



# **Anomalies cytogénomiques et dysfonction télomérique des leucémies chroniques et tumeurs solides**

L Veronese

## **► To cite this version:**

L Veronese. Anomalies cytogénomiques et dysfonction télomérique des leucémies chroniques et tumeurs solides. Sciences du Vivant [q-bio]. ECOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE UCA, 2023. <tel-04796696>

**HAL Id: tel-04796696**

**<https://hal.science/tel-04796696v1>**

Submitted on 21 Nov 2024

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

*ECOLE DOCTORALE DES SCIENCES DE LA VIE, SANTE,  
AGRONOMIE, ENVIRONNEMENT*

## ***Habilitation à Diriger des Recherches***

Présentée à l'Université Clermont Auvergne

Soutenue le 6 décembre 2023

VERONESE Lauren

---

### **Anomalies cytogénomiques et dysfonction télomérique des leucémies chroniques et tumeurs solides**

---

Membres du jury Mme le Pr Fanny Baran-Marszak (rapporteuse)

Mr le Pr Marc-Antoine Belaud-Rotureau (rapporteur)

Mr le Pr Romain Guèze

Mme le Dr Delphine Poncet (rapporteuse)

Mr le Pr Andrei Tchirkov

Mr le Pr Olivier Tournilhac

## Remerciements

**Mme le Pr Fanny Baran-Marszak**, Professeure des Universités-Praticien Hospitalier dans le Laboratoire d'Hématologie de l'Hôpital Avicenne à Bobigny, membre de l'Unité INSERM U978 de l'Université Paris 13. Je vous remercie d'avoir accepté de juger ce travail en tant que rapporteuse et de pouvoir bénéficier de votre expertise en biologie moléculaire des hémopathies malignes.

**Mr le Pr Marc-Antoine Belaud-Rotureau**, Professeur des Universités-Praticien Hospitalier dans le Service de Cytogénétique et Biologie Cellulaire du CHU de Rennes et membre de l'équipe INSERM, EHESP, IRSET, UMR 1085 de l'Université de Rennes 1. Je te suis reconnaissante d'avoir accepté de juger ce mémoire en tant que rapporteur. Ton expertise et tes compétences en cytogénétique vont éclairer l'évaluation de ces travaux et projets.

**Mr le Pr Romain Guièze**, Professeur des Universités-Praticien Hospitalier dans le Service d'Hématologie Clinique Adulte et Thérapie Cellulaire du CHU de Clermont-Ferrand, et membre de l'équipe d'accueil EA 7453 CHELTER de l'Université Clermont-Auvergne. Je te remercie très sincèrement d'avoir accepté d'évaluer ces travaux et de partager ton expertise reconnue et ton enthousiasme communicatif.

**Mme le Dr Delphine Poncet**, Maitresse de Conférences des Universités-Praticien Hospitalier dans le Laboratoire de Pathologie, Neuropathologie et Biologie Moléculaire des Hospices Civils de Lyon et membre de l'équipe INSERM 1052, CNRS 5286, Centre de recherche en cancérologie de Lyon, Université Claude Bernard Lyon 1. Je vous remercie d'avoir accepté de juger ces travaux comme rapporteuse et de pouvoir bénéficier de votre expertise en biologie des télomères et en biologie moléculaire des tumeurs malignes.

**Mr le Pr Andrei Tchirkov**, Professeur des Universités-Praticien Hospitalier dans le Service de Cytogénétique du CHU de Clermont-Ferrand et membre de l'équipe d'accueil EA 7453 CHELTER de l'Université Clermont-Auvergne. Merci infiniment de contribuer à l'accomplissement de ces travaux et merci de me transmettre tes connaissances au quotidien, toujours avec patience et bienveillance. J'exprime ici ma gratitude et mon profond respect.

**Mr le Pr Olivier Tournilhac**, Professeur des Universités-Praticien Hospitalier dans le Service d'Hématologie Clinique Adulte et Thérapie Cellulaire du CHU de Clermont-Ferrand, et membre de l'équipe d'accueil EA 7453 CHELTER de l'Université Clermont-Auvergne. Je te remercie du fond du cœur d'avoir rendu possible la réalisation de ces travaux et d'avoir porté l'héritage de la thématique de la LLC.

Merci à toutes les personnes qui ont contribué à ces travaux. Je pense en particulier à Delphine Voisin, dont l'expertise technique est essentielle et précieuse, et à Farida Godeau, toujours prête à accueillir et former de nouveaux stagiaires. Merci à Manon Garcia et à l'ensemble de l'équipe CHELTER avec qui c'est un plaisir de collaborer.

Merci à toute l'équipe de techniciennes, ingénieur(e)s et secrétaires du Service de Cytogénétique pour votre implication dans l'activité de diagnostic et pour l'aide apportée aux projets de recherche.

Merci au Pr Florence Nguyen-Khac, pour son aide, son expertise et ses conseils précieux. Merci au bureau et à l'ensemble des membres du GFCH pour leur enthousiasme et les partages d'expérience.

Merci à nos collègues d'hématologie clinique et biologique, particulièrement les Pr Jacques-Olivier Bay et Marc Berger.

Merci à Gwendoline pour sa grande expertise cytogénétique et ses conseils avisés.

Merci à tous les étudiant(e)s qui sont volontaires pour un stage chez nous chaque année.

Merci Mathilde, ta présence dans le Service me manque.

Merci Carole, pour l'ensemble de ton œuvre.

Merci aux amis, pour les joies extra-professionnelles et soirées paisibles.

Merci à Bernard et Bichette.

Merci à mes parents et mon frère.

*A la mémoire de Mr le Pr Philippe TRAVADE*



*A Guillaume, Marius, Séléné et Camille*

# Table des matières

CURRICULUM VITAE .....	7
EXPERIENCE MEDICALE .....	7
EXPERIENCE SCIENTIFIQUE.....	8
FINANCEMENTS ET DISTINCTION .....	8
SOCIETE SAVANTES .....	8
ACTIVITES PEDAGOGIQUES ET D'ENCADREMENT.....	9
BIBLIOMETRIE.....	11
LISTE DES PUBLICATIONS .....	12
Liste des publications internationales et nationales.....	12
Liste des communications orales en tant qu'orateur.....	16
Liste des communications orales ou affichées en congrès international .....	17
Liste des communications orales ou affichées en congrès national des 10 dernières années.....	20
ABBREVIATIONS .....	24
TRAVAUX DE RECHERCHE.....	25
1. Avant-propos .....	25
2. Résumé du travail doctoral.....	26
3. Télomères et anomalies cytogénétiques de la LLC.....	27
a. Introduction .....	27
b. Télomères et instabilité génomique de la LLC.....	28
c. Télomères et disruption <i>TP53</i> au cours de la LLC.....	31
4. Télomères et cancers solides.....	33
a. Introduction .....	33
b. Statut télomérique et expression d' <i>ERCC1</i> dans le cancer du sein .....	33
c. Expression de <i>TERT</i> dans le cancer du sein: rôle des mutations du promoteur et du gain de copies du gène <i>TERT</i> .....	36
d. Paramètres télomériques et formes avancées du cancer du poumon non à petites cellules : valeur prédictive et pronostique.....	38
PROJETS DE RECHERCHE .....	40
1. Introduction .....	40
2. Dysfonction télomérique au cours des hémopathies chroniques .....	40
a. Etude longitudinale des télomères et instabilité génomique de la LLC .....	40
b. Dynamique télomérique et leucémies chroniques .....	42

3. Anomalies cytogénomiques des hémopathies chroniques .....	43
a. LLC ou autres syndromes lymphoprolifératifs B et réarrangement de <i>BCL3</i> .....	43
b. Caractérisation clinico-biologiques des SLP-B avec trisomies 12 et 19 .....	46
c. Mécanismes génomiques de résistance thérapeutique de la LLC.....	47
4. Bilan .....	52
REFERENCES .....	55
PUBLICATIONS SELECTIONNEES .....	62

# CURRICULUM VITAE

---

## Dr Lauren VERONESE

Données civiles : née le 16/02/1978  
Nationalité française  
PACSée, 3 enfants

Situation professionnelle : Maître de Conférence des Universités – Praticien Hospitalier (MCU-PH) en Histologie Embryologie Cytogénétique (sous-section 4202 du CNU)

Coordonnées : Service de Cytogénétique Médicale CHU Estaing  
1 place Lucie et Raymond Aubrac F-63003 Clermont-Ferrand Cedex1

Département d’Histologie Embryologie Cytogénétique  
UFR de Médecine et Professions Paramédicales  
28 place Henri Dunant 63000 Clermont Ferrand

Téléphone : +33 (0)4 73 750 443 / (0)4 73 17 81 12

Portable : +33 (0)6 03 42 78 90

Télécopie : +33 (0)4 73 750 704

Courriel : [veronese@chu-clermontferrand.fr](mailto:veronese@chu-clermontferrand.fr) / [lauren.veronese@uca.fr](mailto:lauren.veronese@uca.fr)

## EXPERIENCE MEDICALE

**Depuis 2009 :** MCU-PH, Service de Cytogénétique Médicale et Département Universitaire d’Histologie Embryologie Cytogénétique (Pr Tchirkov – CHU Estaing - UFR Médecine et Professions Paramédicales), dans le secteur d’**onco-hématologie** avec le Dr SOLER (PH) et le Pr TCHIRKOV (PUPH)  
Interprétation des analyses de **cytogénétique conventionnelle** (caryotype et FISH) et de **diagnostics moléculaires** (PCR, RTPCR, analyse de fragment, Sanger, NGS, habilitation Auragen) dans le cadre du diagnostic et du suivi des hémopathies malignes et de certaines tumeurs solides (neuroblastome, tumeurs cérébrales, sarcomes)  
~5500 prélèvements et 8500 analyses dans ce secteur pour l’année 2022

**2013 :** Qualification en Biologie Médicale par l’Ordre des Médecins

**2007 – 2009 :** Assistante hospitalo-universitaire, Service de Cytogénétique Médicale et Département Universitaire d’Histologie Embryologie Cytogénétique (CHU Estaing - UFR Médecine et Professions Paramédicales)

**2007 :** Diplôme d’Etudes Spécialisées en Hématologie Clinique  
Diplôme d’Etudes Spécialisées Complémentaires en Cytogénétique Humaine  
Doctorat d’Etat en Médecine  
CHU Clermont-Ferrand, Université d’Auvergne

**2001–2007 :** Internat de Spécialité, discipline médicale, Hématologie Clinique, CHU Clermont-Ferrand, Université d’Auvergne

**1995-2001 :** Etudes Médicales, Faculté de Médecine de Dijon.

## EXPERIENCE SCIENTIFIQUE

- 2013 :** Doctorat d'Université,  
Ecole Doctorale des Sciences de la Vie, Santé, Agronomie, Environnement  
Université d'Auvergne
- 2006-2007 :** Master 2 Recherche, Spécialité Génétique et Physiologie, Mention Génétique et  
Physiologie  
Université d'Auvergne
- 2001 :** **Maîtrise de Sciences Biologiques et Médicales** (certificat de Biochimie)  
Faculté de Médecine de Dijon, Université de Bourgogne, 2001

## FINANCEMENTS ET DISTINCTION

- 2023** Bourse ACLF Association de Cytogénétique de Langue Française (15 000 euros)  
Mécanisme de résistance au venetoclax dans la leucémie lymphoïde  
chronique (RAVEN) : apport de la cartographie optique du génome
- 2019** Comité Régional de la Ligue Contre le Cancer (27 000 euros)  
Syndromes lymphoprolifératifs B avec réarrangement du locus BCL3 (19q13)
- 2007** Prix de Thèse décerné par le Comité Départemental de la Ligue Nationale Contre le Cancer
- 2006** Comité Régional de la Ligue Contre le Cancer (14 000 euros)  
Leucémie Lymphoïde Chronique : étude des facteurs du pronostic

## SOCIETE SAVANTES

Collège Universitaire et Hospitalier des Histologistes, Embryologistes, Cytologistes et Cytogénéticiens  
(CHEC)

Association de Cytogénétique de Langue Française (ACLF)

Groupe Francophone de Cytogénétique Hématologique (GFCH) au sein de l'ACLF : **membre du bureau  
depuis 2018**

French Innovative Leukemia Organization (FILO) : **membre du Conseil Scientifique depuis 2023**

Groupe de Biologie Moléculaire des Hémopathies Malignes (GBMHM)

Ligue Contre le Cancer : **membre du Conseil Scientifique AURA depuis 2023**

## ACTIVITES PEDAGOGIQUES ET D'ENCADREMENT

**1/ Enseignement** depuis 2006 au sein du Département Universitaire d'Histologie Embryologie Cytogénétique de l'UFR Médecine et Professions Paramédicale de l'Université Clermont Auvergne dans les formations suivantes :

**Premier cycle des études médicales** : PCEM1 puis PACES de 2007 à 2021 ; PASS depuis 2020 ; PASS-Réadaptation (orthoptie) depuis 2021 ; LAS2 depuis 2021 ; DFGSM2 depuis 2007 ; DFGSM3 depuis 2006

**Formations paramédicales** : Orthoptie depuis 2014, Orthophonie depuis 2016, Maïeutique 2<sup>ème</sup> année de 2012 à 2015)

**Odontologie DFGSMO 2<sup>ème</sup> année** (de 2013 à 2015)

**Licence Sciences Pour la Santé** (de 2008 à 2021)

**Troisième cycle des études médicales** : DIU de Cytométrie en Recherche et en Clinique ; DES de cancérologie ou de pédiatrie

volume horaire moyen de **135h par an** depuis ma titularisation en 2009

**2/ Encadrement des internes et étudiants en stage** dans le service de Cytogénétique Médicale (internes de Spécialité, externes en Pharmacie, stages hospitaliers DFGSM3)

## 3/ Encadrement de travaux de recherche

### Doctorat d'Université en Sciences de la vie et de la sante : participation à l'encadrement

**M. GAY-BELLILE (2017)** « Etude de l'instabilité génomique et du statut des télomères dans le cancer du sein » (Directeur, Pr Tchirkov)

### Master 2 Génétique et Physiologie (Université Clermont Auvergne) : participation à l'encadrement

**M.PAGES (2013)** « Rôle des mutations et des délétions du gène *TP53* dans l'instabilité télomérique au cours de la leucémie lymphoïde chronique » (Directeurs, Pr Tchirkov et Pr Tournilhac)

**L. BOUNAIX (2017)** « Dynamique des télomères et complexité génomique dans la leucémie lymphoïde chronique » (Directeur, Pr Tchirkov)

### Master 2 Reproduction et Développement (Université Paris Diderot) : participation à l'encadrement

**L. CHAPUT (2017)** « Détection de la maladie résiduelle dans le tissu ovarien et le tissu testiculaire humain applicable aux enfants prépubères atteints d'un sarcome d'Ewing » (Directeur, Pr Brugnon)

**Master 2 Ingénierie de la Santé/Diagnostic Biomédical (Université Clermont Auvergne) :**  
**participation à l'encadrement**

**E. FAUGERAS (2018)** « Etude du statut télomérique dans le cancer broncho-pulmonaire » (Directeur, Pr Tchirkov)

**Master 1 Génétique et Physiologie**

**J. DISSARD (2008)** « Nouvelles approches d'analyses chromosomiques et moléculaires dans la LLC »

**Master 1 Biologie Santé Option Génétique**

**L. COGNERAS (2010)** « Etude de la relation entre le niveau d'expression des gènes anti-apoptotiques *MCL1*, *BCL2* et *VEGF* et la survenue d'anomalies chromosomiques dans la LLC »

**V. POINTIS (2015)** « Mise au point de la FISH quantitative pour la mesure de la longueur des télomères dans la Leucémie Lymphoïde Chronique (LLC) »

