analysis of C3d deposition



Flow cytometry analysis of two CFH-variant patients revealed a CD59⁻C3d⁺ population - bottom right quadrant (GPI-negative cells with C3 deposition). The C3d deposition on red blood cells surfaces might aggravate hemolysis and contribute to poor response to eculizumab.

Supplemental Table 1: Baseline patients' characteristics

Variables	Modalities	All patients N=84	CFH variant		p
			No (N=75)	Yes (N=9)	
Age at PNH diagnosis	median [IQR]	32.2 [24.3-46.4]	31.6 [22.6-44.2]	41.7 [37.5-48.2]	0.046
	(range)	(7.7-77.8)	(7.7-69.9)	(24.7-77.8)	
Patient sex	Female	52 (61.9)	46 (61.3)	6 (66.7)	1
	Male	32 (38.1)	29 (38.7)	3 (33.3)	
Diag PNH to Eculizumab initiation (years)	median [IQR]	1.3 [0.2-5.8]	1.4 [0.2-6]	0.4 [0.1-2.7]	0.32
	(range)	(0-31.3)	(0-31.3)	(0-19)	
SAA at diagnosis	No	45 (53.6)	40 (53.3)	5 (55.6)	1
	Yes	39 (46.4)	35 (46.7)	4 (44.4)	
SAA severity	nSAA	21 (61.8)	18 (60)	3 (75)	Not done
	SAA	13 (38.2)	12 (40)	1 (25)	
	missing	5 among 39	5 among 35	0 among 35	
Hemolysis at diagnosis	No	13 (15.5)	13 (17.3)	0 (0)	0.34
	Yes	71 (84.5)	62 (82.7)	9 (100)	
Thrombosis before eculizumab loading doses (d+28)	No	56 (66.7)	50 (66.7)	6 (66.7)	1
	Yes	28 (33.3)	25 (33.3)	3 (33.3)	
Regular transfusion before eculizumab loading doses (d+28)	No	37 (44.6)	34 (45.9)	3 (33.3)	0.72
	Yes	46 (55.4)	40 (54.1)	6 (66.7)	
	missing	1	1	0	

PNH: paroxysmal nocturnal hemoglobinuria; CFH: complement factor H; IQR: interquartile range; SAA: severe aplastic anemia; nSAA: non-severe aplastic anemia.

Supplemental Table 2: List of rare variants

UPN	Gene	Variant	Transcript	Status	MAF (%)	CADD score	In vitro functional characterization ¹	Summary of the effect	Classification in this report
PNH078	C3	c.1119+1G>A	IVS10+1	Hz	novel	35	no	Splice site variant	Pathogenic
PNH060	C3	c.749G>T	p.Gly250Val	Hz	novel	19.09	no		VUS
PNH032	C3	c.664T>A	p.Phe222Ile	Hz	0.005	27.3	no		VUS
PNH032	C3	c.4100T>C	p.Ile1367Thr	Hz	0.081	17.41	no		VUS
PNH108	C3	c.4100T>C	p.Ile1367Thr	Hz	0.081	17.41	no		VUS
PNH046	C3	c.4100T>C	p.Ile1367Thr	Hz	0.081	17.41	no		VUS
PNH097	C3	c.3083T>G	p.Leu1028Arg	Hz	novel	26.7	no		VUS
PNH103	CFH	c.388G>A	p.Asp130Asn	Hz	0.014	22.6	yes	Normal C3 and FH levels in plasma, minor defects in complement regulation ¹	Pathogenic
PNH097	CFH	c.472G>A	p.Val158Ile	Hz	0.018	0.001	yes	Any identified functional defect ²	VUS
PNH016	CFH	c.472G>A	p.Val158Ile	Hz	0.018	0.001	yes	Any identified functional defect ²	VUS
PNH073	CFH	c.1366G>A	p.Glu456Lys	Hz	0.0008	2.658	no	Normal C3 level in plasma	VUS
PNH028	CFH	c.2077G>A	p.Asp693Asn	Hz	0.016	20.8	no	Low FH plasma level	Pathogenic
PNH087	CFH	c.2295A>T	p.Leu765Phe	Hz	0.0036	0.035	no	Normal C3 level in plasma	VUS
PNH037	CFH	c.2651C>A	p.Ser884Tyr	Hz	0.032	17.24	yes	Any identified functional defect ²	VUS
PNH102	CFH	c.2867C>T	p.Thr956Met	Hz	0.013	14.99	yes	Any identified functional defect ²	VUS
PNH033	CFH	c.3004G>C	p.Gly1002Arg	Hz	0.078	17.45	no	Low FH plasma level with Alternative pathway C3 consumption	Pathogenic
PNH030	CFI	c.672T>A	p.Asp224Glu	Hz	novel	0.038	yes	Any identified functional defect ³	VUS
PNH032	CFI	c.1246A>C	p.Ile416Leu*	Hz	0.02	18.64	yes	Significantly reduced expression compared to WT ⁴	Pathogenic
PNH038	CFI	c.1534G>A	p.Gly512Ser	Hz	0.0017	33	yes	Any identified functional defect ³	VUS

Note: Allele Frequency is given from the GnomAD database. Hz: Heterozygous; VUS: Variants of undetermined significance. PNH032 carried a pathogenic CFI variant (p.Ile416Leu) and two C3 variants of undetermined significance (p.Ile1367Thr and p.Phe222Ile) and was included in the CFI group; PNH097 carried two VUS variant in C3 (p.Leu1028Arg) and CFH (p.Val158Ile) and is included in the CFH group.

*The allele frequency for p.Ile416Leu in African population is 1.2%.

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Supplemental Table 3: Univariate analysis of response to treatment

Variables	Modalities	Six month transfusion status*		p
		No (N=59)	Yes (N=24)	
Age at PNH diagnosis	median [IQR] (range)	31.8 [23.3-42] (7.7-69.9)	34.1 [26.7-50] (11.3-77.8)	0.31
Patient sex	Female	36 (61)	16 (66.7)	0.8
	Male	23 (39)	8 (33.3)	
Rare variant	No	49 (83.1)	18 (75)	0.54
	Yes	10 (16.9)	6 (25)	
CFH variant	No	56 (94.9)	18 (75)	0.015
	Yes	3 (5.1)	6 (25)	
Diag PNH to Eculizumab initiation (years)	median [IQR] (range)	1.3 [0.1-5.1] (0-26.9)	1.5 [0.3-7.1] (0-31.3)	0.58
SAA at diagnosis	No	35 (59.3)	10 (41.7)	0.16
	Yes	24 (40.7)	14 (58.3)	
Hemolysis at diagnosis	No	9 (15.3)	3 (12.5)	1
	Yes	50 (84.7)	21 (87.5)	
Thrombosis before eculizumab loading doses (d+28)	No	40 (67.8)	15 (62.5)	0.64
	Yes	19 (32.2)	9 (37.5)	
Regular transfusion before eculizumab loading doses (d+28)	No	32 (54.2)	5 (21.7)	0.01
	Yes	27 (45.8)	18 (78.3)	
	missing	0	1	

PNH: paroxysmal nocturnal hemoglobinuria; CFH: complement factor H; IQR: interquartile range; SAA: severe aplastic anemia; nSAA: non-severe aplastic anemia.

Supplemental Table 4: Univariate analysis of EFS

Variable	Modalities	6y EFS (%)	p
Patient sex	Female	29.2 [17-42.5]	0.09
	Male	48.9 [29.4-65.7]	
Age at PNH diagnosis (years)	(7.32]	51.6 [34.8-66]	<0.001
	(32.78]	19.9 [8.6-34.5]	
Rare variant	No	39.7 [27.3-51.8]	0.1
	Yes	19 [3.7-43.4]	
CFH variant	No	39.5 [27.7-51.0]	0.002
	Yes	0	
Hemolysis at diagnosis	no	41.7 [15.2-66.5]	0.73
	yes	35.1 [23.5-46.9]	
SAA at diagnosis	no	37.3 [22.5-52.2]	0.58
	yes	34.6 [19.6-50.1]	
Regular transfusion before eculizumab loading doses (d+28)	no	43.3 [25.3-60.1]	0.045
	yes	27.3 [15-41.2]	
Thrombosis before eculizumab loading doses (d+28)	no	34.8 [22-48]	0.69
	yes	38.7 [19.9-57.1]	

CFH: complement factor H; SAA: severe aplastic anemia; nSAA: non-severe aplastic anemia.

Supplemental Table 5: Clinical evolution of patients carrying a CFH variant

Baseline characteristics			Before landmark ¹			After landmark ¹			
UPN	Gender	Age at PNH diagnosis	Thrombosis	Transfusion	SAA	Total RBC by 6 months	Any RBC transfusion beyond 6 months	Response by 6 months	Thrombosis
PNH016	F	25	No	Yes	no	0	Yes	Partial	No
PNH028	F	43	No	Yes	no	10	Yes	Partial	Yes
PNH033	F	38	No	Yes	no	6	Yes	Good	No
PNH037	F	32	Yes	No	no	0	No	Good	No
PNH073	F	78	No	Yes	no	4	Yes	Partial	No
PNH087	M	42	No	Yes	no	0	Yes	Partial	No
PNH097	M	39	No	Yes	no	3	Yes	No response	Yes
PNH102	F	48	Yes	Yes	no	2	Yes	No response	No
PNH103	M	74	No	Yes	no	8	Yes	No response	No

¹28 days after eculizumab initiation. According to Risitano et al.⁷ UPN: unique patient number; PNH: paroxysmal nocturnal hemoglobinuria; CFH: complement factor H; SAA: severe aplastic anemia.

Annexe II: Clonal hematopoiesis driven by chromosome 1q/MDM4 trisomy defines a canonical route towards leukemia in Fanconi anemia

Our team has defined a pathway of clonal dominance and evolution to AML in FA. We studied and analysed direct patient data and experiments from a *Fancg*^{-/-} murine model expressing a third copy of *Mdm4/Hdmx*^{XI} (for *Fancg*^{-/-} mice refer to [Fanconi Anaemia mouse models](#)). I have contributed to this work, studying the role of PARP inhibition in FA-deficient cell lines. This work is currently under revision in Cell Stem Cell.

Clonal hematopoiesis driven by chromosome 1q/MDM4 trisomy defines a canonical route towards leukemia in Fanconi anemia

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Abstract

Fanconi anemia (FA) is the most frequent inherited DNA-repair disease in human driving hematopoietic stem cell (HSC) failure and poor-prognosis acute myeloid leukemia (AML) in children and young adults. Serial explorations in 62 patients who developed bone marrow clonal evolution from a unique cohort of 335 FA patients revealed a unique structural variants and mutational signature that shares features with BRCA-related cancers. We found a canonical sequence of copy number abnormality acquisition, where chromosome 1q duplication drives early *MDM4* overexpression and clonal hematopoiesis, later followed by secondary-AML genomic lesions. Functionally, *MDM4* trisomy conferred greater fitness to FA stem/progenitor cells *in vitro*, rescued bone marrow failure induced by chronic inflammatory stress, and drove clonal dominance in FA mouse models. Our results identify a linear route towards secondary leukemogenesis wherein early downregulation of basally activated p53 signaling plays a pivotal role, raising monitoring and therapeutic prospects for FA patients.

Methods

EBV cell lines

The cells were maintained at a concentration of 0.5 x 10⁶ cells per milliliter in RPMI + GlutaMAX (Gibco, Grand Island, NY, #61870-010) supplemented with decomplexed 10% foetal bovine serum (Gibco, #10270-106), 1% sodium pyruvate (Eurobio Scientific, France, #CSTVAA00-0U), and 1% penicillin/streptomycin (Gibco, #15070-063) at 37°C and normoxia.

- EBV817-L: healthy (negative control)
- EBV398-L: healthy (negative control)
- EUFA423-L: *BRCA2*^{-/-} (positive control)

^{XI} *Mdm4/Hdmx* is a surrogate marker for 1q+ (refer to [Clonal haematopoiesis in Fanconi Anaemia](#)).

- EGF331-L: *BRCA2*^{-/-} (positive control)
- EGF088-L: *FANCA*^{-/-}
- EGF192-L: *FANCA*^{-/-}
- EGF248-L: *FANCA*^{-/-} *BRCA2*^{+/-}
- EGF409-L: *FANCM*^{-/-}

Survival assay

EBV cell lines were plated at a density of 1×10^4 cells per well in opaque flat bottom 96-well plates and exposed to increasing doses of talazoparib (0, 1n 5, 10nM). Cell viability was assessed after 3 days of culture using the Cell Titer Glo Luminescent Cell Viability Assay (G7573, Promega).

Results and Discussion

The treatment for FA-related myeloid neoplasms, especially AML is an unmet clinical need: in the era of precision medicine, patients with a significant DNA-repair deficiency still depend highly on traditional genotoxic chemotherapy. The sole curative treatment option is allogeneic hematopoietic stem cell transplantation. This modality carries a high treatment-related mortality in this genetically sensitive population.

Therefore, we investigated the underlying mechanisms driving progression towards MDS/AML in Fanconi anaemia. We extracted the FA cases from the National Reference Center for Inherited Bone Marrow Failure Syndromes cohort (n=335, probably all FA patients followed in France over 18 years) and found that nearly one-third developed a hematopoietic clonal evolution (somatic cytogenetic abnormality or myeloid neoplasm). We pair-wise (AML cells and cultured fibroblasts for germline benchmarking) studied samples of 15 patients (10 FA core and 5 *BRCA2/FANCD1*). Interestingly, we found that AML cells from the *BRCA2/FANCD1* and the FA-core genotype patients displayed an HR-deficient, BRCA-like signature (COSMIC 3) compared to a TARGET AML control.

Signature 3 is associated with failure of DNA double-strand break repair by homologous recombination. This signature is strongly associated with cancers with somatic or germline *BRCA1/2* mutations. BRCAness is of clinical interest as it might guide treatment choice in HR-deficient cancers. The poly(ADP-ribose) polymerase (PARP) is an early and versatile player in DNA repair, acting in single and double-strand breaks. It contributes to HR and alternative end-joining and inhibits non-homologous end-joining repair. The inhibition of PARP in HR-deficient cells induces synthetic lethality. Currently, at five four PARP inhibitors have FDA/EMA approval for treating HR-deficient / BRCA-like / COSMIC 3 signature solid cancers – Talazoparib, Olaparib, Rucaparib, and Niraparib.

Studies testing PARP inhibition in Fanconi anemia are scarce and contradictory. Following our observations, we wondered if FA-deficient cells were more sensitive to PARPi than FA-competent cells in vitro. I tested a potent PARP inhibitor, talazoparib, in human immortalized EBV-lymphoblasts bearing biallelic inactivations in *BRCA2* (n=1), *FANCA* (n=2), *FANCA + BRCA2*^{het} (n=1), and *FANCM*, and normal. I found that *FANCA*-deficient cell lines do not seem to present increased sensitivity to PARP inhibition, contrary to *BRCA2* (**Figure 32**). On the other hand, the *FANCM* cell line was very sensitive to PARP inhibition. Adding daunorubicin, cytarabine, or mitomycin is not synergistic in Fanconi lines (data not shown). We will validate our findings in other complementation groups and cell lines. Our preliminary data does not support PARP inhibition as a target in FA-core associated MDS/AML. Diversely, haematopoietic malignancies are not the rule for *FANCM* – for such patients, solid cancers is the major concern.¹⁷²

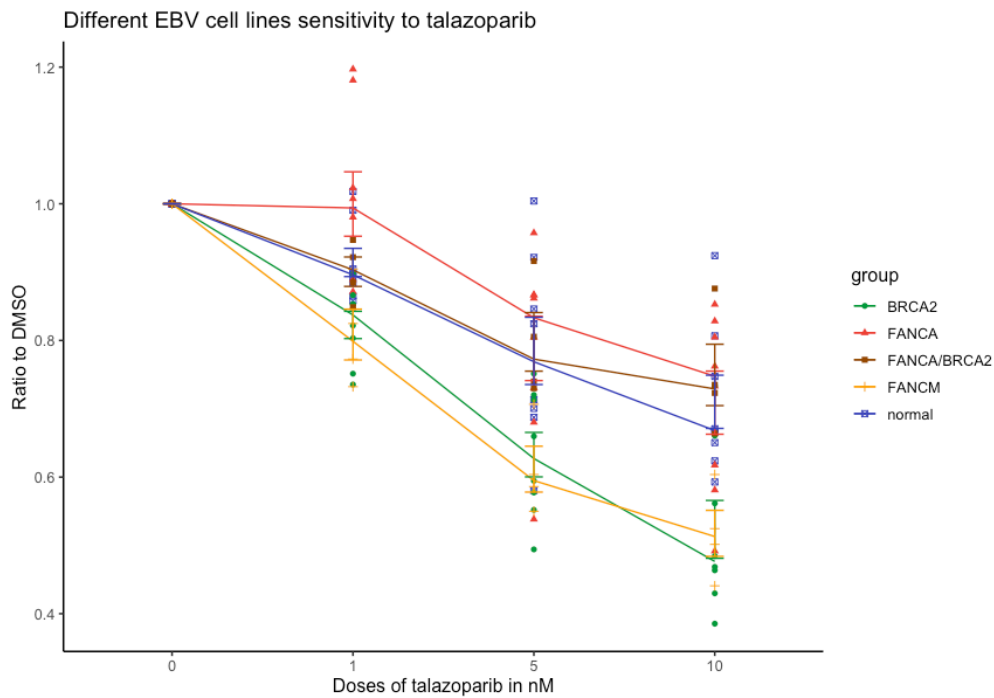


Figure 32: EBV cell line sensitivity to PARP inhibition

Germline heterozygous patients that develop a solid cancer (e.g., breast, ovary), BRCAness is restricted to the neoplastic tissue and PARP inhibition is beneficial. On the other hand, FA patients are present a DNA-repair deficiency in all his / her cells. Thus far, we do not know if toxicity would be prohibitive. The preclinical studies were based on patient-derived xenografts and cell-derived xenografts, *i.e.*, the mice DNA-repair was intact.

We are currently establishing a colony of *BRCA2^{mut}* mice.¹⁷³ These mice's HSPCs present a proliferation defect and spontaneous chromosomal instability but not spontaneous cytopaenias.⁸⁶ Then, we will backcross into the background C57BL/6J CD45.2. Our aim is to develop a *BRCA2*-associated leukaemia using viral transduction (EV1 as presented in Chapter 4), test leukaemia's cells sensitive to PARP inhibition, and toxicity in a constitutively deficient subject. We will investigate a therapeutic window for PARP inhibition in FA-AML.

Annexe III: Haploidentical transplantation and posttransplant cyclophosphamide for treating aplastic anemia patients: a report from the EBMT Severe Aplastic Anemia Working Party

Prata PH, Eikema DJ, Afansyev B, Bosman P, Smiers F, Diez-Martin JL, Arrais-Rodrigues C, Koc Y, Poiré X, Sirvent A, Kröger N, Porta F, Holter W, Bloor A, Jubert C, Ganser A, Tanase A, Ménard AL, Pioltelli P, Pérez-Simón JA, Ho A, Aljurf M, Russell N, Labussiere-Wallet H, Kerre T, Rocha V, Socié G, Risitano A, Dufour C, Peffault de Latour R; SAA WP of the EBMT.

Bone Marrow Transplant. 2020 Jun;55(6):1050-1058.

DOI: [10.1038/s41409-019-0773-0](https://doi.org/10.1038/s41409-019-0773-0)

Abstract

In the absence of an HLA-matched donor, the best treatment for acquired aplastic anemia patients refractory to immunosuppression is unclear. We collected and analyzed data from all acquired aplastic anemia patients who underwent a haploidentical transplantation with posttransplant cyclophosphamide in Europe from 2011 to 2017 (n = 33). The cumulative incidence of neutrophil engraftment was 67% (CI_{95%}: 51-83%) at D +28 and was unaffected by age group, stem cell source, ATG use, or Baltimore conditioning regimen. The cumulative incidence of grades II-III acute GvHD was 23% at D +100, and limited chronic GvHD was 10% (0-20) at 2 years, without cases of grade IV acute or extensive chronic GvHD. Two-year overall survival was 78% (64-93), and 2-year graft-versus-host disease-free survival was 63% (46-81). In univariate analysis, the 2-year OS was higher among patients who received the Baltimore conditioning regimen (93% (81-100) versus 64% (41-87), p = 0.03), whereas age group, stem cell source, and ATG use had no effect. Our results using unmanipulated haploidentical transplantation and posttransplant cyclophosphamide for treating refractory AA patients are encouraging, but warrant confirmation in a prospective study with a larger number of patients and longer follow-up.