**M. MORARD (2018)** « Etude longitudinale de raccourcissement télomérique dans la leucémie lymphoïde chronique en rechute/réfractaire par FISH quantitative »

**C CROIZIER (2018)** « Mesure de la longueur télomérique par FISH quantitative sur une série de leucémie lymphoïde chronique en rechute et/ou réfractaire »

**L LAFFONT (2019)** « Mesure de la longueur télomérique par FISH quantitative sur une série de leucémie lymphoïde chronique en rechute et/ou réfractaire »

**J ANDRAL (2019)** « Etude de la dynamique télomérique sur des échantillons de leucémie lymphoïde chronique »

**C CHOISET (2020)** « Cytogénomique des syndromes lymphoprolifératifs IG-BCL3 »

**E MARCEAU (2022)** « Recherche de mutations de résistance au cours de la LLC »

**A. PAPON (2023)** « Recherche de mutations de résistance au cours de la LLC »

**G. YVON (2023)** « Maladie résiduelle par qRT-PCR et PCR digitale dans le neuroblastome »

**Licence 3 Sciences de la vie : Physiologie et génomique fonctionnelle**

**L. VUILLERMET (2023)**

**BTS Biotechnologie ou IUT Génie Biologique**

IUT Génie Biologique : **L. DEJOUX (2009)**

BTS Biotechnologies : **L. MOUNIER (2009)**

BTS Biotechnologies : **C GYNEYS (2010)**

## **4/ Membre de Jury**

**Master 1 Génétique** : membre de jury tous les ans depuis 2015

**Master 1 Anatomie** : membre de jury en 2015, 2016, 2018, 2019

**Thèse d'exercice, doctorat en médecine** : Fanny CHAMBON, 2012 ; Mélanie PAGES, 2015 ; Caroline Croizier, 2022 ; Gautier CORRE, 2023

**Doctorat d'Université** : Romain GUIEZE, 2018 ; Victoria GREZE, 2019 ; Ludovic JONDREVILLE, 2023

**DESC de Cytogénétique** : Béatrice Grange, 2019

## **BIBLIOMETRIE**

Score SIGAPS : 366

H-index : 14 (Web of Science)

Score fractionné : 14.72

Publications scientifiques dans des revues à comités de lecture : **42** (+4 *acceptés*)

Rangs A : 12

Rangs B : 8

Premier auteur : 6

Deuxième auteur : 4 (+1 *accepté*)

Avant-dernier auteur : 3 (+2 *accepté*)

Dernier auteur : 1 (+1 *accepté*)

Communications orales en congrès en tant qu'orateur : **14**

Communications orales ou affichées en congrès international avec résumé ou support didactique : **25**

Communications orales ou affichées en congrès national avec résumé ou support didactique : **80**



# LISTE DES PUBLICATIONS

---

Les publications cosignées avec un étudiant encadré sont en gras

## Liste des publications internationales et nationales

1. Tchirkov A, Khalil T, Chautard E, Mokhtari K, **Véronèse L**, Irthum B, Vago P, Kemeny JL, Verrelle P. Interleukin-6 gene amplification and shortened survival in glioblastoma patients. *Br J Cancer* 2007; 96(3):474-6 **IF= 4.635**
2. **Véronèse L**, Tournilhac O, Verrelle P, Davi F, Dighiero G, Chautard E, Veyrat-Masson R, Kwiatkowski F, Goumy C, Vago P, Travade P, Tchirkov A. Low MCL-1 mRNA expression correlates with prolonged survival in B-cell chronic lymphocytic leukemia. *Leukemia*. 2008; 22(6): 1291-3 **IF: 8.634**
3. Goumy C, Beaufrère AM, Tchirkov A, Gouas L, Gaspard F, Giollant M, Roucaute T, **Véronèse L**, Lemery D, Vago P. Larsen-like phenotype associated with partial trisomy 3p and monosomy 5p. *Prenat Diagn* 2008; 28(2): 131-4 **IF= 1.596**
4. L. Gouas, C. Goumy, **L. Véronèse**, A. Tchirkov, P. Vago. Gene dosage methods as diagnostic tools for the identification of chromosome abnormalities. *Pathol Biol* 2008; 56(6): 345-353. **IF= 0.96**
5. **Véronèse L**, Tchirkov A, Gouas L, Perissel B, Goumy C et Vago P. Prognosis of chronic lymphocytic leukemia: focus on recent biomarkers. *Ann Biol Clin* 2008; 66(4): 371-7 **IF = 0.286**
6. Goumy C, Gouas L, Tchirkov A, Roucaute T, **Véronèse L**, Giollant M, Francannet C, Vago P. Familial deletion 11q14.3-q22.1 without apparent phenotypic consequences: a haplosufficient 8.5 Mb region. *Am J Med Genet A* 2008; 146A(20): 2668-72. **IF = 2,555**
7. E Chapiro, N Leporrier, I Radford-Weiss, C Bastard, H Mossafa, D Leroux, I Tigaud, M de Braekeleer, C Terré, F Brizard, E Callet-Bauchu, S Struski, **L Véronèse**, S Fert-Ferrer, S Taviaux, C Lesty, F Davi, H Merle-Béral, OA Bernard, L Sutton, SD Raynaud, F Nguyen-Khac. Gain of the short arm of chromosome 2 (2p) is a frequent recurring chromosome aberration in untreated Chronic Lymphocytic Leukemia at advanced stages. *Leuk Res* 2010; 34(1): 63-8. **IF = 2,555**
8. **L. Véronèse**, O. Tournilhac, P. Verrelle, F. Davi, G. Dighiero, E. Chautard, R. Veyrat-Masson, F. Kwiatkowski, C. Goumy, L. Gouas, JO. Bay, P. Vago, A. Tchirkov. Strong correlation between the expression levels of *VEGF* and *MCL-1* genes in B-cell chronic lymphocytic leukaemia. *Leuk Res* 2009; 33(12): 1623-1626. **IF = 2.358**
9. **L. Véronèse**, A.Tchirkov, C. Richard-Pebrel, A. Ledoux-Pilon, J. Fleury, C. Chaletex, C. Goumy, L. Gouas, M. G. Berger, P. Vago P, J. O. Bay, O. Tournilhac. A thrombocytosis occurring in Philadelphia positive CML in molecular response to imatinib can reveal an underlying JAK2V617F myeloproliferative neoplasm. *Leuk Res* 2010; 34(4):e94-6. **IF = 2.555**
10. A Tchirkov, V Sapin, G Marceau, E Chautard, G Narla, **L Véronèse**, P Vago, S Friedman, T Khalil, JL Kemeny and P Verrelle. Increased expression of the oncogenic KLF6-SV1 transcripts in human glioblastoma. *Clin Chem Lab Med*. 2010; 1167-70. **IF = 2.069**
11. Goumy C, Gouas L, Pebrel-Richard C, **Véronèse L**, Eymard-Pierre E, Debost-Legrand A, Haoud K, Tchirkov A, Vago P. Prenatal detection of cryptic rearrangements by MLPA in fetuses with ultrasound abnormalities. *Genet Med*. 2010 Jun;12(6):376-80. **IF = 5.28**
12. Goumy C, Gouas L, Marceau G, Coste K, **Véronèse L**, Gallot D, Sapin V, Vago P, Tchirkov A. Retinoid pathway and congenital diaphragmatic hernia: hypothesis from the analysis of chromosomal abnormalities. *Fetal Diagn Ther*. 2010;28(3):129-39. **IF = 0,84**
13. Debost-Legrand A, Capri Y, Gouas L, Pebrel-Richard C, **Véronèse L**, Tchirkov A, Haoud K, Boespflug-Tanguy O, Goumy C, Vago P. De novo unbalanced translocation 2;4 characterized by metaphase CGH and array CGH in a child with mental retardation and dysmorphic features. *Pathol Biol* 2010 Dec 8. **IF = 0.95**

14. Ravinet A, Gay Belille M, Lemal R, Véronèse L, Bay JO, Prie N. [Physiopathological, diagnostic and therapeutic impacts of chromosomal translocations in hematological malignancies]. *Bull Cancer* 2011 Dec;98(12):1403-18. IF : 0,792
15. Véronèse L, Tournilhac O, Callanan M, Prie N, Kwiatkowski F, Combes P, Chauvet M, Davi F, Gouas L, Verrelle P, Guièze R, Vago P, Bay JO, Tchirkov A. Telomeres and chromosomal instability in chronic lymphocytic leukemia. *Leukemia*. 2013 Feb;27(2):490-3 IF = 9.379
16. Nguyen-Khac F, Lambert J, Chapiro E, Grelier A, Mould S, Barin C, Daudignon A, Gachard N, Struski S, Henry C, Penther D, Mossafa H, Andrieux J, Eclache V, Bilhou-Nabera C, Luquet I, Terre C, Baranger L, Mugneret F, Chiesa J, Mozziconacci MJ, Callet-Bauchu E, Veronese L, Blons H, Owen R, Lejeune J, Chevret S, Merle-Beral H, Leblond V. Chromosomal aberrations and their prognostic value in a series of 174 untreated patients with Waldenstrom macroglobulinemia. *Haematologica* 2013 Apr;98(4):649-54 IF = 5.868
17. Véronèse L, Tournilhac O, Combes P, Prie N, Pierre-Eymard E, Guièze R, Veyrat-Masson R, Bay JO, Vago P, Tchirkov A. Contribution of MLPA to routine diagnostic testing of recurrent genomic aberrations in chronic lymphocytic leukemia. *Cancer Genet*. 2013 Jan-Feb;206(1-2):19-25 IF = 2,417
18. Kemeny S, Pebrel-Richard C, Gouas L, Véronèse L, Lemery D, Tchirkov A, Goumy C, Vago P. Prenatal ultrasound diagnosis of a 48,XXYY syndrome. *Morphologie*. 2013 Jun;97(317):65-7
19. Cosson A, Chapiro E, Belhouachi N, Cung HA, Keren B, Damm F, Algrin C, Lefebvre C, Fert-Ferrer S, Luquet I, Gachard N, Mugneret F, Terre C, Collonge-Rame MA, Michaux L, Rafdord-Weiss I, Talmant P, Véronèse L, Nadal N, Struski S, Barin C, Helias C, Lafage M, Lippert E, Auger N, Eclache V, Roos-Weil D, Leblond V, Settegrana C, Maloum K, Davi F, Merle-Beral H, Lesty C, Nguyen-Khac F; on behalf of the Groupe Francophone de Cytogénétique Hématologique. 14q deletions are associated with trisomy 12, NOTCH1 mutations and unmutated IGHV genes in chronic lymphocytic leukemia and small lymphocytic lymphoma. *Genes Chromosomes Cancer* 2014 Aug;53(8):657-66 IF = 4,041
20. Goumy C, Gay-Bellile M, Eymard-Pierre E, Kemeny S, Gouas L, Déchelotte P, Gallot D, Véronèse L, Tchirkov A, Pebrel-Richard C, Vago P. De novo 2q36.1q36.3 interstitial deletion involving the PAX3 and EPHA4 genes in a fetus with spina bifida and cleft palate. *Birth Defects Res A Clin Mol Teratol* 2014 Jun;100(6):507-11 IF = 2,089
21. Guièze R, Robbe P, Clifford R, de Guibert S, Pereira B, Timbs A, Dilhuydy MS, Cables M, Ysebaert L, Burns A, Nguyen-Khac F, Davi F, Véronèse L, Combes P, Le Garff-Tavernier M, Leblond V, Merle-Béral H, Alsolami R, Hamblin A, Mason J, Pettitt A, Hillmen P, Taylor J, Knight SJ, Tournilhac O, Schuh A. Presence of multiple recurrent mutations revealed by targeted NGS confers poor trial outcome of relapsed/refractory CLL. *Blood* 2015 Oct 29;126(18):2110-7 IF = 11.841
22. Guièze R\*, Pages M\*, Véronèse L, Combes P, Lemal R, Gay-Bellile M, Chauvet M, Callanan M, Kwiatkowski F, Pereira B, Vago P, Bay JO, Tournilhac O, Tchirkov A. Telomere status in chronic lymphocytic leukemia with TP53 disruption. *Oncotarget* 2016 Aug 30;7(35):56976-56985. IF =5.168
23. Gay-Bellile M, Romero P, Cayre A, Véronèse L, Privat M, Singh S, Combes P, Kwiatkowski F, Abrial C, Bignon YJ, Vago P, Penault-Llorca F, Tchirkov A. ERCC1 and telomere status in breast tumours treated with neoadjuvant chemotherapy and their association with patient prognosis. *J Pathol Clin Res* 2016 Jul 13;2(4):234-246. IF = 3.489
24. Gay-Bellile M, Véronèse L, Combes P, Eymard-Pierre E, Kwiatkowski F, Dauplat MM, Cayre A, Privat M, Abrial C, Bignon YJ, Mouret-Reynier MA, Vago P, Penault-Llorca F, Tchirkov A. TERT promoter status and gene copy number gains: effect on TERT expression and association with prognosis in breast cancer. *Oncotarget* 2017 Aug 24;8(44):77540-77551. IF =NC
25. Chapiro E, Lesty C, Gabillaud C, Durot E, Bouzy S, Armand M, Le Garff-Tavernier M, Bougacha N, Struski S, Bidet A, Laharanne E, Barin C, Veronese L, Prié N, Eclache V, Gaillard B, Michaux L, Lefebvre C, Gaillard JB, Terré C, Penther D, Bastard C, Nadal N, Fert-Ferrer S, Auger N, Godon C, Sutton L, Tournilhac O, Susin SA, Nguyen-Khac F; Groupe Francophone de Cytogénétique Hématologique (GFCH) and the French Innovative Leukemia Organization (FILO) group. "Double-

- hit" chronic lymphocytic leukemia: An aggressive subgroup with 17p deletion and 8q24 gain. *Am J Hematol* 2018 Mar;93(3):375-382. **IF = 6.137**
26. Bahashwan S, Moluçon-Chabrot C, Hermet E, Ravinet A, Douge A, **Veronese L**, Tchirkov A, Lemal R, Berger MG, Veyrat-Masson R, Tournilhac O, Bay JO, Guièze R. Outcome and impact of post-remission strategy after MIDAM regimen in patients with relapsing or refractory acute myeloid leukemia. *American Journal of Hematology*. janv 2019;94(1):E32-5 **IF = 6.973**
  27. Bidet A, Dulucq S, Smol T, Marceau-Renaut A, Morisset S, Coiteux V, Noël-Walter MP, Nicolini FE, Tigaud I, Luquet I, Struski S, Gaillard B, Penther D, Tondeur S, Nadal N, Hermet E, **Véronèse L**, Réa D, Gervais C, Theisen O, Terré C, Cony-Makhoul P, Lefebvre C, Gaillard JB, Radford I, Vervaeke AL, Barin C, Chapiro E, Nguyen-Khac F, Etienne G, Preudhomme C, Mahon FX, Roche-Lestienne C. Poor prognosis of chromosome 7 clonal aberrations in Philadelphia-negative metaphases and relevance of potential underlying myelodysplastic features in chronic myeloid leukemia. *Haematologica*. 2019 Jun;104(6):1150-1155. **IF = 7.116**
  28. Chaput L, Grèze V, Halle P, Radosevic-Robin N, Pereira B, **Véronèse L**, Lejeune H, Durand P, Martin G, Sanfilippo S, Canis M, Kanold J, Tchirkov A, Brugnon F. Sensitive and Specific Detection of Ewing Sarcoma Minimal Residual Disease in Ovarian and Testicular Tissues in an In Vitro Model. *Cancers (Basel)*. 2019 Nov 17;11(11). pii: E1807. doi: 10.3390/cancers11111807 **IF = 6.126**
  29. Nguyen-Khac F, Bidet A, **Veronese L**, Daudignon A, Penther D, Troadec MB, Lefebvre C, Lafage-Pochitaloff. Recommendations for cytogenomic analysis of hematologic malignancies: comments from the Francophone Group of Hematological Cytogenetics (GFCH). *Leukemia*. 2020 Jun;34(6):1711-1713. doi: 10.1038/s41375-019-0696-1. Epub 2019 Dec 20. *Leukemia*. 2020. PMID: 31862958 **IF = 11.528**
  30. Bouclet F, Calleja A, Dilhuydy MS, **Véronèse L**, Pereira B, Amorim S, Cymbalista F, Herbaux C, de Guibert S, Roos-Weil D, Hivert B, Aurran T, Dupuis J, Blouet A, Tchernonog E, Laribi K, Dmytruk N, Morel P, Michallet AS, Dartigeas C, Tournilhac O, Nguyen-Khac F, Delmer A, Feugier P, Ysebaert L, Guièze R. Real-world outcomes following venetoclax therapy in patients with chronic lymphocytic leukemia or Richter syndrome: a FILO study of the French compassionate use cohort. *Ann Hematol*. 2021 Jan 25. doi: 10.1007/s00277-021-04419-w. Epub ahead of print. PMID: 33495922. **IF = 4.03**
  31. Lazarian G, Munger M, Quinquenel A, Dilhuydy MS, **Veronese L**, Luque Paz D, Guièze R, Ledoux-Pilon A, Paillassa J, Merabet F, Vial JP, Bidet A, Waultier Rascalou A, Broseus J, Roos-Weil D, Lavaud A, Molina L, Laribi K, Hivert B, Friedrich C, Carpentier B, Ysebaert L, Van Den Neste E, Willems L, Corby A, Poulain S, Eclache V, Maubec E, Martin A, Feugier P, Delmer A, Baran-Marszak F, Leprêtre S, Cymbalista F. Clinical and biological characteristics of leukemia cutis in chronic lymphocytic leukemia: A study of the French innovative leukemia organization (FILO). *Am J Hematol*. 2021 Sep 1;96(9):E353-E356. **IF = 13.26**
  32. Lazarian G, Theves F, Hormi M, Letestu R, Eclache V, Bidet A, Cornillet-Lefebvre P, Davi F, Delabesse E, Estienne MH, Etancelin P, Kosmider O, Laibe S, Lode L, Muller M, Nadal N, Naguib D, Pastoret C, Poulain S, Sujobert P, **Veronese L**, Imache S, Lefebvre V, Cymbalista F, Baran-Marszak F, Soussi T; French Innovative Leukemia Organisation (FILO). TP53 mutations at codon 234 are associated with chlorambucil treatment in chronic lymphocytic leukemia. *Am J Hematol*. 2022 Apr;97(4):E159-E162. **IF = 12.8**
  33. Goumy C, **Veronese L**, Stamm R, Domas Q, Hadjab K, Gallot D, Laurichesse H, Delabaere A, Gouas L, Salaun G, Richard C, Vago P, Tchirkov A. Reduced telomere length in amniocytes: an early biomarker of abnormal fetal development? *Hum Mol Genet*. 2022 Aug 23;31(16):2669-2677. **IF = 3.5**
  34. Zangbéwendé Guy Ouedraogo, Victoria Cacheux, Louis-Thomas Dannus, Nathalie Dupre, **Lauren Veronese**, Benjamin Lebecque. Hemophagocytosis by acute myeloid leukemia blasts associated to poor clinical outcomes. *EJHaem*. 2022 Feb 5;3(2):558-559. **IF=NA**
  35. F Nguyen-Khac, A Bidet, A Daudignon, M Lafage-Pochitaloff, G Ameye, C Bilhou-Nabéra+, E Chapiro, MA Collonge-Rame, W Cuccuini, N Douet-Guilbert, V Eclache, I Luquet, L Michaux, N

- Nadal, D Penther, B Quilichini, C Terre, C Lefebvre, M-B Troadec, **L Véronèse**. The complex karyotype in hematological malignancies: a comprehensive overview by the Francophone Group of Hematological Cytogenetics (GFCH). *Leukemia*. 2022 Jun;36(6):1451-1466. **IF= 11.4**
36. Lemal R, Poulain S, Ledoux-Pilon A, **Veronese L**, Tchirkov A, Lebecque B, Tassin T, Bay JO, Charlotte F, Nguyen-Khac F, Berger M, Godfraind C, Ysebaert L, Davi F, Pereira B, Leblond V, Hermine O, Guièze R, Pagès F, Tournilhac O. Mast cell density and its clinical relevance in Waldenström's macroglobulinemia. *EJHaem*. 2022 Feb 8;3(2):371-378. **IF=NA**
  37. **Faugeras E, Véronèse L, Jeannin G, Janicot H, Bailly S, Bay JO, Pereira B, Cayre A, Penault-Llorca F, Cachin F, Merle P, Tchirkov A. Telomere Status of Advanced Non-Small-Cell Lung Cancer Offers a Novel Promising Prognostic and Predictive Biomarker. *Cancers (Basel)*. 2022 Dec 31;15(1):290. doi: 10.3390/cancers15010290. PMID: 36612286. IF=6.575**
  38. Florence Nguyen-Khac, Audrey Bidet, Marie-Bérengère Troadec, **Lauren Veronese**, Nathalie Auger, Agnes Daudignon, Nathalie Nadal, Dominique Penther, Lucienne Michaux, Marina Lafage-Pochitaloff, Christine Lefebvre. The 5th edition of the WHO classification of haematolymphoid tumors: comments from the Groupe Francophone de Cytogénétique Hématologique (GFCH). *Leukemia*. 2023 Apr;37(4):946-947. **IF= 11.4**
  39. Soler G, Ouedraogo ZG, Goumy C, Lebecque B, Aspas Requena G, Ravinet A, Kanold J, **Véronèse L**, Tchirkov A. Optical Genome Mapping in Routine Cytogenetic Diagnosis of Acute Leukemia. *Cancers (Basel)*. 2023 Apr 3;15(7):2131. **IF=5.2**
  40. Jasneet Kaur Khalsa, Justin Cha, Filippo Utro, Aishath Naeem, Ishwarya Murali, Yanan Kuang, Kevin Vasquez, Liang Li, Svitlana Tyekucheva, Stacey M. Fernandes, **Lauren Veronese**, Romain Guieze, Binu Kandathilparambil Sasi, Zixu Wang, John-Hanson Machado, Harrison Bai1, Maryam Alasfour, Kahn Rhrissorakrai, Chaya Levovitz, Brian P. Danysh, Kara Slowik, Raquel A. Jacobs, Cloud P. Pawelet, Ignaty Leshchiner, Laxmi Parida, Gad Getz, Jennifer R. Brown. Genetic events associated with venetoclax resistance in CLL identified by whole exome and RNA sequencing on longitudinal patient samples. *Blood*. 2023 Aug 3;142(5):421-433. **IF = 20.3**
  41. Grégory Lazarian, Bernard Leroy, Floriane Theves, Myriam Hormi, Rémi Letestu, Virginie Eclache, Giulia Tueur, Adam Ameur, Audrey Bidet, Pascale Cornillet-Lefebvre, Frédéric Davi, Eric Delabesse, Marie-Hélène Estienne, Pascaline Etancelin, Olivier Kosmider, Sophy Laibe, Laurence Lode, Marc Muller, Nathalie Nadal, Dina Naguib, Cédric Pastoret, Stéphanie Poulain, Pierre Sujobert, **Lauren Veronese**, Samia Imache, Valérie Lefebvre, Florence Cymbalista, Fanny Baran-Marszak, Thierry Soussi. On behalf of the French Innovative Leukemia Organization (FILO). The broad spectrum of TP53 mutations in CLL: evidence of multiclonality and novel mutation hotspots. *Human Mutation*, Volume 2023, May 2023. Article ID 4880113, <https://doi.org/10.1155/2023/4880113>. **IF = 4.7**
  42. Goumy C, Guy Ouedraogo Z, Soler G, Eymard-Pierre E, Laurichesse H, Delabaere A, Gallot D, Bouchet P, Perthus I, Pebrel-Richard C, Gouas L, Salaun G, Salse J, **Véronèse L**, Tchirkov A. Optical genome mapping for prenatal diagnosis: A prospective study. *Clin Chim Acta*. 2023 Oct 12;551:117594. doi: 10.1016/j.cca.2023.117594. Online ahead of print. **IF = 5.02**
  43. *Matthieu Decamp, Emilie Klein, Catherine Godon, Valentin Lestringant, Pauline Roynard, Olivier Theisen, Mélanie Jimenez-Pocquet, Catherine Roche-Lestienne, Audrey Bidet, **Lauren Veronese**. Cytogenetics in the management of myeloproliferative neoplasms, mastocytosis and myelodysplastic/myeloproliferative neoplasms: guidelines from the Group Francophone de Cytogénétique Hématologique (GFCH). Accepted for publication in *Current Research in Translational Medicine*, octobre 2023. **IF = 4.513***
  44. *C Lefebvre, **L Veronese**, N Nadal, J-B Gaillard, D Penther, A Daudignon, J Chauzeix, F Nguyen-Khac, E Chapiro. Cytogenetics in the management of mature B-cell non-Hodgkin lymphomas: guidelines from the Groupe Francophone de Cytogénétique Hematologique (GFCH). Accepted for publication in *Current Research in Translational Medicine*, octobre 2023. **IF = 4.513***
  45. *Jean-Baptiste Gaillard, Elise Chapiro, Agnès Daudignon, Nathalie Nadal, Dominique Penther, Jasmine Chauzeix, Florence Nguyen-Khac, **Lauren Veronese**, Christine Lefebvre. Cytogenetics in the management of mature T-cell and NK-cell neoplasms: guidelines from the Groupe*

*Francophone de Cytogénétique Hematologique (GFCH). Accepted for publication in Current Research in Translational Medicine, octobre 2023. IF = 4.513*

46. *F Nguyen-Khac, Z Balogh, J Chauzeix, **L Veronese**, E Chapiro. Cytogenetics in the management of chronic lymphocytic leukemia: guidelines from the Groupe Francophone de Cytogénétique Hematologique (GFCH). Accepted for publication in Current Research in Translational Medicine, octobre 2023. IF = 4.513*

## Liste des communications orales en tant qu'orateur

1. **Véronèse L**, Chaletex C, Magnac C, Vasconcellos Y, Davi F, Tournilhac O, Verrelle P, Dighiero G, Vago P, Travade P, Tchirkov A. Intérêt pronostique de l'expression de la sous-unité catalytique de la télomérase (*hTERT*) dans la Leucémie Lymphoïde Chronique (LLC). **11<sup>ème</sup> journées du Collège Universitaire et Hospitalier des Histologistes, Embryologistes, Cytologistes et Cytogénéticiens**. Tours, France, 16-18 mars 2006
2. **L Véronèse**, O Tournilhac, P Verrelle, A Michel, F Davi, Y Vasconcellos, G Dighiero, C Goumy, P Vago, P Travade, A Tchirkov. Etude de la valeur pronostique de l'expression de Myeloid-Cell-Leukemia-1 (MCL-1) dans la Leucémie Lymphoïde Chronique (LLC). **12<sup>ème</sup> journées du Collège Universitaire et Hospitalier des Histologistes, Embryologistes, Cytologistes et Cytogénéticiens**. Tours, France, 15-17 mars 2007
3. **L Véronèse**. Les plates-formes de génétique moléculaires des cancers. **13<sup>ème</sup> journées du Collège Universitaire et Hospitalier des Histologistes, Embryologistes, Cytologistes et Cytogénéticiens**. Nancy, France, 13-15 mars 2008
4. **L Véronèse**. Prise en charge cytogénétique de la Leucémie Lymphoïde Chronique. **9<sup>th</sup> Euroconference on Clinical Cell Analysis. Saint-Etienne, 9-12 septembre 2009**
5. Vago P, **Véronèse L** et Tchirkov A. Les plates-formes de génétiques moléculaires des cancers. **13<sup>ème</sup> journées du Collège Universitaire et Hospitalier des Histologistes, Embryologistes, Cytologistes et Cytogénéticiens**. Versailles, France, 19-21 mars 2009
6. **Véronèse L**, Combes P, Bay JO, Gouas L, Eymard-Pierre E, Pebrel-Richard C, Goumy C, Vago P, Tournilhac O et Tchirkov A. Mise en évidence des anomalies cytogénétiques de la Leucémie Lymphoïde Chronique : intérêt de la technique de MLPA. **14<sup>èmes</sup> journées du Collège Universitaire et Hospitalier des Histologistes, Embryologistes, Cytologistes et Cytogénéticiens**. Montpellier, France, 11-13 mars 2010
7. **Véronèse L**, Prie N, Bay JO, Combes P, Gouas L, Vago P, Tournilhac O, Tchirkov A. Dysfonction télomérique et instabilité génomique dans la leucémie lymphoïde chronique (LLC). **XX<sup>ème</sup> congrès de l'Association de Cytogénétique de Langue Française**. 20-22 septembre 2010
8. **L. Véronèse**. Etude des mutations de TP53 : déploiement en France. **Réunion de l'Intergroupe Leucémie Lymphoïde Chronique. GCFLC-MW et GOELAMS**, Paris, 17 novembre 2011
9. Télomères et instabilité chromosomique dans la LLC. **Lauren Véronèse. Congrès de la Société Française d'Hématologie**, 2014, Paris, 26-28 mars 2014
10. TP53, instabilité télomérique et anomalies chromosomiques dans la leucémie lymphoïde chronique. **Lauren Véronèse, Romain Guieze, Mélanie Pages, Patricia Combes, Mathilde Gay-Bellile, Gwendoline Soler, Marie Callanan, Fabrice Kwiatkowski, Philippe Vago, Jacques-Olivier Bay, Olivier**. **Congrès de la Société Française d'Hématologie 2015**, Paris, 1-3 avril 2015
11. Altération de la fonction télomérique dans le cancer. Andreï Tchirkov, **Lauren Véronèse**. **20<sup>èmes</sup> journées du Collège Universitaire et Hospitalier des Histologistes, Embryologistes, Cytologistes et Cytogénéticiens**. Paris, 12-14 mars 2015
12. **L. Véronèse** & A. Bidet Analyses cytogénomiques des hémopathies malignes : Réponse du GFCH aux recommandations européennes. **Congrès de la Société Française d'Hématologie**, Paris, 9 septembre 2020

13. **L. Véronèse.** Profil mutationnel des LLC et SLP-B avec réarrangement BCL3 : une cohorte nationale de 113 patients GFCH/FILO. **Congrès de la Société Française d'Hématologie**, Paris, 30 mars 2022
14. **L. Véronèse.** Nouvelle classification OMS 2022 : point de vue du cytogénéticien et commentaires du GFCH. Focus sur les hémopathies lymphoïdes. **Congrès de la Société Française d'Hématologie**, Paris, 29 mars 2023