Contenu retiré pour des raisons de droit d'auteur

Annexe IV: Published manuscripts as a co-author (2020-2022)

Long-term outcomes and risk factor analysis of steroid-refractory graft versus host disease after hematopoietic stem cell transplantation

Pagliuca S, Prata PH, Xhaard A, Frieri C, Giannoni L, Sutra Del Galy A, Brignier A, Sicre de Fontbrune F, Michonneau D, Dhedin N, Peffault de Latour R, Socié G, Robin M.

Bone Marrow Transplant. 2021 Jan;56(1):38-49.

DOI: [10.1038/s41409-020-0977-3](https://doi.org/10.1038/s41409-020-0977-3)

Abstract

Steroid-refractory graft versus host disease (GVHD) represents a fearsome complication after allogeneic hematopoietic stem cell transplantation (HSCT). We conducted a retrospective study on outcomes and risk factors associated with acute and chronic steroid-refractory GVHD in a large cohort of 1207 patients receiving HSCT in Saint Louis Hospital between 2007 and 2017. Among patients who developed an acute and/or a chronic GVHD, the cumulative incidences of acute and chronic steroid-refractory disease were 31% and 48%, respectively, at day +100 and 1-year post-HSCT. Through a multivariable analysis we selected several risk factors associated with the development of a steroid-refractory disease. For acute GVHD steroid refractoriness, we identified (1) a very high disease risk index, (2) an unrelated donor, (3) the absence of in vivo T-depletion as GVHD prophylaxis, and (4) a reduced intensity conditioning regimen. For chronic GVHD, (1) the use of peripheral blood stem cells, (2) unrelated donors, and (3) absence of in vivo T-depletion were more likely associated with a steroid-refractory disease. After the construction of a multistate dynamic model, we found that the probability of being alive without relapse after the resolution of all GVHD episodes was about 36% in the long term.

Contenu retiré pour des raisons de droit d'auteur

Categorizing hematological response to eculizumab in paroxysmal nocturnal hemoglobinuria: a multicenter real-life study

Debureaux PE, Kulasekararaj AG, Cacace F, Silva BGP, Calado RT, Barone F, Sicre de Fontbrune F, Prata PH, Soret J, Sica M, Notaro R, Scheinberg P, Mallikarjuna V, Gandhi S, Large J, Risitano AM, Peffault de Latour R, Frieri C; Severe Aplastic Anemia Working Party of the European Group for Bone Marrow Transplantation.

Bone Marrow Transplant. 2021 Oct;56(10):2600-2602.

DOI: [10.1038/s41409-021-01372-0](https://doi.org/10.1038/s41409-021-01372-0)

Correspondence. No abstract available

2022 international clinical practice guidelines for the treatment and prophylaxis of venous thromboembolism in patients with cancer, including patients with COVID-19



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The International Initiative on Thrombosis and Cancer is an independent academic working group of experts aimed at establishing global consensus for the treatment and prophylaxis of cancer-associated thrombosis. The 2013, 2016, and 2019 International Initiative on Thrombosis and Cancer clinical practice guidelines have been made available through a free, web-based mobile phone application. The 2022 clinical practice guidelines, which are based on a literature review up to Jan 1, 2022, include guidance for patients with cancer and with COVID-19. Key recommendations (grade 1A or 1B) include: (1) low-molecular-weight heparins (LMWHs) for the initial (first 10 days) treatment and maintenance treatment of cancer-associated thrombosis; (2) direct oral anticoagulants for the initial treatment and maintenance treatment of cancer-associated thrombosis in patients who are not at high risk of gastrointestinal or genitourinary bleeding, in the absence of strong drug–drug interactions or of gastrointestinal absorption impairment; (3) LMWHs or direct oral anticoagulants for a minimum of 6 months to treat cancer-associated thrombosis; (4) extended prophylaxis (4 weeks) with LMWHs to prevent postoperative venous thromboembolism after major abdominopelvic surgery in patients not at high risk of bleeding; and (5) primary prophylaxis of venous thromboembolism with LMWHs or direct oral anticoagulants (rivaroxaban or apixaban) in ambulatory patients with locally advanced or metastatic pancreatic cancer who are treated with anticancer therapy and have a low risk of bleeding.

Introduction

Cancer-associated venous thromboembolism (VTE), which includes deep vein thrombosis, pulmonary embolism, and central venous catheter-related VTE, is the second leading cause of death in patients with cancer after progression. Patients with cancer-associated thrombosis are at high risk of recurrent VTE and anticoagulant-related bleeding,¹ which are associated with high morbidity and resource use. The prevalence of cancer-associated thrombosis is increasing because of multiple factors, including longer patient survival, anticancer therapies, increased detection of incidental VTE during surveillance imaging, and wider use of central venous catheters.²

Monotherapy with low-molecular-weight heparins (LMWHs) for at least 3 up to 6 months was the standard of care for the treatment of cancer-associated thrombosis,³ with vitamin K antagonists (after LMWH) providing a secondary treatment option, until direct oral anticoagulants emerged as alternative first-line treatment options in 2016.^{4,5} Anticoagulant choice for cancer-associated thrombosis is based on evidence from well designed clinical trials, but should also incorporate a personalised medicine approach that considers cancer type, VTE and bleeding risk factors, drug–drug interactions, and patient preferences. Thromboprophylaxis can be used selectively in patients with cancer at high risk of VTE, for example in patients with pancreatic cancer.^{3–5} Risk assessment models and computerised tools can identify patients at greatest risk of cancer-associated thrombosis, to allow for an appropriate, personalised approach for thromboprophylaxis.⁵

The International Initiative on Thrombosis and Cancer (ITAC) first developed evidence-based clinical practice guidelines in 2013,³ using Grading of Recommendations Assessment Development and Evaluation (GRADE) methodology.⁶ The 2016 and 2019 updates^{4,5} to the ITAC guidelines were made available through a free, companion, web-based mobile application. Since December 2019, the COVID-19 pandemic has been raising specific issues in patients with both cancer and SARS-CoV-2 infection, such as an increased risk of hypercoagulability, which results in both macrovascular and microvascular thrombosis.⁷ A meta-analysis of observational, cohort, and cross-sectional studies reported the pooled incidences of VTE in patients with COVID-19 admitted to the ward (7·1%) or to the intensive care unit (27·9%).⁸ Because patients with cancer have a baseline increased risk of VTE compared with patients without cancer, the combination of both COVID-19 and cancer—and its effect on VTE risk and treatment—is of concern. The 2022 ITAC guidelines cover new evidence on the treatment and prophylaxis of cancer-associated thrombosis, including in patients with cancer and with COVID-19.

Guideline development

Development of the 2022 ITAC guidelines followed the same process and methods as with previous iterations,^{3–5} and also received support from the *Institut National du Cancer*. Guideline development is based on GRADE methodology⁶ (panel 1; fully detailed in appendix pp 3–23). The panel, which comprised 19 independent international academic experts from various specialties,

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For more on ITAC-CME Treatment Guidelines mobile app see <https://www.itacme.com>

See Online for appendix

Panel 1: Grading of Recommendations Assessment, Development, and Evaluation scale and additional economic considerations

Levels of evidence

- High (A): further research is very unlikely to change our confidence in the estimate of effect
- Moderate (B): further research is likely to have an important impact on our confidence in the estimate of effect and could change the estimate
- Low (C): further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate
- Very low (D): any estimate of effect is very uncertain

Levels of recommendation

- Strong (grade 1): the panel is confident that the desirable effects of adherence to a recommendation outweigh the undesirable effects
- Weak (grade 2): the panel concludes that the desirable effects of adherence to a recommendation probably outweigh the undesirable effects, but is not confident
- Best clinical practice (guidance): in the absence of any clear scientific evidence and because of an undetermined balance between desirable and undesirable effects, judgment was based on the professional experience and consensus of the international experts within the working group

Additional economic considerations considered during the development and ranking of the recommendations

- The price of a drug varies in different countries and in different regions of the world
- In the case of a strong recommendation, the benefit to the patient outweighs health economics considerations
- Costs of anticoagulants are negligible compared with the cost of cancer treatment

used the PICO (Population, Intervention, Comparator, and Outcomes) model to formulate specific clinical questions and to determine outcomes of interest. An updated literature search was done from Jan 1, 1996, to Jan 1, 2022, with MEDLINE, Embase, and the Cochrane Central Register of Controlled Trials. The literature search strategy used keywords including all VTE and cancer types, patients treated by all cancer-associated therapies, and all anticoagulant drugs and devices. Antiplatelet therapy was beyond the scope of this Review. Keywords did not include specific items on other patient-related outcomes or quality of life. The main outcomes were the rates of VTE (de-novo or recurrent VTE), major and minor bleeding, thrombocytopenia, and death. The 2022 ITAC guidelines were critically reviewed by an independent, multi-disciplinary advisory panel of 87 members (appendix pp 120–22), and were endorsed by the International Society on Thrombosis and Haemostasis.

Guideline recommendations

Treatment of established VTE

Recommendations for the treatment of incidental or symptomatic cancer-associated thrombosis are shown in panel 2. Since the 2019 ITAC guidelines,⁵ three randomised clinical trials^{8–11} and 12 meta-analyses^{12–23} assessed the efficacy and safety of LMWHs or direct oral anticoagulants for the treatment of cancer-associated thrombosis.¹²

Initial treatment of established VTE (up to 10 days)

LMWHs, unfractionated heparin, or fondaparinux (followed by a vitamin K antagonist)

An increased number of patients with cancer-associated thrombosis receiving LMWHs (n=1840) in six randomised clinical trials comparing direct oral anticoagulants with LMWH^{9–11,24–26} resulted in an upgrade from 1B to 1A for LMWHs as an initial treatment in the first 5–10 days. Fondaparinux and unfractionated heparin remain acceptable alternative treatment options without new evidence.