## Liste des communications orales ou affichées en congrès international

1. F Nguyen-Khac , A Cosson, E Chapiro, N Belhouachi, H Cung, B Keren, F Damm , C Algrin, C Lefebvre, S Fert-Ferrer, I Luquet, N Gachard, F Mugneret, C Terre, MA Collonge-Rame, L Michaux, I Rafdord-Weiss, P Talmant, **L Véronèse**, N Nadal, S Struski, C Barin, C Helias, M Lafage, E Lippert, N Auger, V Eclache, C Settegrana, K Maloum, C Lesty, F Davi, H Merle-Beral. Notch1 mutations are associated with the 14q deletion in chronic lymphocytic leukemia (CLL) and lymphocytic lymphoma (LL). **Congrès de l'European Hematology Association, Stockholm, 13-16 juin 2013**
2. **Role of TP53 mutations and deletions in telomeric instability in chronic lymphoid leukemia. Pagès M , Véronèse L, Combes P, Guièze R, G. Soler, Prie N, M. Callanan, Vago P, Bay J.O, Tournilhac O, Tchirkov A. International Workshop on CLL, Cologne, 2013**
3. p53 functional assessment and correlation with 17p deletion and/or TP53 mutation status. A preliminary report of the ICLL001 BOMP trial. Le Garff-Tavernier M., **Veronese L.**, Nguyen-Khac F., Dilhuydy M.S., Combes P., Leblond V., Feugier P., Mahé B., Sanhès L., Delmer A., Ysebaert L., Truchan-Graczyk M., Dreyfus B., Leprêtre S., Davi F., Pereira B., Delepine R., Guieze R., Merle-Béral H., De Guibert S., Tournilhac O., on Behalf of the French CLL intergroup (GCFLLC/MW – GOELAMS) **International Workshop on CLL, Cologne, 2013**
4. F Nguyen-Khac , A Cosson, E Chapiro, N Belhouachi, H Cung, B Keren, F Damm , C Algrin, C Lefebvre, S Fert-Ferrer, I Luquet, N Gachard, F Mugneret, C Terre, MA Collonge-Rame, L Michaux, I Rafdord-Weiss, P Talmant, **L Véronèse**, N Nadal, S Struski, C Barin, C Helias, M Lafage, E Lippert, N Auger, V Eclache, C Settegrana, K Maloum, C Lesty, F Davi, H Merle-Beral. Notch1 mutations are associated with the 14q deletion in chronic lymphocytic leukemia (CLL) and lymphocytic lymphoma (LL) **12<sup>TH</sup> international conference on malignant lymphoma, Lugano, juin 2013**
5. P53 Functional Assessment and Correlation with 17p Deletion and/Or TP53 Mutation Status In Chronic Lymphocytic Leukemia (CLL). A Preliminary Report of The ICLL001 Bomp Trial On Behalf Of The French CLL Intergroup (GCFLLC/MW - GOELAMS). Magali Le Garff-Tavernier, MD, PhD1,2\*, **Lauren Veronese**, MD, PhD3\*, Florence Nguyen-Khac, MD, PhD1,2\*, Marie-Sarah Dilhuydy, MD4\*, Patricia Combes3\*, Véronique Leblond, MD, PhD5, Pierre Feugier, MD, PhD6\*, Beatrice Mahe7\*, Laurence Sanhès, MD8\*, Alain Delmer, MD9, Loic Ysebaert, MD PhD10\*, Aurore Grelier1\*, Malgorzata Truchan-Graczyk, MD11\*, Brigitte Dreyfus, MD12\*, Emmanuelle Ferrant, MD13\*, Stéphane Leprêtre, MD14\*, Frederic Davi, MD, PhD2,15\*, Bruno Pereira16,17\*, Roselyne Delepine18\*, Romain Guièze, MD16,17\*, Jacques-Olivier Bay, MD, PhD16,17, Helene Merle-Beral, MD, PhD1,2, Sophie De Guibert, MD19\* and Olivier Tournilhac; **American Society of Hematology Annual Meeting La Nouvelle Orléans, décembre 2013**
6. **Pagès M 1, Véronèse L, Combes P, Guièze R, Soler G, Prie N, Callanan M, Vago P, Bay JO, Tournilhac O, Tchirkov A. TP53 disruption and telomere instability in chronic lymphocytic leukemia. American Society of Hematology Annual Meeting, La Nouvelle Orléans, décembre 2013**
7. Characterization and significance of rare balanced immunoglobulin translocations in chronic lymphocytic leukemia; Florence Van Obbergh\*, Natalie Put, Francine Mugneret, Audrey Bidet,

- Lauren Veronese**, Mia Janssen, Peter Vandenberghe, Lucienne Michaux. **19ème congrès de l'EHA European Hematology Association, Milan, 12-14 juin 2014**
8. Bendamustine, Ofatumumab and high-dose MethylPrednisolone (BOMP) in high-risk R/R CLL : interim results of the French CLL Intergroup (GCFLLC/MW - GOELAMS) ICLL01 phase 2 trial. Sophie de Guibert, Marie-Sarah Dilhuydy, Loïc Ysebaert, Laurence Sanhès, Sylvain Choquet, Thérèse Aurran-Schleinitz, Romain Guieze, Béatrice Mahé, Nicolas Daguindau, Carla Araujo, Hussan Saad, Emmanuelle Ferrant, Bruno Cazin, Pierre Feugier, Brigitte Dreyfus, Alain Delmer, Stephane Lepretre, Magali Le Garff-Tavernier, **Lauren Véronèse**, Veronique Leblond, Olivier Tournilhac for the French intergroup GCFLLC/MW-GOELAMS. **19ème congrès de l'EHA European Hematology Association, Milan, 12-14 juin 2014**
  9. Mutational landscape of 118 relapsed chronic lymphocytic leukemia clinical trial samples, evidence for a multiple-hit profile using targeted next generation sequencing. Pauline Robbe\*, Romain Guieze\*, Ruth Clifford, Sophie de Guibert, Adele Timbs, Marie-Sarah Dilhuydy, Maïte Cables, Loïc Ysebaert, Adam Burns, Florence Nguyen-Khac, Frédéric Davi, **Lauren Véronèse**, Patricia Combes, Magali Le Garff-Tavernier, Véronique Leblond, Hélène Merle-Béral, Reem Alsolami, Susanne Weller, Angela Hamblin, Joanne Mason, Andrew Pettitt, Peter Hillmen, Jenny Taylor, Samantha JL Knight, Anna Schuh, Olivier Tournilhac. **American Society of Hematology Annual Meeting, San Francisco, 4-9 décembre 2014**
  10. Bendamustine, Ofatumumab and high-dose MethylPrednisolone (BOMP) in high-risk relapsed/refractory CLL : interim results of the French CLL Intergroup (GCFLLC/MW - GOELAMS) ICLL01 phase 2 trial. Sophie de Guibert, Marie-Sarah Dilhuydy, Loïc Ysebaert, Laurence Sanhès, Sylvain Choquet, Thérèse Aurran-Schleinitz, Romain Guieze, Béatrice Mahé, Nicolas Daguindau, Carla Araujo, Hussan Saad, Emmanuelle Ferrant, Bruno Cazin, Pierre Feugier, Brigitte Dreyfus, Alain Delmer, Stephane Lepretre, Magali Le Garff-Tavernier, **Lauren Véronèse**, Anna Schuh, Bruno Pereira, Veronique Leblond, Olivier Tournilhac for the French intergroup GCFLLC/MW-GOELAMS. **American Society of Hematology Annual Meeting, San Francisco, 2014**
  11. Very long term (10-yr) molecular follow-up of minimal residual disease in patients with neuroblastoma A Tchirkov, E Merlin, **L Véronèse**, N Rouel, F Chambon, P Vago and J Kanold. **SIOPEN Annual General Meeting, Vienne (Autriche), 8-10 octobre 2014**
  12. RIC allogeneic stem cell transplantation for high risk CLL followed by preemptive MRD-based immunointervention -intermediate results from the phase II ICLL03 RICAC-PMM trial (GCFLLC/MW - GOELAMS - SFGMTC French intergroup). Olivier Tournilhac\*, Magali Le Garff-Tavernier, Reza Tabrizi, Stephanie NGuyen Quac, Faezeh Legrand-Izadifar, Patrice Chevallier, Oumedaly Reman, Cécile Tomowiak, Cécile Borel, Sylvie Francois, Dominique Bordessoule, Sebastien Maury, Gaëlle Guillermin, Laure Vincent, Karin Bilger, Richard Lemal, Claire Quiney4, Carole Bellanger, Laure Calvet, **Lauren Veronese**, Aurélie Cabrespine, Mathieu Coudert, Hélène Merle-Béral, Nathalie Dhedin. **42nd Annual Meeting EBMT European Society For Blood and Marrow Transplantation, Valencia, Spain, 3-6 avril 2016**
  13. Isochromosome 17q, unbalanced translocations and 8q gain represent adverse prognostic factors in chronic lymphocytic leukemia (CLL) with 17p deletion. A GFCH study. Elise Chapiro, Claude Lesty, Clémentine Gabillaud, Eric Durot, Stéphanie Struski, Audrey Bidet, Elodie Laharanne, Carole Barin, **Lauren Veronese**, Virginie Eclache, Baptiste Gaillard, Lucienne Michaud, Christine Lefebvre, Jean-Baptiste Gaillard, Christine Terre, Dominique Penther, Christian Bastard, Nathalie Nadal, Sandra Fert-Ferrer, Nathalie Auger, Catherine Godon, Olivier Tournilhac, Laurent Sutton, Florence Nguyen-Khac. **International Workshop on Chronic Lymphocytic Leukemia, New-York, 13-15 Mai 2017**
  14. TP53 mutational profile in CLL: some Ariadne's threads in a complex landscape? A retrospective study of the FILO group. Fanny Baran-Marszak, Valérie Vidal, Virginie Eclache, **Lauren Véronèse**, Olivier Tournilhac, Frederic Davi, Florence Nguyen-Khac, Veronique Leblond, Eric Delabesse, Loïc Ysebaert, Audrey Bidet, Marie Sarah Dilhuydy, Stephanie Poulain, Charles Herbaux, Marie-Helene Estienne, Caroline Dartigeas, Cedric Pastoret, Sophie De Guibert Stephane Giraudier, Jehan Dupuis, Pascale Cornillet-Lefebvre, Anne Quinquenel, Sophy Laibe, Therese Aurran, Dina

- Naguib, Xavier Troussard, Pierre Sujobert, Anne Sophie Michallet, Catherine Thieblemont, Remi Letestu, Gregory Lazarian, Vincent Levy, Thierry Soussi, Florence Cymbalista. **International Workshop on Chronic Lymphocytic Leukemia, New-York, 13-15 Mai 2017**
15. Isochromosome 17q, unbalanced translocations and 8q gain represent adverse prognostic factors in chronic lymphocytic leukemia (CLL) with 17p deletion. A GFCH study. Elise Chapiro, Claude Lesty, Clémentine Gabillaud, Eric Durot, Stéphanie Struski, Audrey Bidet, Elodie Laharanne, Carole Barin, Lauren Veronese, Virginie Eclache, Baptiste Gaillard, Lucienne Michaud, Christine Lefebvre, Jean-Baptiste Gaillard, Christine Terre, Dominique Penther, Christian Bastard, Nathalie Nadal, Sandra Fert-Ferrer, Nathalie Auger, Catherine Godon, Olivier Tournilhac, Laurent Sutton, Florence Nguyen-Khac. **22nd Congress of European Hematology Association, Madrid, 22-25 Juin 2017**
  16. ERCC1 and telomere status in breast cancer patients treated with neoadjuvant chemotherapy. Mathilde Gay-Bellile\*, Pierre Romero\*, Anne Cayre, Lauren Véronèse, Maud Privat, Shalini Singh, Patricia Combes, Fabrice Kwiatkowski, Catherine Abrial, Yves-Jean Bignon, Philippe Vago, Frédérique Penault-Llorca\*\* & Andreï Tchirkov\*\*. **3rd International Cancer Symposium, 25-27 septembre 2017, Lyon**
  17. Outcome after Midam Regimen and Impact of Post-Remission Strategy in Patients with Relapsing or Refractory Acute Myeloid Leukemia. Salem Bahashwan, MD, Cécile Moluçon-Chabrot, Eric Hermet, Aurélie Ravinet, Aurore Douge, Lauren Veronese, Andrei Tchirkov, Richard Lemal, Marc G. Berger, Olivier Tournilhac, Jacques-Olivier Bay and Romain Guieze. **59<sup>th</sup> American Society of Hematology Annual Meeting, Atlanta, 9-12 décembre 2017**
  18. Bounaix, L., Tchirkov, A., Nguyen-Khac, F., Combes, P., Feugier, P., Sanhes, L., Dilhuydy, M.-S., Saad, H., Ysebaert, L., Sylvain, C., Vilque, J.-P., Brion, A., Araujo, C., Ferrant, E., Dreyfus, B., Delmer, A.J., Truchan-Graczyk, M., Lepretre, S., de Guibert, S., Guieze, R., Veronese, L., Lemal, R., Vago, P., Bay, J.-O., Berger, M.G., Schuh, A., Ferrand, C., Delepine, R., Leblond, V., Tournilhac, O., 2017. Dynamic of Telomeric Parameters in Relapsing or Refractory (R/R) Chronic Lymphocytic Leukemia (CLL), an Analysis of the Filo. ICLL001 Bomp Trial. **Blood 130. Society of Hematology Annual Meeting, Atlanta, 9-12 décembre 2017**
  19. Mast Cells Density and Its Clinical Relevance in Waldenström's Macroglobulinemia. Richard Lemal, Stéphanie Poulain, Albane Ledoux-Pilon, Lauren Veronese, Andrei Tchirkov, Benjamin Lebecque, Jacques-Olivier Bay, Frédéric Charlotte, Marc G. Berger, Catherine Godfraind, Frédéric Davi, Véronique Leblond, Olivier Hermine, Romain Guièze, Franck Pagès, Olivier Tournilhac. **59<sup>th</sup> American Society of Hematology Annual Meeting, Atlanta, 9-12 décembre 2017**
  20. A retrospective analysis of 450 TP53 mutations in a real life cohort of CLL from the French Innovative Leukemia Organization (FILO) group. Fanny Baran-Marszak, Valérie Vidal, Myriam Hormi, Virginie Eclache, Lauren Véronèse, Olivier Tournillac, Frederic Davi, Florence Nguyen-Khac, Veronique Leblond, Eric Delabesse, Loic Ysebaert, Audrey Bidet, Marie Sarah Dilhuydy, Stephanie Poulain, Charles Herbaux, Marie-Helene Estienne, Caroline Dartigeas, Cedric Pastoret, Sophie De Guibert, Sophie Raynaud, Stephane Giraudier, Jehan Dupuis, Pascale Cornillet-Lefebvre, Anne Quinquenel, Sophy Laibe, Therese Aurran, Dina Naguib, Xavier Troussard, Pierre Sujobert, Anne Sophie Michallet, Catherine Thieblemont, Remi Letestu, Gregory Lazarian, Vincent Levy, Thierry Soussi, Florence Cymbalista. **59<sup>th</sup> American Society of Hematology Annual Meeting, Atlanta, 9-12 décembre 2017**
  21. Validation of a sensitive and specific method to detect Ewing sarcoma minimal residual disease in testicular and ovarian tissue by an *in vitro* model. L. Chaput, V. Grèze, P. Halle, N. Robin, B. Pereira, A. Tchirkov, L. Véronèse, H. Lejeune, P. Durand, AS. Grémeau, M. Canis and F. Brugnon. **ESHRE 2018**
  22. Dynamic of telomeric parameters in relapsing or refractory (r/r) chronic lymphocytic leukemia (CLL), an analysis of the FILO ICLL001 BOMP TRIAL. Laura Bounaix\*, Andrei Tchirkov, Florence Nguyen-Khac, Patricia Combes, Pierre Feugier, Laurence Sanhes, Marie- Sarah Dilhuydy, Hussam Saad, Loic Ysebaert, Sylvain Choquet, Jean-Pierre Vilque, Annie Brion, Carla Araujo,



- Emmanuelle Ferrant, Brigitte Dreyfus, Alain Jacques Delmer, Malgorzata Truchan-Graczyk, Stéphane Leprêtre, Sophie de Guibert, Romain Guièze, Lauren Véronèse, Richard Lemal, Philippe Vago, Jacques-Olivier Bay, Marc Berger, Anna Schuh, Christophe Ferrand, Roselyne Delepine, Veronique Lebond, Olivier Tournilhac. **European Hematology Association, Stockholm, 2018**
23. p53 Functional Assessment and Correlation with 17p Deletion and/or TP53 Mutation Status: Final Report of the ICLL001 Bomp Trial. Magali Le Garff-Tavernier, Claire Quiney, **Lauren Veronese**, Florence Nguyen-Khac, Pauline Robbe, Patricia Combes, Marie-Sarah Dilhuydy, Pierre Feugier, Beatrice Mahe, Laurence Sanhes, Alain Jacques Delmer, Loic Ysebaert, Malgorzata Truchan-Graczyk, Brigitte Dreyfus, Choquet Sylvain, Thérèse Aurran, Emmanuelle Ferrant, Caroline Dartigeas, Stéphane Leprêtre, Gian Matteo Pica, Frederic Davi, Bruno Pereira, Roselyne Delepine, Anna Schuh, Romain Guièze, Jacques-Olivier Bay, Véronique Leblond, Helene Merle-Beral, Sophie de Guibert, Olivier Tournilhac; *Blood* 2018; 132 (Supplement 1): 3127. doi: <https://doi.org/10.1182/blood-2018-99-116722>. **American Society of Hematology Annual Meeting, San Diego, 2018**
  24. The Broad Spectrum of TP53 Variants in CLL: NGS Analysis of 573 Pathogenic TP53 Variants. Gregory Lazarian, Floriane Theves, Myriam Hormi, Virginie Eclache, Stéphanie Poulain, Audrey Bidet, Cedric Pastoret, **Lauren Veronese**, Eric Delabesse, Marie-Hélène Estienne, Nathalie Nadal, Frederic Davi, Florence Nguyen-Khac, Laurence Lode, Pierre Sujobert, Dina Naguib, Sophie Raynaud, Stephane Giraudier, Olivier Kosmider, Pascaline Etancelin, Pascale Cornillet-Lefebvre, Laurent Miguët, Veronique Leblond, Remi Letestu, Thierry Soussi, Florence Cymbalista, Fanny Baran-Marszak. *Blood* 2019; 134 (Supplement\_1): 3021. doi: <https://doi.org/10.1182/blood-2019-130907> **American Society of Hematology Annual Meeting, Orlando, 2019**
  25. Leukemia cutis in chronic lymphocytic leukemia: a retrospective study of the french innovative leukemia organization (FILO). Gregory Lazarian<sup>\*1</sup>, Michaël Munger<sup>2</sup>, Anne Quinquenel<sup>3</sup>, Marie-Sarah Dilhuydy<sup>4</sup>, **Lauren Veronese**<sup>5</sup>, Damien Luque Paz<sup>6</sup>, Romain Guièze<sup>7</sup>, Albane Ledoux-pilon<sup>8</sup>, Mathilde Hunault<sup>9</sup>, Fatiha Merabet<sup>10</sup>, Jean-Philippe Vial<sup>11</sup>, Audrey Bidet<sup>12</sup>, Agathe Waultier Rascalou<sup>13</sup>, Julien Broseus<sup>14</sup>, Anne Lavaud<sup>15</sup>, Lysiane Molina<sup>16</sup>, Kamel Laribi<sup>17</sup>, Benedicte Hivert<sup>18</sup>, Chloé Friedrich<sup>19</sup>, Benjamin Carpentier<sup>18</sup>, Loïc Ysebaert<sup>20</sup>, Eric Van Den Neste<sup>21</sup>, Lise Willems<sup>22</sup>, Anne Corby<sup>23</sup>, Pierre Feugier<sup>24</sup>, Véronique Leblond<sup>15</sup>, Delmer Alain<sup>3</sup>, Fanny Baran-Marszak<sup>1</sup>, Stéphane Leprêtre<sup>25</sup>, Florence Cymbalista. **European Hematology Association 2021**

## Liste des communications orales ou affichées en congrès national des 10 dernières années

1. Pagès M, Calvet L, Véronèse L, Vago P, Tchirkov A, Bay JO, Guièze R, Tournilhac. Leucémie aiguë myéloïde de novo avec chromosome Philadelphie (LAM Ph+). XVIIIème Journées du Collège des Histologistes, Embryologistes, Cytogénéticiens, Paris, 14-16 mars 2013
2. Pagès M, Calvet L, Véronèse L, Vago P, Tchirkov A, Bay JO, Guièze R, Tournilhac O. Leucémie aiguë myéloïde de novo avec chromosome Philadelphie (LAM Ph+). De novo acute myeloid leukemia with Philadelphia chromosome. Hématologie, numéro spécial, mars 2013. Congrès de la Société Française d'Hématologie, Paris, 27-29 mars 2013
3. TP53 et instabilité télomérique dans la leucémie lymphoïde chronique. Pagès M, Guièze R, Véronèse L, Combes P, G. Soler, M. Callanan, Vago P, Bay J.O, Tournilhac O, Tchirkov A. 19e journées du Collège d'Histologie Embryologie et Cytogénétique, Amiens, 2014
4. Gay-bellile M, Véronèse L, Combes P, Eymard-Pierre E, Kwiatkowski F, Dauplat MM, Privat M, Abrial C, Soler G, Bignon YJ, Nabholz JM, Vago P, Penault-Llorca F & Tchirkov A. Mécanismes moléculaires de la surexpression de hTERT dans le cancer du sein. (Communication affichée). Volume des résumés, 20e journées du Collège d'Histologie Embryologie et Cytogénétique / Paris, 12-14 mars 2015

5. Spectre mutationnel de la leucémie lymphoïde chronique réfractaire ou en rechute : un profil à plusieurs événements. Romain Guièze, Pauline Robbe, Ruth Clifford, Sophie de Guibert, Adèle Timbs, Marie-Sarah Dilhuydy, Maite Cabes, Loic Ysebaert, Adam Burns, Florence Nguyen Khac, Frédéric Davi, Lauren Véronèse. **Congrès de la Société Française d'Hématologie 2015**, Paris, 1-3 avril 2015 (communication orale)
6. **Eradication de la maladie résiduelle par immunointervention préemptive après allogreffe de cellules souches hématopoïétiques à conditionnement d'intensité réduite pour le traitement des LLC de haut risque. Résultats préliminaires. Protocole ICLL03 RICAC-PMM (GROUPES SFGM-TC, FILO).** O. Tournilhac\* 1, M. Le Garff-Tavernier 2, R. Tabrizi3, S. NGuyen Quoc 4, F. Legrand-Izadifar 5, P. Chevallier 6, O. Reman 7, C. Tomowiak 8, C. Borel 9, S. Francois 10, P. Turlure 11, S. Maury12, G. Guillermin 13, L. Vincent14, K. Bilger15, R. Lemal 1, C. Quiney2, C. Bellanger1, L. Calvet1, L. Veronese16, A. Cabrespine1, M. Coudert17, Jo. Bay1, H. Merle-Beral2, N. Dhedin **SFGM 2015**
7. **Clonal chromosomal abnormalities in Philadelphia-negative hematopoiesis in chronic myeloid leukemia: prognostic implication of monosomy 7/ deletion 7q and assessing for possible second hematologic malignancy.** Catherine Roche-Lestienne, Stéphanie Dulucq, Anne Laure Vervaeke, Audrey Bidet, Alice Marceau, Lauren Véronèse, Eric Hermet, Delphine Réa, Marie-Pierre Noël-Walter, Olivier Nibourel, Valérie Coiteux, Claude Preudhomme, François Xavier Mahon. On the Behalf of France Intergroupe sur la Leucémie Myéloïde Chronique (Fi-LMC) and Groupe Francophone de Cytogénétique Onco-hématologique (GFCH). **8<sup>ème</sup> assise de Génétique Humaine et Médicale, 4-5 février 2016, Lyon**
8. **Les altérations de TP53 et la sous-expression des gènes du complexe shelterin contribuent à l'instabilité chromosomique dans la leucémie lymphoïde chronique.** Véronèse L<sup>1</sup>, Guièze R<sup>2</sup>, Pagès M<sup>1,3</sup>, Combes P<sup>1</sup>, Lemal R<sup>2</sup>, Gay-Bellile M<sup>1</sup>, Chauvet M<sup>4</sup>, Callanan M<sup>4</sup>, Kwiatkowski F<sup>5</sup>, Pereira B<sup>6</sup>, Vago P<sup>1</sup>, Bay JO<sup>2</sup>, Tournilhac O<sup>2</sup>, Tchirkov A<sup>1</sup>. **21<sup>ème</sup> Journées du CHECC, 17-19 mars 2016, Toulouse**
9. **Profil des mutations de TP53 dans la Leucémie Lymphoïde Chronique (LLC) : une étude FILO.** F. Baran-Marszak; L. Veronese; P. Sujobert ; A. Bidet ; F. Davi; S. Poulain; MH. Estienne; E. Delabesse; S. Giraudier ; C. Pastoret ; P. Cornillet-Lefebvre ; S. Laibe; L. Lodé; D. Naguib; F. Nguyen Khac; O. Kosmider ; M. Muller; T. Soussi; F. Cymbalista. **37<sup>ème</sup> congrès de la Société Française d'Hématologie, 15-17 mars 2017, Paris** (communication orale)
10. **Diagnostic moléculaire prospectif des LAL-B pour une intervention thérapeutique ciblée des LAL BCR-ABL1-like : bilan à 2 ans.** I. Ba; S. Quentin ; P. Ruminy ; C. Pastoret ; P. Ballerini ; H. Cavé ; V. Saada ; L. Veronese ; JM. Cayuela ; V. Lheritier ; Y. Hicheri ; F. Isnard ; C. Bonmati ; S. Maury ; T. Braun; E. Raffoux ; P. Rousselot; N. Boissel ; J. Soulier ; H. Dombret; E. Clappier. **37<sup>ème</sup> congrès de la Société Française d'Hématologie, 15-17 Mars 2017, Paris** (communication orale)
11. **Chaput L, GrèzeV, Kanold J, Halle P, Grémeau AS, Pouly JL, Robin N, Pereira B, Tchirkov A, Véronèse L, Canis M, Brugnion F. Détection de la maladie résiduelle dans le tissu ovarien et testiculaire humain, applicable aux enfants pré-pubères atteints d'un sarcome d'Ewing. 22<sup>ième</sup> journées de la Fédération Française d'Etude de la Reproduction (Tours), 2017.**
12. **Mise en place en routine diagnostique du séquençage d'un panel myéloïde par NGS et premiers apports cliniques.** C. Bourgne; N. Tribalat ; O. Tournilhac ; L. Veronese; JO. Bay; P. Flandrin-Gresta ; M. Berger ; A. Janel. **38<sup>ème</sup> congrès de la Société Française d'Hématologie, Paris 2018**
13. **Observatoire LMC : un Outil pour une Etude de Cohorte Multicentrique dans la "vraie vie"** A. Janel (1) ; S. Saugues (2) ; D. Guyotat (3) ; A. Turhan (4) ; A. Genthon (5) ; D. Hamroun (6) ; A. Tchirkov (7) ; L. Veronese (7) ; E. Hermet (8) ; M. Berger. **38<sup>ème</sup> congrès de la Société Française d'Hématologie, Paris 2018**
14. **Arrêt des ITK pour Intolérance ou Inefficacité dans la "vraie vie"** A. Janel (1) ; S. Saugues (2) ; D. Guyotat (3) ; A. Turhan (4) ; A. Genthon (5) ; D. Hamroun (6) ; A. Tchirkov (7) ; L. Veronese (7) ; E. Hermet (8) ; M. Berger **38<sup>ème</sup> congrès de la Société Française d'Hématologie, Paris 2018**

15. Les arrêts des ITK pour réponse optimale dans la LMC dans la "vraie vie" A. Janel (1) ; S. Saugues (2) ; D. Guyotat (3) ; A. Turhan (4) ; A. Genthon (5) ; D. Hamroun (6) ; A. Tchirkov (7) ; **L. Veronese** (7) ; E. Hermet (8) ; M. Berger **38ème congrès de la Société Française d'Hématologie, Paris 2018**
16. Relevance clinique de la densité mastocytaire dans la Maladie de Waldenström. Richard Lemal, Stéphanie Poulain, Albane Ledoux-Pilon, **Lauren Veronese**, Andrei Tchirkov, Benjamin Lebecque, et al. **38ème congrès de la Société Française d'Hématologie, Paris, 2018 (communication orale)**
17. Le Garff-Tavernier M, Quiney C, **Véronèse L**, Nguyen-Khac F, Robbe P, Combes P et al. Test fonctionnel de la p53 et corrélation avec la délétion 17p et/ou les mutations de TP53 dans la leucémie lymphoïde chronique. Résultats du protocole ICLL001 BOMP sous l'égide du groupe FILO. **Congrès de la Société Française d'Hématologie, Paris, 2019 (communication orale)**
18. Etude de la longueur des télomères dans les amniocytes et les villosités chorionales en cas de malformation congénitale et d'hypotrophie fœtale. C. Goumy, R. Stamm, **L. Veronese**, F. Godeau, L. Gouas, G. Salaun, C. Richard, A. Tchirkov, P. Vago. **Congrès de l'Association de Cytogénétique de Langue française, Saint Malo, 2018**
19. Andrei Tchirkov, Eve Faugeras, **Lauren Véronèse**, Gaëlle Jeannin, Henri Janicot, Lise Thibonnier et al. **Le statut télomérique dans le cancer broncho-pulmonaire : un nouveau biomarqueur du pronostic et de la résistance à l'immunothérapie. 24ème Journées du CHEC, 2019, Rennes**
20. OBSERVATOIRE LMC : un Outil Unique pour une Etude Multicentrique dans la « vraie vie » en France S. Saugues ; D. Guyotat ; H. Johnson-Ansah ; A. Turhan ; F. Huguet ; A. Guerci ; A. Tchirkov ; **L. Veronese** ; D. Hamroun ; E. Hermet ; M. Berger. **Congrès de la Société Française d'Hématologie, Paris, 2020**
21. Leucémie lymphoïde chronique avec atteinte cutanée spécifique : une étude rétrospective du groupe FILO. Grégory Lazarian, Mickaël Munger, Anne Quinquenel, Marie-Sarah Dilhuydy, **Lauren Veronese**, Damien Luque Paz, Romain Guïèze, Albane Ledoux-Pilon, Mathilde Hunault, Fatiha Merabet, Jean Philippe Vial, Audrey Bidet, Agathe Waultier, Julien Broseus, Anne Lavaud, Lysiane Molina, Kamel Laribi, Bénédicte Hivert, Chloe Friedrich, Benjamin Carpentier, Loïc Ysebaert, Eric Van Den Neste, Lise Willems, Stéphanie Poulain, Anne Corby, Damien Roos-Weil, Fanny Baran-Marszak, Pierre Feugier, Alain Delmer, Stephane Lepretre, Florence Cymbalista. **Congrès de la Société Française d'Hématologie, Paris, 11/09/2021**
22. Amplification intrachromosomique du chromosome 21 (iAMP(21)) et leucémie aiguë myéloïde : à propos d'un cas de diagnostic difficile. **VERONESE Lauren**, TASSIN Thomas, CAVALIERI Doriane, RAVINET Aurélie, LEBECQUE Benjamin, BOURGNE Céline, DANNUS Louis-Thomas, LEDOUX-PILON Albane, SOLER Gwendoline, VOISIN Delphine, TCHIRKOV Andrei. **Journées du Collège Universitaire et Hospitalier des Histologistes, Embryologistes, Cytologistes et Cytogénéticiens. Annecy, 2022**
23. Faisabilité de la cartographie optique en prénatal et en foetopathologie : évaluation de l'extraction d'ADN de haut poids moléculaire pour différents types de matrices. Carole Goumy, Claude Darcha, Hélène Laurichesse, Michaela West, Charles Poncet, Jérôme Salse, Delphine Voisin, Gaëlle Salaun, Laetitia Gouas, Céline Pebrel-Richard, **Lauren Véronèse**, Andrei Tchirkov, Gwendoline Soler, Philippe Vago. **Journées du Collège Universitaire et Hospitalier des Histologistes, Embryologistes, Cytologistes et Cytogénéticiens. Annecy, 2022 (communication orale)**
24. Télomères significativement plus courts en période prénatale en cas de malformation congénitale et d'hypotrophie fœtale. Carole Goumy, **Lauren Véronèse**, Laetitia Gouas, Gaëlle Salaun, Philippe Vago, Andrei Tchirkov. **Journées du Collège Universitaire et Hospitalier des Histologistes, Embryologistes, Cytologistes et Cytogénéticiens. Annecy, 2022**
25. Hétérogénéité intraclonale de la dynamique télomérique dans les cellules primaires de leucémie myéloïde chronique au diagnostic. Andrei Tchirkov, Thomas Tassin, Céline Bourgne, Louis Thomas Dannus, Benjamin Lebecque, Sandrine Saugues, Juliette Berger, **Lauren Véronèse**, Philippe Vago, Gwendoline Soler, Eric Hermet, Marc Berger. **Journées du Collège Universitaire et Hospitalier des Histologistes, Embryologistes, Cytologistes et Cytogénéticiens. Annecy, 2022**