Direct oral anticoagulants

Rivaroxaban or edoxaban were recommended (grade 1B) in 2019,⁵ as initial treatment options in patients with cancer-associated thrombosis who are not at high risk of gastrointestinal or genitourinary bleeding. New evidence from four randomised clinical trials (ADAM-VTE,²⁶ CARAVAGGIO,⁹ CASTA-DIVA,¹⁰ and CANVAS¹¹) found that direct oral anticoagulants are non-inferior to LMWHs for the outcomes of recurrent VTE and mortality. The recommendation for direct oral anticoagulants, including apixaban, is upgraded from 1B to 1A. Starting doses of apixaban (10 mg twice daily for the first 7 days) and rivaroxaban (15 mg twice daily for the first 21 days) are used. Edoxaban requires 5 days of parenteral anticoagulant, typically LMWHs, before initiating treatment with the standard 60 mg daily dose.

Inferior vena cava filters

The recommendation for inferior vena cava filters is unchanged from the 2019 ITAC guidelines.⁵ A new retrospective database study, which used propensity score matching and competing risk analysis, compared 33740 patients with cancer and concomitant deep vein thrombosis who had inferior vena cava filters with 54845 patients without inferior vena cava filters, showing improved pulmonary embolism-free survival (hazard ratio [HR] 0.69, 95% CI 0.64–0.75; p<0.001), without increased risk of recurrent deep vein thrombosis.²⁷ Another propensity-matched retrospective study compared the use of inferior vena cava filters in 247 patients with cancer, in whom anticoagulant therapy was contraindicated, with 247 matched patients with cancer but without inferior vena cava filters, reporting a non-significant lower risk of death (12.2% vs 17.0%, p=0.13), and a significantly lower risk of pulmonary embolism-related mortality (0.8% vs 4.0%; p=0.04).²⁸

Panel 2: Treatment of incidental or symptomatic established venous thromboembolism (VTE) in patients with cancer**Initial treatment of established VTE (up to 10 days of anticoagulation)***International Advisory Panel ranking: 8.67 out of 9.00*

- 1 Low-molecular-weight heparin (LMWH) is recommended for the initial treatment of established VTE in patients with cancer when creatinine clearance is ≥ 30 mL/min (grade 1A). Values and preferences: LMWH is easier to use than unfractionated heparin. A regimen of LMWH, taken once per day, is recommended, unless a twice-per-day regimen is required because of patients' characteristics (eg, risk of bleeding or moderate renal failure) or the need for technical intervention (eg, surgery or changing regimen). When a twice-per-day regimen is required, only enoxaparin (1 mg/kg, twice-daily) can be used.
- 2 For patients who do not have a high risk of gastrointestinal or genitourinary bleeding, rivaroxaban or apixaban (in the first 10 days), or edoxaban (started after at least 5 days of parenteral anticoagulation) can also be used for the initial treatment of established VTE in patients with cancer when creatinine clearance is ≥ 30 mL/min (grade 1A).
- 3 Unfractionated heparin can be also used for the initial treatment of established VTE for patients with cancer when LMWH or direct oral anticoagulants are contraindicated, or not available (grade 2C).
- 4 Fondaparinux can be also used for the initial treatment of established VTE in patients with cancer (grade 2D). Values and preferences: fondaparinux is easier to use than unfractionated heparin.
- 5 Thrombolysis in patients with cancer and with established VTE can only be considered on a case-by-case basis, with specific attention paid to contraindications, especially bleeding risk—eg, brain metastasis (guidance, based on evidence of very low quality and the high bleeding risk of thrombolytic therapy). Values and preferences: an expert opinion is recommended before using thrombolytics, and the procedure should be done in centres with health-care practitioners who have appropriate expertise.
- 6 In the initial treatment of VTE, inferior vena cava filters might be considered when anticoagulant treatment is contraindicated or, in the case of pulmonary embolism, when recurrence occurs under optimal anticoagulation. Periodic reassessment of contraindications for anticoagulation is recommended, and anticoagulation should be resumed when safe (guidance, based on evidence of very low quality and an unknown balance between desirable and undesirable effects).

Early (up to 6 months) and long-term (beyond 6 months) maintenance*International Advisory Panel ranking: 8.61 out of 9.00*

- 1 LMWHs are preferred over vitamin K antagonists for the treatment of VTE in patients with cancer when creatinine

clearance is ≥ 30 mL/min (grade 1A). Values and preferences: daily subcutaneous injection can represent a burden for patients.

- 2 Direct oral anticoagulants (edoxaban, rivaroxaban, or apixaban) are recommended for patients with cancer when creatinine clearance is ≥ 30 mL/min in the absence of strong drug-drug interactions or gastrointestinal absorption impairment (grade 1A). Use caution in patients with gastrointestinal tract malignancies, especially upper gastrointestinal tract malignancies, as the available data show increased risk of gastrointestinal tract bleeding with edoxaban and rivaroxaban.
- 3 LMWH or direct oral anticoagulants should be used for a minimum of 6 months to treat established VTE in patients with cancer (grade 1A).
- 4 After 6 months, termination or continuation of anticoagulation (LMWH, direct oral anticoagulants, or vitamin K antagonists) should be based on individual evaluation of the benefit-risk ratio, tolerability, drug availability, patient preference, and cancer activity (guidance, in the absence of data).

Treatment of VTE recurrence in patients with cancer under anticoagulation*International Advisory Panel ranking: 8.43 out of 9.00*

- 1 In the event of VTE recurrence, three options can be considered: (1) increase LMWH by 20–25% or switch to direct oral anticoagulants; (2) for direct oral anticoagulants, switch to LMWH; and (3) for vitamin K antagonist, switch to LMWH or direct oral anticoagulants (guidance, based on evidence of very low quality and an unknown balance between desirable and undesirable effects). Values and preferences: individual decision. Effect of therapy should be monitored by improvement of symptoms.

Treatment of established catheter-related thrombosis*International Advisory Panel ranking: 8.61 out of 9.00*

- 1 For the treatment of symptomatic catheter-related thrombosis in patients with cancer, anticoagulant treatment is recommended for a minimum of 3 months and as long as the central venous catheter is in place; in this setting, LMWHs are suggested and direct comparisons between LMWHs, direct oral anticoagulants, and vitamin K antagonists have not been made (guidance).
- 2 In patients with cancer and with catheter-related thrombosis, the central venous catheter can be kept in place if it is functional, well positioned, and not infected, with good resolution of symptoms under close surveillance while anticoagulation therapy is administered. No standard approach in terms of duration of anticoagulation is established (guidance).

Thrombolysis

Data on the benefits and risks of thrombolytic therapy in patients with cancer-associated thrombosis are scarce. The 2019 ITAC recommendation on thrombolysis is unchanged.⁵ A new retrospective database study compared two matched groups of 1297 patients with lower-extremity proximal or vena cava deep vein thrombosis undergoing catheter-directed thrombolysis or anticoagulation alone, finding no significant difference in mortality (2.6% vs 1.9%, $p=0.23$), but an increased risk of intracranial haemorrhage (1.3% vs 0.4%, $p=0.017$) with thrombolytic therapy.²⁹

Early (up to 6 months) and long-term (beyond 6 months) maintenance

Unchanged from the 2019 ITAC guidelines,⁵ LMWHs (which are preferred over vitamin K antagonists when creatinine clearance ≥ 30 mL/min) or direct oral anticoagulants as first-line treatment in patients without contraindications (strong drug–drug interactions, impaired gastrointestinal absorption, or high bleeding risk), are recommended (grade 1A) for early maintenance and long-term treatment of cancer-associated thrombosis. Three new randomised controlled trials showed that direct oral anticoagulants were non-inferior to LMWHs for the outcome of VTE recurrence with apixaban (CARAVAGGIO),⁹ rivaroxaban (CASTA-DIVA),¹⁰ or any direct oral anticoagulant (CANVAS).¹¹ New data since the 2019 guidelines have supported LMWH or direct oral anticoagulants treatment for up to 12 months.^{30,31}

LMWHs versus vitamin K antagonists

Since the 2016 ITAC guidelines,⁴ there have been no new randomised controlled trials comparing LMWHs with vitamin K antagonists. Five pivotal randomised controlled trials (CANTHANOX,³² CLOT,³³ LITE,³⁴ CATCH,³⁵ and ONCENOX³⁶) showed that LMWHs for 6 months are more effective than vitamin K antagonists in patients with cancer-associated thrombosis, without an increase in bleeding risk.³⁴ Data from the LMWH control group of the HOKUSAI-VTE cancer trial,²⁴ two prospective single-arm cohorts,^{37,38} and one retrospective study³⁰ showed no increase in major bleeding at 6 months and 12 months when a 12-month LMWH regimen was used as treatment.

Direct oral anticoagulants versus vitamin K antagonists

Comparisons between direct oral anticoagulants and vitamin K antagonists are limited to post-hoc analyses of patients with cancer included in randomised controlled trials comparing direct oral anticoagulants with warfarin in patients with and without cancer, which reported no difference in the risks of recurrent VTE, major bleeding, or clinically relevant non-major bleeding between direct oral anticoagulants and vitamin K antagonists.^{4,5}