26. Les mutations de TP53 au codon 234 sont associées au traitement par chlorambucil dans la Leucémie Lymphoïde Chronique. Grégory Lazarian (Bobigny), Floriane Theves (Paris), Myriam Hormi (Bobigny), Rémi Letestu (Bobigny), Virginie Eclache (Bobigny), Pierre Sujobert (Lyon), **Lauren Veronese** (Clermont-Ferrand), Audrey Bidet (Bordeaux), Pascale Cornillet-Lefebvre (Reims), Frederic Davi (Paris), Delabesse Eric (Toulouse), Marie-Helene Estienne (Tours), Pascaline Etancelin (Rouen), Olivier Kosmider (Paris), Sophie Laibe (Marseille), L. Lodé (Montpellier), Marc Muller (Vandœuvre Lès-Nancy), Nathalie Nadal (Dijon), Dina Naguib (Caen), Cédric Pastoret (Rennes), Florence Cymbalista (Bobigny), Fanny Baran-Marszak (Bobigny), Thierry Soussi (Solna, SUÈDE). **Congrès de la Société Française d'Hématologie, Paris, 30 mars – 1er avril 2022**
27. **Profil mutationnel des LLC et SLP-B avec réarrangement BCL3 : résultats préliminaires.** Hedi Bensaber, Florence Nguyen-Khac, Andrei Tchirkov, Olivier Tournilhac, Bruno Pereira, Louis-Thomas Dannus, Romain Guieze et **Lauren Véronèse**. **Jeunes experts dans les Leucémies/Journées du FILO, Dijon, 23-25 novembre 2022 (récompensé du prix FILO-ELLYE)**
28. **Etude GFCH/FILO du profil mutationnel des leucémies lymphoïdes chroniques et autres syndromes lymphoprolifératifs B avec translocation *IG::BCL3*.** **Lauren Véronèse**, Florence Nguyen-Khac, Hedi Bensaber, Olivier Tournilhac, Bruno Pereira, Louis-Thomas Dannus, Romain Guieze et Andrei Tchirkov. **Journées du Collège Universitaire et Hospitalier des Histologistes, Embryologistes, Cytologistes et Cytogénéticiens. Lille, 9-11 mars 2023**

## ABBREVIATIONS

---

ATM	ATM Serine/Threonine Kinase
BCL2	BCL2 Apoptosis Regulator
BCL3	BCL3 Transcription Coactivator
BCLXL	BCL2-like 1 (= BCL2L1)
BIRC3	Baculoviral IAP repeat containing 3
BRCA1	BRCA1 DNA Repair Associated
BTk	Bruton Tyrosine Kinase
CXCR4	chemokine (C-X-C motif) receptor 4
ERCC1	excision repair cross-complementing rodent repair deficiency, complementation group 1
FBXW7	F-box and WD repeat domain containing 7, E3 ubiquitin protein ligase
FILO	French Innovative Leukemia Organization
GFCH	Groupe Francophone de Cytogénétique Hématologique
hTERC	Human telomerase RNA Component
hTERT	Human telomerase reverse transcriptase
IGHV	Région variable du gène des chaînes lourdes des immunoglobulines
LDH	Lactico-déshydrogénase
MAPK	Mitogen activated protein kinase
MCL1	Myeloid Cell Leukemia 1
MLPA	Multiplex ligation probe amplification
MYD88	Myeloid differentiation primary response gene 88
NOTCH1	Notch receptor 1
PCR	Polymerase chain reaction
PEST	Proline (P), acide glutamique (E), sérine (S) et thréonine (T)
PLCγ2	Phospholipase Cγ 2
POT1	Protection of telomeres 1
RAP1	Repressor/Activator Protein 1 (actuellement nommé TERF2IP pour TERF2 Interacting Protein)
SF3B1	Splicing factor 3b, subunit 1
TIN2	TERF1 interacting Nuclear protein 2 (actuellement nommé TINF2)
TP53	Tumour protein p53
TPP1	Tripeptidyl-peptidase 1
TRF1-TRF2	Telomeric repeat binding factor 1 et 2 (actuellement nommé TERF1 et TERF2)
XPO1	Exportine 1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

# TRAVAUX DE RECHERCHE

---

## 1. *Avant-propos*

L'étude de la leucémie lymphoïde chronique (LLC) constitue une thématique identifiante en Hématologie à Clermont-Ferrand, concentrant la majeure partie des efforts de recherche clinique et biologique. Elle constitue l'héritage scientifique du Pr Travade, qui est entretenu grâce à l'investissement durable du Pr Tournilhac et du Pr Guieze, localement et dans les groupes nationaux.

Le Pr Tchirkov, reconnu pour son expertise dans le domaine des télomères, a construit à Clermont-Ferrand une thématique télomère et cancers, déclinée en hématologie initialement dans la LLC, en collaboration avec le Pr Travade puis le Pr Tournilhac, et plus récemment dans la leucémie myéloïde chronique (LMC), avec le Pr Berger.

J'ai intégré ces thématiques dès mon master 2 dans l'équipe d'accueil EA 3846 ("Thérapie ciblée combinatoire en onco-hématologie", Université d'Auvergne/Centre Jean Perrin) sous la direction du Pr Tchirkov : mon intérêt s'est porté sur l'évaluation de marqueurs pronostique au cours de la LLC, en particulier la valeur pronostique et des mécanismes moléculaires de l'expression de *MCL1*. J'ai poursuivi ces travaux sur la LLC dans le cadre de mon Doctorat d'Université réalisé sous la co-direction du Pr P. Vago et du Pr A. Tchirkov et portant sur l'étude des marqueurs du pronostic et de l'instabilité génomique au cours de la LLC. Ces travaux ont été réalisés dans le cadre de l'équipe d'accueil 4677 ERTiCa « Equipe de Recherche sur le Traitement Individualisé du Cancer » en étroite collaboration avec l'équipe d'accueil 7283 CREaT « Cancer Resistance Exploring and Targeting ». L'équipe ERTiCa a évolué en UMR1240 IMOST « Imagerie Moléculaire et Stratégies Théranostiques » (Université Clermont Auvergne, INSERM) et l'équipe CREaT est devenue après restructuration l'équipe d'accueil 7453 CHELTER « Clonal Heterogeneity, Leukemic Environment, Therapy Resistance of Chronic Leukemias », équipe à laquelle j'appartiens actuellement, dirigée par le Pr Berger.

D'autre part, j'ai développé des interactions scientifiques au sein du Groupe Francophone de Cytogénétique Hématologique (GFCH) pour participer à des travaux collaboratifs centrés sur la cytogénétique de la LLC, qui constitue le lien entre les thématiques clermontoises et mon activité hospitalière.

## 2. *Résumé du travail doctoral*

**Titre** : « Leucémie lymphoïde chronique : Etude des marqueurs du pronostic et de l'instabilité génomique »

La leucémie lymphoïde chronique (LLC), hémopathie lymphoïde fréquente, se caractérise par une évolution clinique extrêmement variable. Bien que les marqueurs de pronostic soit nombreux dans la LLC, aucun n'est univoque. Dans ce contexte, identifier de nouveaux facteurs prédictifs et comprendre la pathophysiologie de marqueurs pronostiques déjà établis constituent deux objectifs importants pour améliorer la prise en charge thérapeutique de cette hémopathie.

Nous avons tout d'abord choisi d'étudier la valeur pronostique et les mécanismes de régulation de l'expression du gène anti-apoptotique *MCL1*. Nous avons montré que l'expression de *MCL1* est un marqueur prédictif de la survie globale parmi l'ensemble des patients et parmi les stades précoces ; ce marqueur est également prédictif de la survie sans traitement des patients en stade A. Ainsi, l'expression de *MCL1* permet d'identifier précocement les formes de LLC à haut risque et faible risque d'évolution défavorable. Nous avons également démontré que l'expression de *MCL1* est fortement corrélée à l'expression de *VEGF*, confirmant le rôle de cette voie de signalisation dans la survie des lymphocytes tumoraux et suggérant que VEGF pourrait réguler positivement l'expression de *MCL1* selon un mode autocrine.

**VERONESE L**, TOURNILHAC O, VERRELLE P, DAVI F, DIGHIERO G, CHAUTARD E, VEYRAT-MASSON R, KWIATKOWSKI F, GOUMY C, VAGO P, TRAVADE P, TCHIRKOV A. Low *MCL-1* mRNA expression correlates with prolonged survival in B-cell chronic lymphocytic leukemia. *Leukemia*. 2008; 22(6): 1291-3. **cf page 63**

Encadrement d'une étudiante en **Master 1** : **L. COGNERAS** (2010)

Nous avons évalué l'intérêt de la technique de MLPA (multiplex ligation probe amplification) pour la mise en évidence des anomalies cytogénétiques récurrentes à valeur pronostique de la LLC. Nous avons montré qu'il existe une bonne concordance entre la technique de référence et la MLPA, qui constitue une approche rapide et peu coûteuse pour la recherche d'anomalies génomiques présentes dans une majorité de cellules malignes. Nous avons cependant mis en évidence des cas intéressants de faux-positifs et de faux-négatifs avec la MLPA, indiquant que cette méthode ne peut pas remplacer les techniques classiques, mais constitue une approche complémentaire permettant une évaluation simultanée de divers déséquilibres.

**VERONESE L**, TOURNILHAC O, COMBES P, PRIE N, PIERRE-EYMARD E, GUIEZE R, VEYRAT-MASSON R, BAY JO, VAGO P, TCHIRKOV A. Contribution of MLPA to routine diagnostic testing of recurrent genomic aberrations in chronic lymphocytic leukemia. *Cancer Genet*. 2013 Jan-Feb;206(1-2):19-25

Encadrement d'un étudiant en **Master 1** : **J. DISSARD** (2008)

Enfin, nous avons ensuite exploré la fonction télomérique en rapport avec les anomalies chromosomiques à valeur pronostique, reflets de l'instabilité génomique.

### **3. Télomères et anomalies cytogénétiques de la LLC**

#### **a. Introduction**

En 1990, Juliusson *et al.* fut le premier à démontrer que les aberrations chromosomiques mises en évidence par le caryotype présentent une valeur pronostique indépendante dans la LLC (1). Par la suite, l'utilisation d'agents mitogènes spécifiques des lymphocytes B a permis d'obtenir un caryotype analysable dans plus de 90% des cas avec des anomalies clonales dans 53% à 83% des cas selon les séries (2–4). La description des anomalies cytogénétiques de la LLC s'est donc enrichie, avec notamment des translocations réciproques rares et des caryotypes complexes ( $\geq 3$  anomalies) voire hautement complexe ( $\geq 5$  anomalies) (5–8). Parallèlement, la FISH interphasique, qui s'affranchit de l'obtention d'une division cellulaire *in vitro*, permet de détecter des déséquilibres chromosomiques dans 82% des LLC, de stratifier les malades en groupe de risque (9) et, pour certaines, comme la délétion 17p ou 11q, de guider la stratégie thérapeutiques (10,11). En pratique, ces 2 approches de cytogénétique conventionnelle sont actuellement combinées pour mettre en évidence l'éventail des anomalies chromosomiques à valeur pronostique comportant les anomalies récurrentes classiques (délétion 13q, trisomie 12, délétion 11q et délétion 17p), les caryotypes complexes et les remaniements plus rares.

Plus récemment, les techniques de séquençage, d'abord par technique de Sanger, puis par séquençage haut débit, ont permis de décrire le paysage moléculaire de la LLC. Des mutations du gène suppresseur de tumeur *TP53* ont d'abord été rapportées, généralement en association avec une délétion de l'autre allèle en 17p (12). La disruption de *TP53* constitue le facteur de chimiorésistance le plus important dans la LLC (13). De plus, la valeur pronostique péjorative majeure de cette inactivation de *TP53*, largement démontrée à l'époque de l'immunochimiothérapie (14–16), persiste à l'ère des thérapies ciblées (17–21). Par la suite, l'étude de l'exome ou du génome entier a identifié diverses mutations, soit initiatrices et spécifiques de la transformation des cellules B, soit au contraire passagères (22).

Au total, au lieu de révéler un événement cytogénomique unique à l'origine de la leucémogène, l'ensemble de ces données cytogénétiques et moléculaires met en évidence une importance hétérogénéité inter-patients, qui semble sous-tendre en partie la diversité de l'évolution des patients. De plus, l'évolution clonale, qui survient de façon linéaire ou non selon la nature des sous-



clones et des traitements administrés au patient, est un élément clé de la rechute et de la progression au cours de la LLC (22,23).

Les télomères sont des complexes nucléoprotéiques protégeant les extrémités chromosomiques linéaires chez les eucaryotes de la dégradation ou de réparations aberrantes (24) : ils permettent par conséquent le maintien de l'intégrité du génome. Ils comprennent des séquences nucléotidiques répétitives (TTAGGG) et le complexe protéique shelterin constitué des protéines TRF1 et TRF2 (ou TERF1 et TERF2), TIN2 (ou TINF2), POT1, TPP1 et RAP1 (encore nommé TERF2IP) (25,26). La fonction protectrice des télomères requiert une quantité suffisante de répétitions télomériques ainsi que l'intégrité du complexe shelterin. L'étude des télomères, qui raccourcissent à chaque division cellulaire par réplication incomplète de l'ADN télomérique, a fourni des arguments en faveur de l'existence d'un compartiment prolifératif dans la population tumorale de la LLC ; l'érosion télomérique importante observée dans certains cas de LLC indique notamment que le clone leucémique peut avoir une activité proliférative accrue (27). Sur le plan physiopathologique, cette érosion télomérique satellite de la croissance tumorale pourrait favoriser la survenue des aberrations chromosomiques et promouvoir l'évolution de la leucémie : les cellules de LLC subiraient une crise télomérique durant la progression de la maladie, qui serait responsable de l'instabilité génomique (27).