Direct oral anticoagulants versus LMWHs

Three new randomised controlled trials comparing direct oral anticoagulants with LMWHs (CARAVAGGIO,⁹ CASTA-DIVA,¹⁰ and CANVAS;¹¹ appendix p 110–12), and 11 meta-analyses^{13–23} pooling results from HOKUSAI-VTE Cancer,²⁴ SELECT-D,²⁵ ADAM-VTE,²⁶ and CARAVAGGIO (2894 patients),⁹ showed that direct oral anticoagulants confer a reduced risk of recurrent VTE (relative risk [RR] 0.62, 95% CI 0.43–0.91), without an increase in major bleeding (1.31, 0.83–2.08). Direct oral anticoagulants were associated with a substantial increase in the risk of clinically relevant non-major bleeding in all, but one, meta-analyses.²⁰ One meta-analysis,¹³ focusing on gastrointestinal cancers (483 patients), reported a significantly higher risk of major bleeding in patients receiving direct oral anticoagulants (RR 2.3, 95% CI 1.08–4.88) than in patients receiving LMWHs. The published ADAM-VTE trial (300 patients)²⁶ reported that apixaban for 6 months was safe, with no major bleeds in 145 patients assigned apixaban, and two (1.4%) events in 142 assigned dalteparin (HR not estimable, $p=0.138$), with recurrent VTE in one (0.7%) of 145 assigned apixaban and nine (6.3%) of 142 assigned dalteparin (HR 0.099, 95% CI 0.013–0.78; $p=0.0281$), without any difference in overall survival at 6 months. In the CARAVAGGIO trial,⁹ 1170 patients with cancer and with symptomatic or incidental VTE received apixaban (10 mg twice-daily for the first 7 days, followed by 5 mg twice-daily) or dalteparin (200 IU/kg daily for 1 month, followed by 150 IU/kg daily) for 6 months, with stratification by symptomatic or incidental VTE and active cancer or a history of cancer. For the primary outcome of recurrent VTE at 6 months, apixaban was non-inferior to LMWH (5.6% of patients receiving apixaban vs 7.9% of patients receiving dalteparin; HR 0.63, 95% CI 0.37–1.07; $p<0.001$ for non-inferiority). Major bleeding events (3.8% under apixaban vs 4.0% under dalteparin) did not differ between groups (HR 0.82, 95% CI 0.40–1.69; $p=0.60$). Clinically relevant non-major bleeding events were not significantly different between groups (9.0% under apixaban vs 6.0% under dalteparin, HR 1.42, 95% CI 0.88–2.30). Overall survival was also similar between groups. In the CASTA-DIVA trial,¹⁰ 158 patients with cancer and with symptomatic or incidental VTE at high risk of recurrence (modified Ottawa score ≥ 1) received rivaroxaban (15 mg twice-daily for 3 weeks, and then 20 mg daily) or dalteparin (200 IU/kg daily for 1 month, and then 150 IU/kg daily) for 3 months. Rates of recurrent VTE within 3 months did not differ between groups (6.4% under rivaroxaban vs 10.1% under dalteparin). The study did not fulfil the predefined criteria for non-inferiority (HR 0.75, 95% CI 0.21–2.66; $p=0.13$) due to a lower-than-expected VTE rate with dalteparin. Rates of major bleeding (1.4% with rivaroxaban vs 3.7% with dalteparin; HR 0.36, 95% CI 0.04–3.43), major or clinically relevant non-major bleeding (12.2% with rivaroxaban vs 9.8% with dalteparin; 1.27, 0.49–3.26) or death from any cause were similar in both

groups. Preliminary results from the CANVAS trial,¹¹ with randomised and preference cohorts, were presented at the 2021 American Society of Clinical Oncology meeting. In the randomised cohort, 671 patients with symptomatic or incidentally diagnosed VTE received either a direct oral anticoagulant (type determined by the treating investigator) or LMWHs with or without transition to warfarin for 6 months. The primary outcome of recurrent VTE occurred in 6.1% of patients in the direct oral anticoagulant group versus 8.8% in the LMWH group (difference -2.7, 90% CI -6.1 to 0.7), meeting non-inferiority criteria. The proportion of secondary outcomes of major bleeding (5.2% with direct oral anticoagulants vs 5.6% with LMWHs), clinically relevant non-major bleeding (5.8% with direct oral anticoagulants vs 2.6% with LMWHs), and all-cause deaths (21.5% with direct oral anticoagulants vs 18.4% with LMWHs) were similar for both groups. Results from an updated meta-analysis pooling the results from HOKUSAI-VTE Cancer,²⁴ SELECT D,²⁵ ADAM-VTE,²⁶ CARAVAGGIO,⁹ CASTA-DIVA,¹⁰ and CANVAS (3690 patients)¹¹ were presented at the 2021 American Society of Hematology meeting.³⁹ After a follow-up of 3 to 6 months, use of direct oral anticoagulants significantly decreased the risk of recurrent VTE compared with LMWHs (RR 0.67, 95% CI 0.52-0.85), with a non-significant increase in the risk of major bleeding (1.17, 0.82-1.67), but a significant increase in the risk of clinically relevant non-major bleeding events (1.66, 1.31-2.09).³⁹

The randomised controlled trials comparing direct oral anticoagulants with LMWHs for the treatment of cancer-associated thrombosis (appendix pp 110-12) included a substantial proportion of patients with incidental VTE, with location varying from deep vein thrombosis to pulmonary embolism. In a systematic review and meta-analysis including 774 patients with cancer and with incidental VTE,²⁰ the risk of recurrent VTE was not different in patients receiving direct oral anticoagulants, compared with those receiving LMWHs (RR 0.54, 95% CI 0.26-1.11), nor was the risk of major bleeding (1.29, 0.74-2.28). These findings further support managing patients with incidental cancer-associated thrombosis in a similar manner as symptomatic cancer-associated thrombosis. Randomised controlled trials comparing direct oral anticoagulants with LMWHs were heterogeneous in terms of sample size, cancer types, study design, primary outcomes, treatment duration, and compliance to long-term treatment (appendix pp 110-112).

Duration of anticoagulation

Five randomised controlled trials,^{9,11,24-26} totalling 3532 patients treated for at least 6 months, further support the use of either LMWH or direct oral anticoagulants for at least 6 months with a grade 1A recommendation. No new studies on either treatment of VTE recurrence while on anticoagulation or treatment of established central venous catheter-associated

thrombosis have been identified since the 2019 ITAC guidelines, which remain unchanged.⁵

VTE prophylaxis in patients with cancer

Recommendations for VTE prophylaxis are shown in panel 3. Risk factors and stratification scores to identify high-risk patients who could benefit from primary VTE prophylaxis are summarised in the appendix (pp 113-16).

VTE prophylaxis in patients undergoing cancer surgery

Unchanged since the 2019 ITAC guidelines,⁵ anticoagulant prophylaxis should be started between 12 h and 2 h preoperatively and continued for at least 7-10 days postoperatively with once-daily low-dose LMWHs or low-dose unfractionated heparin thrice-daily. New data support the grade 1A recommendation for extended-duration LMWHs prophylaxis for 4 weeks after major cancer abdominal or pelvic surgery (laparotomy or laparoscopy) in patients without a high-bleeding risk.⁴⁰ There are no new data for fondaparinux and there is insufficient evidence to support the use of apixaban, despite one small randomised controlled trial.⁴¹ Similarly, no evidence currently exists to support the use of other direct oral anticoagulants in this setting.

LMWHs versus unfractionated heparin

A new network meta-analysis of 20 randomised controlled trials in 1693 patients with gynaecological cancer undergoing major abdominopelvic surgery treated with LMWH or unfractionated heparin showed no difference in rates of VTE (RR 1.16, 95% CI 0.85-1.56) or major bleeding (0.62, 0.32-1.23).⁴² Daily LMWH is associated with a lower risk of heparin-induced thrombocytopenia and is more convenient than twice-daily or thrice-daily unfractionated heparin. Regarding patients with previous heparin-induced thrombocytopenia (definite or possible), the literature search did not retrieve any specific data in patients with cancer concerning the common practice on the use of fondaparinux.

Preoperative pharmacological thromboprophylaxis

A new meta-analysis of 12 studies (14273 patients),⁴³ mostly retrospective studies including patients with cancer, found that preoperative pharmacological prophylaxis reduced the risk of VTE after major gynaecological and gynaecological cancer surgery compared with no preoperative pharmacological prophylaxis (odds ratio [OR] 0.59, 95% CI 0.39-0.89), without increased bleeding (1.26, 0.98-1.62).

Comparison between doses of LMWHs

No new studies were identified comparing different doses of LMWHs.

Extended-duration (4 weeks) thromboprophylaxis

The 2019 ITAC recommendation for extended-duration prophylaxis with LMWH in patients undergoing cancer

Panel 3: Prophylaxis of venous thromboembolism (VTE) in patients with cancer**Prophylaxis of VTE in surgically-treated patients with cancer***International Advisory Panel ranking: 8.62 out of 9.00*

- 1 Use of low-molecular-weight-heparin (LMWH) once per day (when creatinine clearance is ≥ 30 mL/min) or low-dose unfractionated heparin three times per day is recommended to prevent postoperative VTE in patients with cancer; pharmacological prophylaxis should be started 2–12 h preoperatively and continued for at least 7–10 days; there are no data allowing conclusions regarding the superiority of one type of LMWH over another (grade 1A). Values and preferences: LMWH once per day is more convenient.
- 2 There is insufficient evidence to support fondaparinux (grade 2C) or direct oral anticoagulants (grade 2B) as an alternative to LMWH for the prophylaxis of postoperative VTE in patients with cancer. Values and preferences: as per the first recommendation.
- 3 Use of the highest prophylactic dose of LMWH to prevent postoperative VTE in patients with cancer is recommended (grade 1A).
- 4 Extended prophylaxis (4 weeks) with LMWH to prevent postoperative VTE after major abdominal or pelvic surgery (either laparotomy or laparoscopy) is recommended in patients with cancer who do not have a high risk of bleeding (grade 1A). Values and preferences: longer duration of injections.
- 5 Mechanical methods are not recommended as monotherapy except when pharmacological methods are contraindicated (grade 2A). Values and preferences: no injection.
- 6 Inferior vena cava filters are not recommended for routine prophylaxis (grade 1A).

Prophylaxis of VTE in medically-treated patients with cancer*International Advisory Panel ranking: 8.44 out of 9.00*

- 1 We recommend prophylaxis with LMWH or fondaparinux when creatinine clearance is ≥ 30 mL/min, or with unfractionated heparin in medically-treated patients with cancer and reduced mobility who are admitted to hospital (grade 1B). In this setting, direct oral anticoagulants are not recommended routinely (guidance). Values and preferences: subcutaneous injections. Costs: in some countries, price differences between LMWH, unfractionated heparin, or fondaparinux might affect the choice.
- 2 Primary pharmacological prophylaxis of VTE with LMWH (grade 1A) or with direct oral anticoagulants (rivaroxaban or apixaban; grade 1B) is indicated in ambulatory patients with locally advanced or metastatic pancreatic cancer treated with systemic anticancer therapy and who have a low risk of bleeding. Values and preferences: subcutaneous injections.
- 3 Primary pharmacological prophylaxis of VTE with LMWH is not recommended outside of a clinical trial for patients with locally advanced or metastatic lung cancer treated with systemic anticancer therapy, including patients who have a low risk of bleeding (guidance).
- 4 Primary prophylaxis with direct oral anticoagulant (rivaroxaban or apixaban) is recommended in ambulatory patients who are receiving systemic anticancer therapy and are at intermediate-to-high-risk of VTE, identified by a validated risk assessment model (ie, a Khorana score ≥ 2), and not actively bleeding or not at a high risk for bleeding (grade 1B).
- 5 In patients with myeloma treated with immunomodulatory drugs combined with steroids or other systemic anticancer therapies, VTE primary pharmacological prophylaxis is recommended (grade 1A); in this setting, oral anticoagulants (vitamin K antagonists at low or therapeutic doses and apixaban at prophylactic doses), LMWH at prophylactic doses, or low-dose aspirin (100 mg daily) can be used, and have shown similar effects with regard to preventing VTE (grade 2B). Values and preferences: subcutaneous injections.

Prophylaxis of catheter-related thrombosis*International Advisory Panel ranking: 8.52 out of 9.00*

- 1 Use of anticoagulation for routine prophylaxis of catheter-related thrombosis is not recommended (grade 1A). Values and preferences: bleeding risk with anticoagulants.
- 2 Catheters should be inserted on the right side, in the jugular vein, and the distal extremity of the central catheter should be located at the junction of the superior vena cava and the right atrium (grade 1B).
- 3 In patients requiring central venous catheters, we suggest the use of implanted ports over peripherally inserted central catheter lines (guidance).

surgery (laparotomy and laparoscopic) is unchanged (grade 1A).⁵ A new meta-analysis (18 studies, 7495 patients)⁴⁰ showed a significantly reduced risk of symptomatic VTE (1.0% vs 2.0%; RR 0.48, 95% CI 0.31–0.74), without increased risk of clinically relevant non-major bleeding (4.0% vs 4.9%; 1.00, 0.66–1.50). One randomised controlled trial compared apixaban, 2.5 mg twice-daily, with enoxaparin, 40 mg daily, for 28 days for postoperative prophylaxis (400 patients, 19.3% of benign tumours).⁴¹ The proportion of patients in the apixaban and enoxaparin groups with major bleeding (0.5% in both groups; OR 1.04, 95% CI

0.07–16.76), clinically relevant non-major bleeding (5.4% vs 9.7%; 1.88, 0.87–4.10), and VTE (1.0% vs 1.5%; 1.57, 0.26–9.50) was similar.⁴¹

Mechanical methods of prophylaxis

Unchanged since the 2019 ITAC guidelines,⁵ monotherapy with mechanical methods of prophylaxis is not recommended, except when pharmacological methods are contraindicated. Five small Japanese randomised controlled trials assessed mechanical methods of thromboprophylaxis in surgical patients with cancer, with inconsistent findings.^{44–48} In a network meta-analysis

of patients with gynaecological cancer undergoing major abdominopelvic surgery, comparing different methods of thromboprophylaxis, intermittent pneumatic compression plus LMWH was best for VTE prevention.⁴²

Inferior vena cava filters placement

No additional studies have been available since the 2019 ITAC guidelines.⁵ The recommendation against routine use of inferior vena cava filters as primary VTE prophylaxis is unchanged.

VTE prophylaxis in medically-treated patients with cancer who are hospitalised

The 2022 ITAC guidelines for medical prophylaxis in hospitalised patients have remained unchanged from the 2019 publication. A phase 2 trial randomly assigned 50 patients with solid tumours, myeloma, or lymphoma at high risk for VTE based on the Padua risk score to fixed-dose enoxaparin (40 mg daily) or weight-adjusted-dose enoxaparin (1 mg/kg daily) during hospitalisation.⁴⁹ No symptomatic VTE or bleeding events were observed in either group within 14 days after randomisation.

VTE prophylaxis in ambulatory patients with cancer receiving systemic anticancer therapy

The risks of VTE and bleeding vary by cancer type, treatment, and patient characteristics, ranging from 3% to 5% in patients with early stage cancer to 30% in patients with metastatic disease.⁵⁰ The ITAC guidelines have remained unchanged since 2019 and do not recommend routine primary prophylaxis with LMWHs, vitamin K antagonists, or direct oral anticoagulants for ambulatory patients with cancer.⁴⁵

Ten new meta-analyses (1415–15 678 patients),^{50–59} one subgroup analysis of a randomised controlled trial (273 patients with pancreatic cancer),⁶⁰ and three observational studies^{61–63} compared anticoagulant prophylaxis with no intervention or placebo. In one updated meta-analysis of 24 randomised controlled trials with VTE or death as primary outcomes, thromboprophylaxis conferred a 50% reduction in the incidence of VTE, with similar reductions in studies with LMWHs or direct oral anticoagulants.⁵³ VTE risk reduction was found in patients with pancreatic cancer (OR 0.26, 95% CI 0.14–0.48) and lung cancer (0.42, 0.26–0.67).⁵³ Another meta-analysis of six randomised controlled trials (4626 patients) showed that primary thromboprophylaxis (with LMWHs or direct oral anticoagulants) compared with placebo or standard care reduced the risk of VTE by 55% (95% CI 0.28–0.67) in patients with a Khorana score of at least 3, and by 42% (0.36–0.83) in patients with a Khorana score of at least 2, without increased risk of major bleeding.⁵⁹ Since 2019,⁵ the use of LMWHs or direct oral anticoagulants (apixaban and rivaroxaban) for thromboprophylaxis has gained evidence in subgroups of ambulatory patients with cancer receiving chemotherapy at high risk of VTE (Khorana score ≥ 2) and low risk of bleeding, with optimal net

clinical benefit for LMWHs (grade 1A) or direct oral anticoagulants (grade 1B).

LMWH

A meta-analysis from 14 randomised controlled trials (8278 patients) comparing parenteral thromboprophylaxis with placebo or standard care in ambulatory patients with cancer receiving chemotherapy found no difference in mortality at 1 year (RR 0.99, 95% CI 0.93–1.06).⁵⁷ LMWH reduced the risk of symptomatic deep vein thrombosis and pulmonary embolism (RR 0.58, 95% CI 0.47–0.71), with the most certain benefit in patients with lung cancer (0.59, 0.42–0.81) dominating the overall reduction, with no increase in major bleeding (1.27, 0.92–1.74), and a significant increase in minor bleeding (1.34, 1.19–1.59).⁵⁷ In a second meta-analysis that included 14 randomised controlled trials with VTE as primary outcome in ambulatory patients with cancer receiving chemotherapy (8226 patients),⁵³ anticoagulant prophylaxis was compared with placebo (eight studies), no treatment (five studies), or aspirin treatment (one study), and it was associated with a reduced risk of VTE (OR 0.45, 95% CI 0.36–0.56; $p < 0.0001$) and a significant increase in major bleeding risk (1.43, 1.01–2.04). According to an updated Cochrane meta-analysis,⁵⁰ with 11 randomised controlled trials (3931 patients) comparing LMWH with no prophylaxis, LMWHs reduced the rate of symptomatic VTE (RR 0.62, 95% CI 0.46–0.83), with a significant increase in major bleeding (1.63, 1.12–2.35). In patients with multiple myeloma, LMWHs were associated with a significantly decreased rate of symptomatic VTE compared with vitamin K antagonists (RR 0.33, 95% CI 0.14–0.83; 439 patients), whereas the difference between LMWH and aspirin was not significant (0.51, 0.22–1.17; 781 patients).⁵⁰ Two other meta-analyses reported consistent findings.^{52,56}

Direct oral anticoagulants

One new meta-analysis pooling the results from CASSINI⁶⁴ and AVERT⁶⁵ found that direct oral anticoagulants significantly reduced the rate of overall VTE (RR 0.56, 95% CI 0.35–0.89) but not the rate of symptomatic VTE (0.58, 0.29–1.13) compared with no prophylaxis, with no differences in major bleeding (1.96, 0.8–4.82) or clinically relevant non-major bleeding (1.28, 0.74–2.2) observed between treatment groups.⁵³ Three additional meta-analyses reported similar findings.^{50,52,56}

Anticoagulant thromboprophylaxis in selected patients according to tumour type

Patients with pancreatic cancer

The 2022 ITAC recommendation for VTE primary prophylaxis with LMWHs (grade 1A) or direct oral anticoagulants (grade 1B) in ambulatory patients with locally advanced or metastatic pancreatic cancer receiving chemotherapy and with a low bleeding risk is supported

by two randomised controlled trials,^{66,67} and subgroup analyses of three other trials.^{60,68,69} Subgroup analysis of the 273 patients with pancreatic cancer included in the CASSINI study⁶⁰ showed a significant decrease in a composite endpoint of symptomatic deep vein thrombosis, asymptomatic proximal deep vein thrombosis, any pulmonary embolism, and VTE-related death (HR 0.35, 95% CI 0.13–0.97), without an increase in major bleeding or clinically relevant non-major bleeding during treatment with rivaroxaban compared with placebo. In one meta-analysis of five randomised controlled trials (1003 patients with pancreatic cancer),⁵⁵ thromboprophylaxis compared with placebo decreased the VTE rate by 69% (RR 0.31, 95% CI 0.19–0.51; $p < 0.0001$), with similar reductions of VTE in studies with LMWHs (0.30, 0.17–0.53) or direct oral anticoagulants (0.37, 0.14–0.99), without excess in bleeding.

Patients with lung cancer

The 2019 ITAC guidelines for patients with lung cancer is unchanged, because the benefit from thromboprophylaxis is offset by the risk of bleeding.⁵ In one new meta-analysis of nine randomised controlled trials (5443 patients),⁵⁴ LMWH prophylaxis reduced the risk for VTE (RR 0.54, 95% CI 0.43–0.69), without an increase in overall survival (1.02, 0.83–1.26);⁵⁴ this meta-analysis did not assess risk for bleeding.