### ***b. Télomères et instabilité génomique de la LLC***

**L. VERONESE, O. TOURNILHAC, M. CALLANAN, N. PRIE, F. KWIATKOWSKI, P. COMBES, M. CHAUVET, F. DAVI, L. GOUAS, P. VERRELLE, R. GUIEZE, P. VAGO, J.O. BAY, A. TCHIRKOV. *Telomeres and chromosomal instability in chronic lymphocytic leukemia. Leukemia* 2013 Feb;27(2):490-3. Cf page 65**

L'intégrité des télomères dépend du complexe protéique shelterin intervenant dans la structure des télomères et dans l'activité de la télomérase, enzyme capable d'assurer l'élongation des séquences d'ADN télomériques (24). Un raccourcissement télomérique est fréquent dans divers types de cellules tumorales où il est responsable par le biais d'une crise télomérique et d'une instabilité génomique d'un effet sur la progression tumorale selon plusieurs modèles murins (28). De plus, un tel raccourcissement induit normalement une réponse aux dommages à l'ADN, réponse qui peut s'avérer inefficace -notamment en cas d'anomalie de TP53- aggravant encore l'instabilité génomique (29). La protéine hTERT est une composante clef de la télomérase qui peut régénérer et stabiliser les télomères courts ce qui peut permettre à une cellule une prolifération sans limite. La dysfonction télomérique, l'altération de des voies de réparation de l'ADN et l'activation de hTERT coopèrent dans l'oncogenèse en permettant l'émergence de clones génétiquement instables et dotés de capacités de prolifération infinie (29–31).

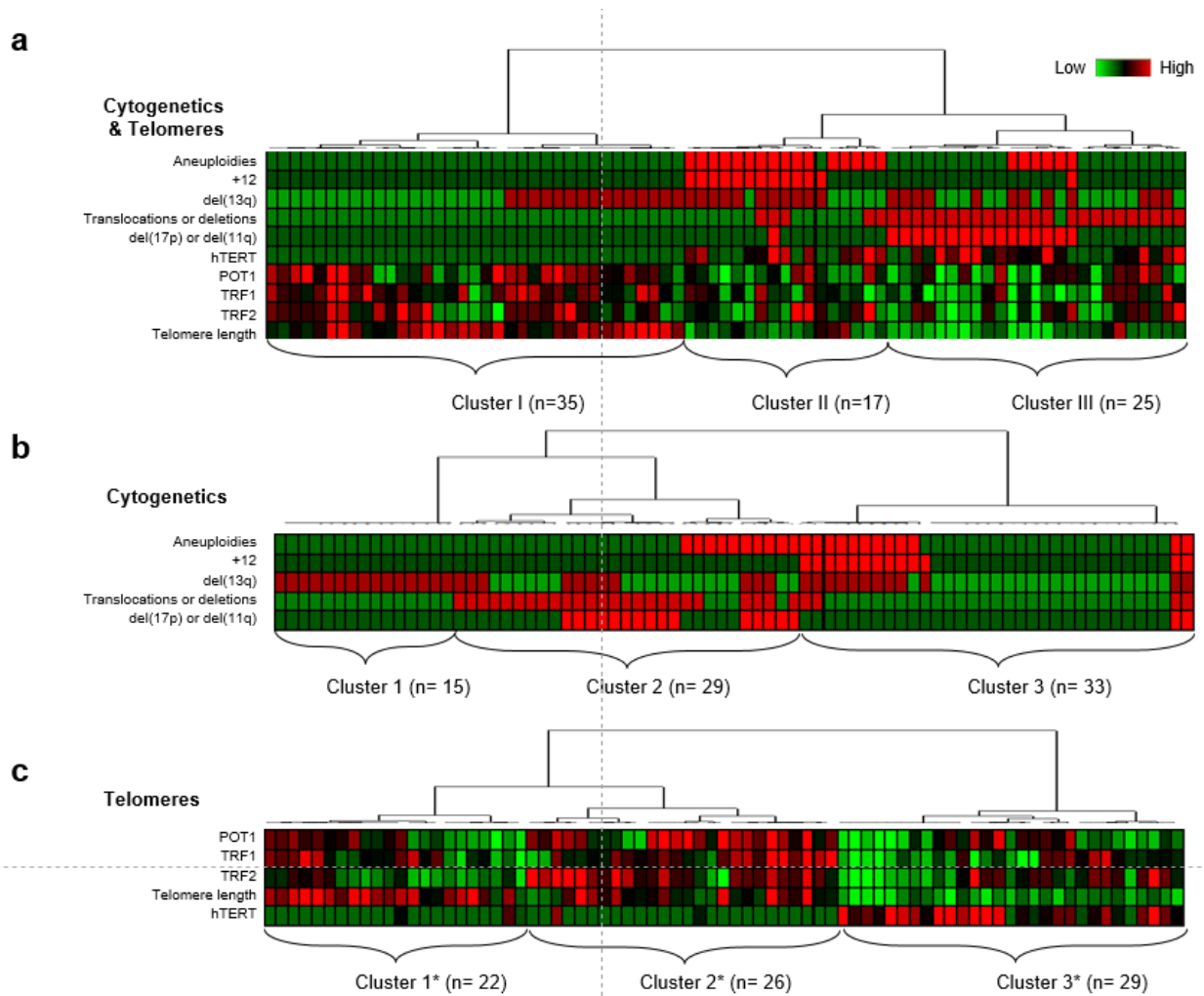
Dans la LLC, les cellules tumorales ont les télomères les plus courts que l'on peut observer dans des tissus humains (27). La longueur des télomères et le niveau d'expression de la sous-unité catalytique de la télomérase *hTERT* constituent des facteurs de pronostic corrélés à la survie des patients (27,32–38). L'existence de variations dans l'expression de gènes impliqués dans le maintien des télomères (39) suggère l'existence d'une dysfonction télomérique globale pouvant participer à la survenue d'anomalies cytogénétiques à valeur pronostique (27,33,40).

L'objectif de ce travail était d'étudier de façon globale et descriptive la relation entre la présence d'une dysfonction télomérique -évaluée par l'étude combinée de la longueur des télomères par PCR quantitative, de l'expression de *hTERT*, de l'expression des gènes du complexes shelterin *TRF1*, *TRF2* et *POT1* par RT-PCR quantitative- et l'existence d'anomalies cytogénétiques, reflet de l'instabilité génomique dans une série de 77 patients suivis au CHU de Clermont-Ferrand.

Une analyse de clustering hiérarchique non supervisé (**Figure 1**) combinant ou non les caractéristiques cytogénétiques et télomériques nous a permis de mettre en évidence 3 sous-groupes de patients présentant des profils cytogénétiques et télomériques distincts : le premier groupe associe une cytogénétique favorable, des télomères longs, une expression faible ou absente de *hTERT* et une expression forte des gènes du complexe shelterin ; le troisième groupe se caractérise par de multiples aberrations chromosomiques (en particulier délétion 17p et 11q), une augmentation de l'expression de *hTERT* et une diminution de la longueur des télomères et de l'expression de *TRF1*, *TRF2* et *POT1* ; le deuxième groupe est intermédiaire. L'altération du statut télomérique est par ailleurs associée à des facteurs de pronostic défavorable, comme l'absence de mutation des IGHV, l'expression de CD38 et le doublement rapide de la lymphocytose.

Le raccourcissement des télomères et les modifications d'expression des gènes du complexe shelterin et de *hTERT* sont associés à l'augmentation du nombre d'anomalies chromosomiques, en particulier les anomalies cytogénétiques à valeur pronostique péjorative. Ces résultats confirment l'existence d'un lien entre statut télomérique et instabilité génomique au cours de la LLC et soulignent le rôle de la perte de *TP53* ou *ATM* dans cette dysfonction télomérique. Cette étude vient compléter les données précédemment publiées, qui montraient soit l'impact clinique du raccourcissement des télomères ou de l'expression de *hTERT*, soit l'existence d'une association entre raccourcissement télomérique et nature des anomalies génomiques, soit enfin l'existence d'altérations d'expression du complexe shelterin, sans que ces divers paramètres télomériques ne soient confrontés les uns aux autres (32,33,39–41).

**Figure 1.** Clustering hiérarchique de 77 patients atteints de LLC en fonction du profil cytogénétique, du niveau d'expression de *hTERT*, du niveau d'expression des gènes du complexe shelterin (*POT1*, *TRF1* et *TRF2*) et de la longueur des télomères.



### c. Télomères et disruption TP53 au cours de la LLC

GUIEZE R\*, PAGES M\*, **VERONESE L**, COMBES P, LEMAL R, GAY-BELLILE M, CHAUVET M, CALLANAN M, KWIATKOWSKI F, PEREIRA B, VAGO P, BAY JO, TOURNILHAC O, TCHIRKOV A. **Telomere status in chronic lymphocytic leukemia with TP53 disruption**. *Oncotarget* 2016 Aug 30;7(35):56976-56985. \*equal contribution. Cf. page 69

**Participation à l'encadrement** d'une étudiante en Master 2 : **M. PAGES** (2013)

Encadrement d'un étudiant en **Master 1** : **V. POINTIS** (2015)

D'après les données de la littérature, on peut émettre l'hypothèse qu'une érosion télomérique sévère ne peut avoir lieu dans les cellules de LLC que si les mécanismes de contrôle des télomères sont défaillants (30). En ce sens, la disruption TP53 entraîne la disparition d'une des voies de signalisation majeure responsable de l'apoptose en cas de raccourcissement extrême des télomères, et favorise par conséquent la survie cellulaire, la poursuite des divisions cellulaires, l'érosion télomérique et la survenue d'instabilité génomique. Les travaux de Roos *et al.* et de Rampazzo *et al.* ont d'ailleurs montré que la longueur des télomères est corrélée à la nature des anomalies génomiques, les télomères courts étant particulièrement associés à la survenue d'anomalies cytogénétiques défavorables (délétion 17p et 11q, caryotypes complexes) (33,40).

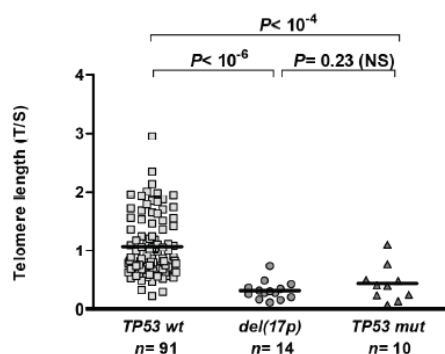
Nous avons donc poursuivi le travail précédent par la recherche de mutations du gène *TP53* par séquençage Sanger, l'étude de l'expression des autres membres du complexe shelterin avec la quantification des transcrits des gènes *RAP1*, *TPP1* et *TINF2* et la recherche de signes de fragilité télomérique par FISH pantélomérique dans une série de 115 patients suivis au CHU de Clermont-Ferrand.

Nous avons montré qu'une relation similaire existe entre mutation/délétion *TP53* et perturbation du statut télomérique, avec raccourcissement des télomères, forte expression de *hTERT*, diminution d'expression des gènes du complexe shelterin chez les patients *TP53-mut/del* et signes de fragilité télomérique en FISH (**Figure 2**). La perte de signal télomérique (plus fréquente chez les patients *TP53-mut/del*) témoigne d'une érosion télomérique avancée. Enfin, l'expression des gènes *TRF1* et *TPP1* est significativement diminuée dans le groupe des patients *TP53-mut/del*.

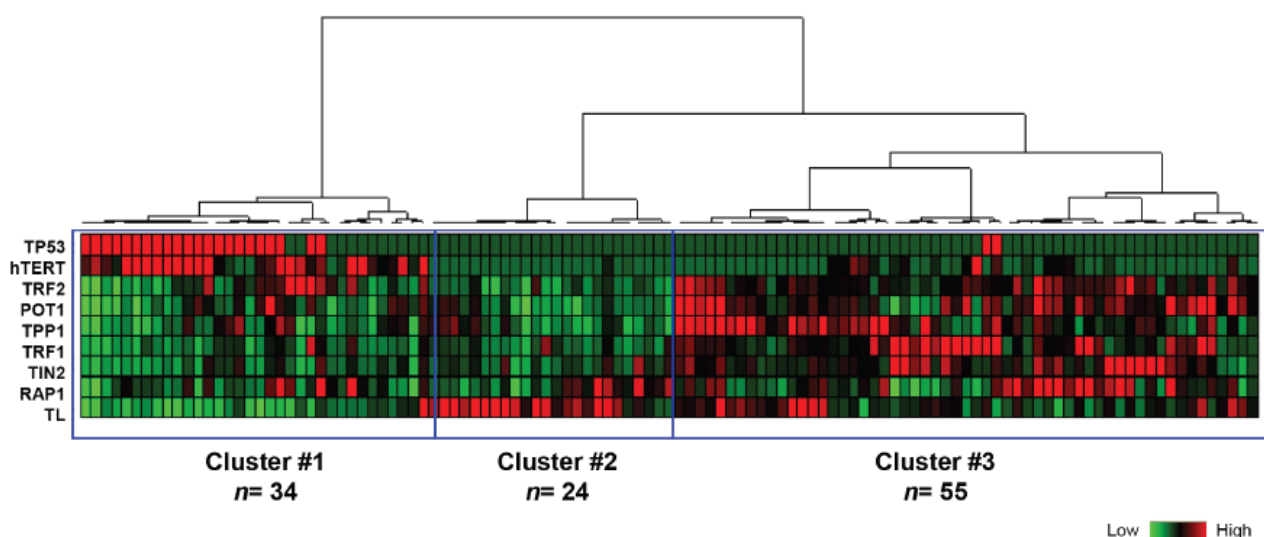
Ces résultats démontrent que les paramètres télomériques et les aberrations chromosomiques sont affectés par la présence d'une dysfonction de la voie TP53, qu'il s'agisse indifféremment de la conséquence d'une délétion 17p ou d'une mutation *TP53*. Ceci conforte nos précédents résultats concluant à l'impact des délétions 17p et 11q sur l'instabilité génomique. Ces résultats suggèrent que

**Figure 2** : Estimation de la taille des télomères en fonction du statut *TP53* (A), clustering hiérarchique de 115 patients atteints de LLC en fonction du statut *TP53*, de la taille des télomères et du niveau d'expression de *hTERT* et des membres du complexe shelterin (*TRF1*, *TRF2*, *POT1*, *TPP1*, *RAP1*, *TIN2*) (B) et analyse télomérique qualitative (C). Les patients avec une diruption de *TP53* (en rouge) sont regroupés dans un cluster caractérisé par des télomères courts, une expression de *hTERT* et un faible niveau d'expression des gènes du complexe shelterin.

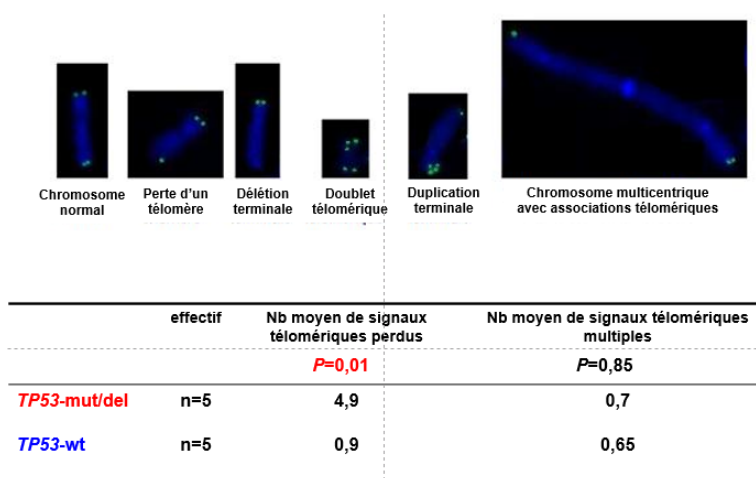
A/



B/



C/



la LLC pourrait suivre le modèle de coopération entre perte de TP53 et dysfonction télomérique tel que décrits dans des modèles murins d'oncogenèse (29). La survenue concomitante de ces 2 lésions génomiques pose toutefois la question de leur lien de causalité. D'autres coopérations sont possibles, qui pourraient impliquer par exemple l'altération d'*ATM* dont le rôle dans la maintenance télomérique et la réponse aux dommages à l'ADN est bien connu. D'autre part, ces résultats soulignent le rôle de la dérégulation de l'expression des gènes *TPP1* et *TINF2* dans le désassemblage du complexe shelterin et l'altération des télomères.