Patients with multiple myeloma treated with immunomodulatory drugs

The 2022 ITAC guidelines have remained unchanged from the 2019 publication.⁵ A meta-analysis of ten studies (1964 patients) comparing thromboprophylaxis with aspirin or LMWH with no intervention found that aspirin reduced the risk of VTE compared with no intervention (OR 0.20, 95% CI 0.07–0.61), but increased the VTE risk compared with LMWH (2.60, 1.08–6.25).⁷⁰ Intervention with either aspirin or LMWH did not increase the risk of bleeding. Two prospective, small, single-arm studies reported that apixaban, 2.5 mg twice-daily, was safe and well tolerated.^{61,63}

Patients with acute lymphoblastic leukaemia

One Cochrane systematic review in patients with acute lymphoblastic leukaemia receiving asparaginase-based therapy identified 23 non-randomised studies of anti-coagulant thromboprophylaxis, but methodological limitations precluded treatment effect estimates.⁵⁸ In a retrospective study of thromboprophylaxis in 125 patients with acute lymphoblastic leukaemia, LMWH compared with no prophylaxis in 99 historical controls was associated with a reduced incidence of VTE (OR 0.42, 95% CI 0.21–0.83) without an increase in major bleeding risk.⁶²

Prophylaxis of central venous catheter-related VTE

The 2022 ITAC guidelines against routine primary prophylaxis of central venous catheter-related VTE have

remained unchanged from the 2016 publication.⁴ In a pilot randomised controlled trial⁷¹ of 105 patients with cancer and with new central venous catheters receiving rivaroxaban 10 mg daily, or no intervention, VTE occurred in 5.8% of patients in the rivaroxaban group and 9.4% patients in the control group (HR 0.58; 95% CI 0.14–2.5). One patient (1.9%) on rivaroxaban had major bleeding.⁷¹ One meta-analysis of 22 studies (4131 cases, 5272 controls) in patients with breast cancer showed that arm ports were associated with a higher VTE risk than chest ports (RR 2.23, 95% CI 1.04–4.79; $p = 0.041$).⁷² In one randomised controlled trial (399 patients), peripherally inserted central catheters were associated with a higher risk of VTE and adverse events than implanted port catheters (HR 10.2, 95% CI 2.3–44.6, $p = 0.0002$).⁷³ Another prospective, non-randomised study of 423 patients treated with chemotherapy via a peripherally inserted central catheter reported substantially lower rates of upper extremity VTE when patients received prophylaxis with rivaroxaban (10 mg daily; 3.76%) or enoxaparin (40 mg daily; 3.03%), compared with no prophylaxis (12.4%).⁷⁴ Conversely, peripherally inserted central catheter–central venous catheter were associated with a lower risk of catheter-related deep vein thrombosis compared with centrally inserted central venous catheter (RR 0.34, 95% CI 0.12–0.98, $p = 0.03$) in another randomised controlled trial of 93 untreated patients receiving induction therapy for acute myeloid leukaemia.⁷⁵

Prevention and treatment of VTE in special cancer situations

Recommendations on prevention and treatment of VTE in special clinical situations are shown in panel 4.

Patients with brain tumours

The 2022 ITAC guidelines recommend the use of LMWHs or direct oral anticoagulants for the treatment of established VTE in patients with a brain tumour (grade 2A). Since the 2019 ITAC guidelines,⁵ one meta-analysis of seven retrospective studies (1291 patients) showed that patients with glioma receiving full-dose anticoagulants (LMWH, unfractionated heparin, or vitamin K antagonist) for cancer-associated thrombosis have an increased risk of intracerebral haemorrhage compared with patients without anticoagulants (OR 3.66, 95% CI 1.84–7.29).⁷⁶ Similarly, a matched retrospective study of 291 patients with brain metastasis reported that anticoagulation conferred a non-significant increased risk of intracerebral haemorrhage (HR 1.31, 95% CI 0.96–1.79; $p = 0.09$).⁷⁷ In a retrospective cohort of 79 patients with metastatic brain tumours who developed intracerebral haemorrhage on anticoagulation for VTE, the cumulative incidence of recurrent VTE was significantly lower in patients restarting anticoagulation compared with patients who did not (8.1% vs 35.3%; $p = 0.003$).⁷⁸ Data from randomised controlled trials

Panel 4: Treatment of venous thromboembolism (VTE) in unique situations**International Advisory Panel ranking: 8-32 out of 9-00**

- 1 For the treatment of established VTE in patients with a brain tumour, low-molecular-weight heparin (LMWH) or direct oral anticoagulants can be used (grade 2A).
- 2 We recommend the use of LMWH or unfractionated heparin commenced postoperatively for the prevention of VTE in patients with cancer undergoing neurosurgery (grade 1A).
- 3 Primary pharmacological prophylaxis of VTE in medically-treated patients with a brain tumour who are not undergoing neurosurgery is not recommended (grade 1B).
- 4 In the presence of severe renal failure (creatinine clearance <30 mL/min), we suggest using unfractionated heparin followed by early vitamin K antagonists (possible from day 1) or LMWH adjusted to anti-Xa concentration for the treatment of established VTE (guidance, in the absence of data and an unknown balance between desirable and undesirable effects).
- 5 In patients with severe renal failure (creatinine clearance <30 mL/min), an external compression device can be applied, and pharmacological prophylaxis could be considered on a case-by-case basis; in patients with severe renal failure (creatinine clearance <30 mL/min), unfractionated heparin can be used on a case-by-case basis (guidance, in the absence of data and a balance between desirable and undesirable effects depending on the level of VTE risk).
- 6 In patients with cancer and with thrombocytopenia, full doses of anticoagulant can be used for the treatment of established VTE if the platelet count is $>50 \times 10^9$ per L and there is no evidence of bleeding; for patients with a platelet count $<50 \times 10^9$ per L, decisions on treatment and dose should be made on a case-by-case basis with the utmost caution (guidance, in the absence of data and a balance between desirable and undesirable effects depending on the bleeding risk vs VTE risk).
- 7 In patients with cancer and with mild thrombocytopenia, platelet count $>80 \times 10^9$ per L, pharmacological prophylaxis could be used; if the platelet count is $<80 \times 10^9$ per L, pharmacological prophylaxis can only be considered on a case-by-case basis and careful monitoring is recommended. In the CASSINI⁶⁴ and AVERT⁶⁵ trials, patients with a platelet count as low as 50×10^9 per L were allowed to receive thromboprophylaxis (guidance, in the absence of data and a balance between desirable and undesirable effects depending on the bleeding risk vs VTE risk).
- 8 In patients with cancer who are pregnant, we suggest the use of LMWH for treatment of established VTE and for VTE prophylaxis and avoidance of vitamin K antagonists and direct oral anticoagulants (guidance, in the absence of data and based on the contraindication of vitamin K antagonist and direct oral anticoagulants during pregnancy).
- 9 In patients with cancer who are obese, consideration for a higher dose of LMWH should be given for cancer surgery (guidance).
- 10 For the treatment of symptomatic catheter-related thrombosis in children with cancer, anticoagulant treatment is recommended for a minimum of 3 months and as long as the central venous catheter is in place; in this setting, direct comparisons between unfractionated heparin, LMWHs, direct oral anticoagulants, and vitamin K antagonists have not been done (guidance).
- 11 In children with acute lymphoblastic leukaemia undergoing induction chemotherapy, we recommend LMWH as thromboprophylaxis (grade 2A).
- 12 In children requiring central venous catheters, we suggest the use of implanted ports over peripherally inserted central catheter lines (guidance).

comparing the efficacy and safety of direct oral anticoagulants versus LMWHs in patients with brain tumours were limited to three patients included in the SELECT-D trial²⁵ and 74 patients included in HOKUSAI-VTE Cancer trial.²⁴ Since the 2019 ITAC guidelines,⁵ three retrospective studies assessed the safety of direct oral anticoagulants versus LMWHs in patients with primary or metastatic brain tumours treated for VTE. In the first retrospective study, after reviewing all intracerebral haemorrhage radiographic images for eligible patients (n=172), the 12-month cumulative incidence of any intracerebral haemorrhage with direct oral anticoagulants was 0%, compared with 36.8% with LMWHs (95% CI 22.3–51.3; p=0.007) in patients with primary brain tumours, and 27.8% (5.5–56.7) versus 52.9% (37.4–66.2; p=0.38) in patients with brain metastases.²⁹ In the second chart review study (n=125), the rate of major bleeding was 9.6% under direct oral anticoagulants versus 26% under LMWHs (p=0.03), and the respective rates of intracerebral haemorrhage were 5.8% and 15%

(p=0.09), with no difference in minor bleeding and recurrent VTE.³⁰ In the third study (n=111), the 6-month cumulative incidence of intracerebral haemorrhage was 4.3% (95% CI 0.74–13.2) with direct oral anticoagulants versus 5.9% (1.5–14.9) with LMWHs, and the 6-month cumulative incidence of bleeding was 14.3% (6.2–25.8) versus 27.8% (15.5–41.6), respectively.³¹ Rates of recurrent VTE did not differ between groups.³¹ The recommendation for VTE prophylaxis in brain tumours patients has remained unchanged since 2019.⁵

Patients with thrombocytopenia

The 2022 ITAC guidelines for patients with cancer and with thrombocytopenia have remained unchanged from the 2019 publication.⁵ In a prospective non-randomised study of patients with VTE and thrombocytopenia (platelets $<100 \times 10^9$ per L, n=121), the 60-day incidence of major bleeding was 12.8% (95% CI 4.9–20.8) with full-dose anticoagulation, and 6.6% (95% CI 2.4–14.7) with a lower dose anticoagulation (HR 2.18, 95% CI

Panel 5: Treatment and prophylaxis of venous thromboembolism (VTE) for patients with cancer and with COVID-19

International Advisory Panel ranking: 8-48 out of 9-00

- 1 Recommendations for the treatment of established VTE for patients with cancer are similar, independent of whether or not they have COVID-19 (guidance).
 - 2 Recommendations for the prophylaxis of VTE in patients with cancer are similar in those with and without COVID-19 (guidance).
- Patients with cancer and with COVID-19, whether they are hospitalised, post-discharge, or ambulatory, should be assessed for risk of VTE as any other patient with COVID-19 (guidance)
 - Pharmacological prophylaxis during hospitalisation should be given, with the same dose and anticoagulant type as in patients with cancer who do not have COVID-19, based on current institutional practice (guidance).
 - Post-discharge VTE prophylaxis is not advised in patients with cancer and with COVID-19; as with any patient with cancer, individual assessment of benefit-risk ratio should be done (guidance).
 - Primary pharmacological prophylaxis of VTE in ambulatory patients with cancer who have COVID-19 is not recommended routinely (guidance).

1.21–3.93).⁸² The incidence of recurrent VTE was 5.6% (95% CI 0.2–11%) with full-dose anticoagulation and 0% with modified-dose anticoagulation.⁸² One retrospective study of 15 337 patients with cancer reported that patients with severe thrombocytopenia (platelets $<50 \times 10^9$ per L, $n=166$) compared with patients who had a normal platelet count had a similar risk for major bleeding at 10 days (OR 0.84, 95% CI 0.20–3.49) and 30 days (0.90, 0.32–2.49), regardless of the LMWH dose used.⁸³

Children with cancer

Since the 2019 ITAC guidelines,⁵ a new randomised study ($n=949$) compared low-dose unfractionated heparin, prophylactic LMWH, and antithrombin supplementation in children with acute lymphoblastic leukaemia during induction therapy.⁸⁴ Low-dose unfractionated heparin was associated with a higher VTE rate (8.0%) compared with prophylactic LMWH (3.5%; $p=0.011$) or antithrombin (1.9%; $p<0.001$),⁸⁴ with no difference in major bleeding.⁸⁴ One network meta-analysis of primary pharmacological thromboprophylaxis in children with acute lymphoblastic leukaemia ($n=1318$) reported that LMWH was the only agent associated with a significantly decreased risk of VTE compared with standard of care (OR 0.23, 95% CI 0.06–0.81).⁸⁵ In children with acute lymphoblastic leukaemia undergoing induction chemotherapy, we recommend LMWHs as thromboprophylaxis (grade 2A). In a multicentre, prospective cohort study (including 41% of patients with cancer), children with peripherally inserted central catheters had a significantly higher incidence of catheter-related VTE than children with centrally inserted central catheters (HR 8.5, 95% CI 3.1–23.0; $p<0.001$).⁸⁶ The predefined EINSTEIN-Jr randomised controlled trial analysis comparing rivaroxaban versus standard of care

Search strategy and selection criteria

The updated literature search was done by the *Institut National du Cancer* using MEDLINE, Embase, and Cochrane Central Register of Controlled Trials with the following subject headings: “exp cancer” AND “exp venous thromboembolism” AND “anticoagulant drugs and devices” AND “COVID-19”. The literature search was restricted to articles in English published from Dec 15, 2018, to Jan 1, 2022. Meta-analyses, systematic reviews, randomised clinical trials, or non-randomised prospective or retrospective studies in the absence of randomised clinical trials, were included. Articles were selected for potential inclusion based on article critical appraisal grids designed by the Institut National du Cancer for each clinical question. For inclusion in the analysis, studies had to focus on the treatment of established venous thromboembolism (VTE) in patients with cancer, prophylaxis of VTE in patients with cancer (surgical and medical settings), and treatment and prophylaxis of central venous catheter related VTE in patients with cancer. When data from studies specific to patients with cancer were not available, studies done in the general population (non-cancer specific data) were included if they fulfilled the inclusion criteria. Members of the working group had the opportunity to add any additional references for the individual questions that might have been missed in the literature search. Studies in patients with VTE related to tumour material or a history of cancer in remission for more than 5 years were excluded from the analysis. Studies that did not report VTE or side effects of anticoagulation as outcomes were also excluded. The main study outcomes were rates of VTE (first event or recurrence), major bleeding, clinically relevant non-major bleeding, and death.

in children with central venous catheter-VTE showed no recurrent VTE or major bleeding in both groups.⁸⁷ Together, the data on central venous catheter-VTE prompted two new ITAC guidelines for the treatment and prophylaxis of VTE in children requiring a central venous catheter.

Since the 2019 ITAC guidelines,⁵ no new studies have addressed the treatment and prevention of VTE in patients with cancer and with renal failure, or in obese patients.

VTE treatment and prophylaxis in patients with cancer and with COVID-19

Studies reporting the incidence of venous and arterial thrombosis in patients with cancer who have COVID-19 are scarce.^{88–90} No large differences in the rates of VTE between patients with COVID-19 with and without cancer were found. In two studies that assessed risks factors for VTE,^{89,90} cancer-specific factors, such as cancer type or chemotherapy, did not correlate with increased risk, rather obesity, renal failure, and severity of COVID-19 were associated. A large cancer and COVID-19

registry of 2804 patients found similar VTE rates in patients who had been hospitalised with cancer compared with reported rates in patients without cancer.⁹¹ There are no specific data regarding the benefit and risk of different anticoagulants for the treatment or prevention of VTE in patients with cancer and with COVID-19. Available data in the general population are summarised in the appendix (pp 81–83).

Recommendations for the treatment and prophylaxis of VTE in patients with cancer and with COVID-19 are shown in panel 5. Patients with cancer and with COVID-19 should be assessed for risk of VTE like any patient with COVID-19. Pharmacological prophylaxis during hospitalisation should be given, with the same dose and type of anticoagulant as in patients with cancer who do not have COVID-19. Post-discharge VTE prophylaxis is also not advised; however, as with any patient with cancer, individual assessment of benefit-risk ratio is warranted, as one randomised controlled trial found that rivaroxaban 10 mg daily for 35 days improved clinical outcomes in patients with COVID-19 at high risk of VTE.⁹²

Conclusion

Cancer-associated VTE remains an important clinical problem, associated with increased morbidity and mortality. The 2022 updated ITAC guidelines incorporate emerging data within established approaches for the prevention and treatment of cancer-associated thrombosis. The ITAC guidelines' companion free web-based mobile application will assist the practising clinician with decision making at various levels to provide optimal care of patients with cancer to treat and prevent VTE.

Contributors

The *Institut National du Cancer* designed the methods used to develop the clinical practice guidelines and provided logistical support by doing the MEDLINE OVID reference searches. The guidelines were developed by an independent working group of academic clinicians, researchers, and experts (all authors of this Review). DF and JD were the acting coordinators for the working group; they coordinated the preparation of the manuscript, and the contribution of the authors. CF (lead methodologist) and PHP assessed the methodological strength and clinical relevance of the articles identified by the literature search (critical appraisal), selected the articles, and did the extraction of the data into evidence tables. All authors reviewed and approved the *Institut National du Cancer* literature search, the critical appraisal of articles, the article selection, the data extraction, and the evidence tables. DF, CF (equal contribution), JMC, and JD wrote the first draft of the literature review. All working group members edited and contributed to the development of the literature review. Guideline consensus was achieved during four meetings, at which the working group collectively drafted and ranked the recommendations. The manuscript was reviewed by a multidisciplinary advisory panel of 87 experts. All working group members approved the final recommendations and the manuscript.

Declaration of interests

DF is the founding cochair of the International Initiative on Thrombosis and Cancer. CF reports honoraria for lectures from Bayer, Bristol Myers Squibb, and LEO Pharma; and support for attending meetings from LEO Pharma and Pfizer, outside the submitted work. JMC reports research funding to her institution from CSL Behring; consulting fees from Abbott; honoraria for lectures from Bristol Myers Squibb, Roche, and Sanofi; and participated in the advisory board of Abbott, Alnylam, Anthos, Bristol

Myers Squibb, Sanofi, and Takeda, outside the submitted work. AAK reports consulting fees from Anthos, Bayer, Bristol Myers Squibb, Janssen, Pfizer, and Sanofi; honoraria for lectures, presentations, speakers bureaus, manuscript writing, or educational events from Anthos, Bayer, Bristol Myers Squibb, Janssen, Pfizer, and Sanofi; payment for expert testimony to his institution; and support for attending meetings or travel from Bayer, Janssen, Pfizer, and Sanofi, outside the submitted work. AK reports consulting fees from Verseeon; and payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing, or educational events from CCL Pharma, outside the submitted work. CA reports payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing, or educational events from Bayer, Bristol Myers Squibb, Daiichi-Sankyo, Pfizer, and Sanofi, outside the submitted work. AM reports royalties or licenses for risk assessment model in venous thromboembolism in patients with cancer; consulting fees from AstraZeneca, Bristol Myers Squibb, Celgene, Daiichi Sankyo, Incyte, Lilly, LEO Pharma, MSD, Pfizer, Roche, Sanofi, and Servier; honoraria for lectures from Amgen, AstraZeneca, Bayer, Bristol Myers Squibb, Daiichi Sankyo, Lilly, LEO Pharma, Menarini, Pfizer, Rovi, Sanofi, and Stada; and support for attending meetings or travel from Amgen, AstraZeneca, Celgene, Merck Serono, and Roche, outside the submitted work. BB reports payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing, or educational events from Horiba Medical, Johnson & Johnson, Rovi, and Sanofi, outside the submitted work. DB reports honoraria for lectures from Bayer and Sanofi; support for attending meetings or travel from LEO Pharma and Sanofi; and participated in the advisory board of Apixabano (Pfizer), outside the submitted work. DA reports payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing, or educational events from Roche, Janssen, Takeda, and Pfizer; participated in the advisory board of AstraZeneca, Bayer, Janssen, Pfizer, Roche, and Takeda, outside the submitted work; and is the president of the Serbian Lymphoma Group. PC reports support for attending meetings from Sanofi-Aventis, outside the submitted work, and is a member of the guidelines and guidance Committee of the International Society on Thrombosis and Haemostasis. MCGE reports support for attending meetings from Nolver Uruguay, outside the submitted work. TI reports honoraria for lectures from Asahi Kasei Pharma, Bristol Myers Squibb, Daiichi Sankyo, Nippon Shinyaku, and Pfizer, outside the submitted work. SAA reports grants or contract from AstraZeneca; support for attending meetings from Curoscience; and a leadership or fiduciary role in AstraZeneca, ASTCT, and ASSCO POS, outside the submitted work. LAM-G reports receiving consulting fees from Novartis; and honoraria for lectures from Novartis, Amgen, Astellas, AstraZeneca, Janssen, Roche, and Teva, outside the submitted work. HB participated in the data safety monitoring board of the COVID-HEP and the Dawn-Antico COVID-19 studies; is a member of the steering committee of the GARFIELD VTE Study; is the President of the Swiss Academy of Medical Science, the President of the Société Académique de Genève, and participated to the foundation board of the Promotion Santé Suisse outside the submitted work. IP reports honoraria for lectures and advisory boards from Bayer, Daiichi Sankyo, Pfizer, and Sanofi, outside the submitted work; and is the Chair of the European Thrombosis and Haemostasis Association. JD reports consulting fees from Sanofi; honoraria for lectures from LEO Pharma, Pfizer, and Servier, outside the submitted work; and is the President of Thrombosis Canada and the cochair of the International Initiative on Thrombosis and Cancer. PHP declares no competing interests.

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