## **4. Télomères et cancers solides**

### **a. Introduction**

Le thème de l'équipe de recherche ERTICa (Equipe de Recherche sur les Traitements Individualisés du Cancer) était l'optimisation de la prise en charge thérapeutique des tumeurs avec une forte instabilité génétique, à travers le modèle du cancer du sein triple négatif (TNBC). En particulier, aucune thérapeutique ciblée (telle que le pembrolizumab, autorisé en 2022) n'était disponible pour ces tumeurs triple-négatives lors de la réalisation de nos travaux, et le but de l'équipe était d'identifier les meilleures options thérapeutiques, adaptées à la biologie des différents sous-groupes de TNBC.

Parallèlement, l'objectif principal de nos travaux était d'identifier des biomarqueurs pronostiques et prédictifs de la réponse thérapeutique dans le cancer du sein et le cancer du poumon non à petites cellules.

### **b. Statut télomérique et expression d'*ERCC1* dans le cancer du sein**

GAY-BELLILE M, ROMERO P, CAYRE A, VERONESE L, PRIVAT M, SINGH S, COMBES P, KWIATKOWSKI F, ABRIAL C, BIGNON YJ, VAGO P, PENAULT-LLORCA F, TCHIRKOV A. **ERCC1 and telomere status in breast tumours treated with neoadjuvant chemotherapy and their association with patient prognosis.** *J Pathol Clin Res* 2016 Jul 13;2(4):234-246. Cf. page 79

Participation à l'encadrement d'une étudiante en **thèse de sciences** : M. GAY-BELLILE (2015-2017)

Les télomères dysfonctionnels et la réparation des dommages à l'ADN jouent un rôle important dans la progression du cancer de façon générale. Des corrélations ont été décrites entre ces facteurs et l'agressivité de la tumeur et la survie dans le cancer du sein (42–51).

L'hétérodimère ERCC1-XPF (excision repair cross complementation group 1 – xeroderma pigmentosum factor) est une endonuclease connue pour son rôle dans les voies de réparation de l'ADN, notamment la réparation par excision de nucléotide (NER nucleotide excision repair) (52). ERCC1-XPF participe également à la maintenance télomérique. Les souris KO pour ERCC1 présentent un phénotype télomérique unique caractérisé par la présence de chromosomes double minutes contenant de l'ADN télomérique (53). ERCC1 a été largement étudié comme biomarqueur de la réponse au traitement (54). Les études immunohistochimiques du statut ERCC1 dans le cancer du sein ont mis en évidence des corrélations entre expression forte et facteurs de pronostics favorables (55), mais les mécanismes sous-jacents restent mal connus. Les interactions entre ERCC1 et les télomères n'ont pas été étudiées dans des cellules tumorales de cancer du sein.

Ce travail a donc porté sur les caractéristiques télomériques et l'expression d'ERCC1, une protéine impliquée dans un certain nombre de voies de réparation de l'ADN et dans l'homéostasie des télomères, afin d'étudier leur valeur pronostique, seuls ou en combinaison, dans 90 tumeurs mammaires résiduelles après traitement par chimiothérapie néoadjuvante.

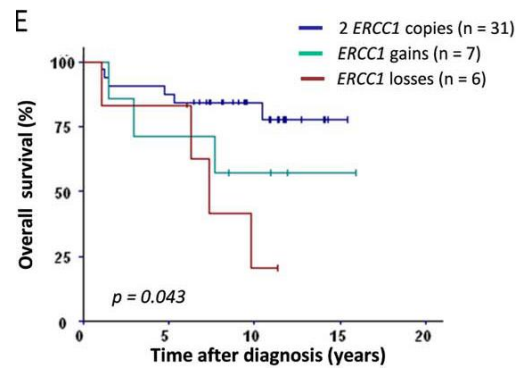
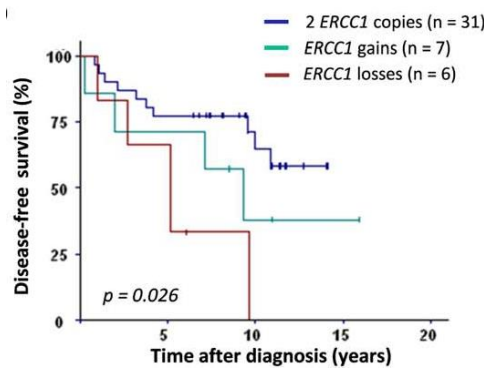
Le statut d'ERCC1 a été étudié par l'évaluation de l'expression protéique (immunohistochimie), de l'expression génique (RT-PCR quantitative) et de la variation du nombre de copies du gène (PCR multiplex quantitative fluorescente (QMF-PCR)). Une analyse complète des caractéristiques des télomères a été réalisée avec évaluation de la longueur des télomères par PCR quantitative, de l'expression de la télomérase (*hTERT*), la tankyrase 1 (*TNKS*, une enzyme de la famille PARP impliquée dans l'élongation des télomères) et du complexe shelterin (*TRF1*, *TRF2*, *POT1*, *TPP1*, *RAP1* et *TIN2*) par RT-PCR quantitative.

Des télomères courts, une forte expression de *hTERT* et de *TNKS* et une faible expression de la protéine ERCC1 étaient indépendamment associés à une plus mauvaise survie. Les gains et les pertes d'*ERCC1* ont été corrélés à des survies sans maladie et globale raccourcie par rapport aux patients sans anomalie du nombre de copie du gène *ERCC1* (Figure 3).

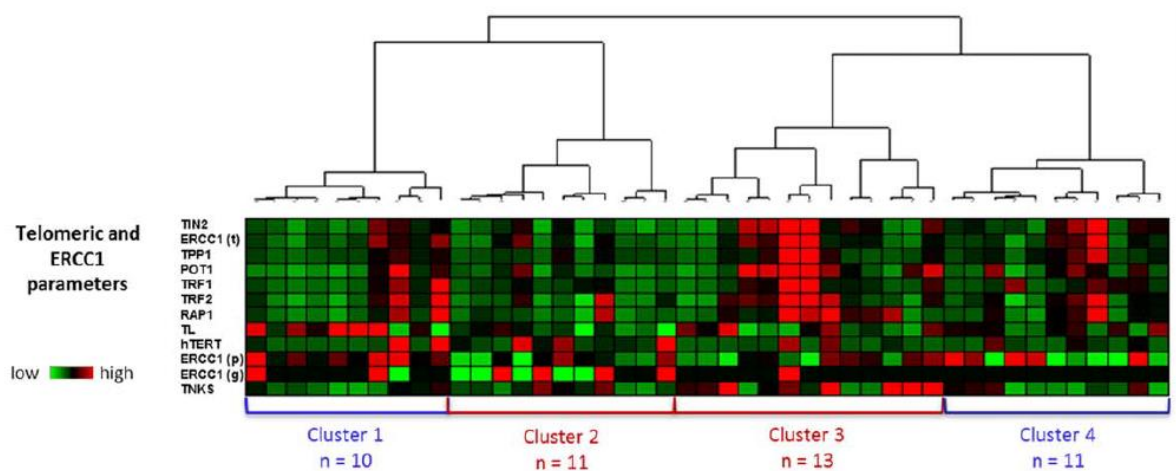
Une analyse de clustering hiérarchique non supervisée de tous les paramètres (*ERCC1* et télomères) a mis en évidence quatre sous-groupes de pronostic différent. En particulier, le groupe 2 combinant un faible niveau d'expression d'*ERCC1*, des altérations du gène *ERCC1*, des télomères dysfonctionnels et un niveau élevé d'*hTERT*, et le groupe 3 présentant un niveau d'expression élevé de *TNKS* et de shelterin présentait une survie sans maladie et une survie globale raccourcie, indépendamment du stade et du grade de la tumeur.

**Figure 3** : Courbes de survie des patientes selon le nombre de copie du gène *ERCC1* (**A**), clustering hiérarchique de 90 patientes en fonction des paramètres télomériques et du statut *ERCC1* évalué sur reliquat tumoral post NCT (**B**), courbes de survie des patientes selon le cluster (**C**).

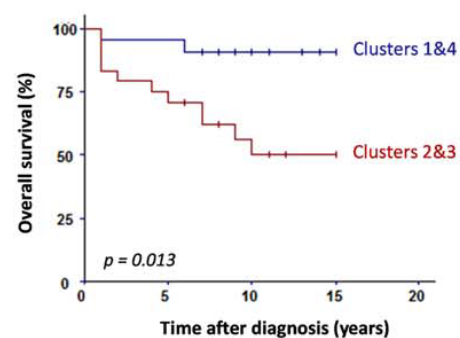
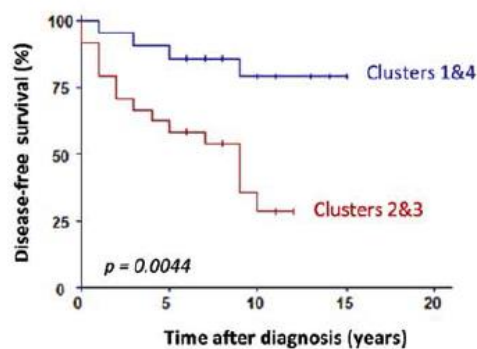
**A/**



**B/**



**C/**





Cette étude descriptive globale démontre que le dysfonctionnement des télomères et la réparation des dommages à l'ADN pourraient contribuer de manière synergique à la progression tumorale et à la chimiorésistance. Ces paramètres sont prédictifs de l'évolution chez les patientes atteintes d'un cancer du sein et traitées par chimiothérapie néoadjuvante et pourraient ainsi être utiles cliniquement comme biomarqueurs pronostiques pour adapter la chimiothérapie adjuvante.

### ***c. Expression de TERT dans le cancer du sein: rôle des mutations du promoteur et du gain de copies du gène TERT***

GAY-BELLILE M, **VÉRONÈSE L**, COMBES P, EYMARD-PIERRE E, KWIATKOWSKI F, DAUPLAT MM, CAYRE A, PRIVAT M, ABRIAL C, BIGNON YJ, MOURET-REYNIER MA, VAGO P, PENALT-LLORCA F, TCHIRKOV A. **TERT promoter status and gene copy number gains: effect on TERT expression and association with prognosis in breast cancer.** *Oncotarget* 2017 Aug 24;8(44):77540-77551. Cf. page 92

Participation à l'encadrement d'une étudiante en **thèse de sciences** : M. GAY-BELLILE (2015-2017)

Le gène de la transcriptase inverse de la télomérase (TERT) code la sous-unité catalytique de la télomérase qui permet le maintien des télomères. L'activité de la télomérase, retrouvée dans 90% des cancers, contribue à la croissance et la survie des cellules tumorales (56). La régulation positive du gène de la transcriptase inverse de la télomérase (TERT) constitue le mécanisme principal d'activation de la télomérase dans les cancers humains (57). Dans le cancer du sein, la surexpression de TERT est associée à l'agressivité de la tumeur (58) et à une survie courte après chimiothérapie néoadjuvante et adjuvante (43,59).

Les mécanismes de régulation de *TERT* sont complexes et tumeur-spécifique : des mutations somatiques et des SNP ont été rapportés dans le promoteur de *TERT*, pouvant influencer sur le niveau d'expression (60). La surexpression de MYC peut activer directement ce promoteur (61). Enfin, des gains de copie ou des amplifications du gène *TERT* peuvent être à l'origine d'une surexpression (60,62,63).

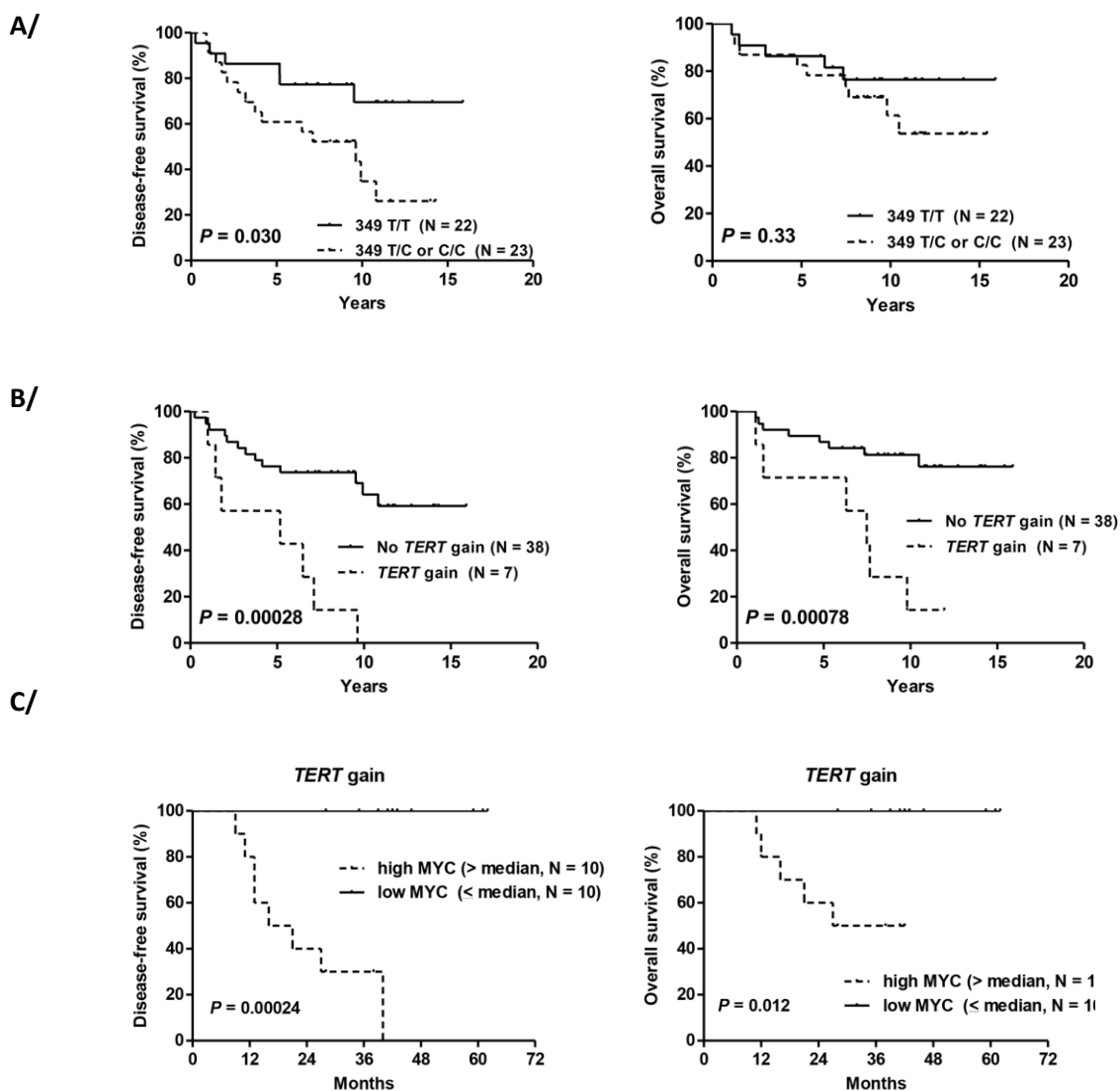
Pour explorer ces mécanismes dans le cancer du sein, nous avons séquencé le promoteur de *TERT*, évalué le nombre de copie du gène *TERT* par QMF-PCR et évalué l'expression de MYC et *TERT* par RT-PCR quantitative dans 2 cohortes comportant au total 122 patients.

Aucune mutation du promoteur de *TERT* n'a été mise en évidence, suggérant que ce mécanisme n'est pas impliqué dans la régulation de *TERT* dans le cancer du sein. La présence du polymorphisme T349C du promoteur de *TERT*, retrouvé chez quasi 50% des patients, n'est pas corrélée à l'expression de *TERT*. Cependant, les porteurs de ce polymorphisme présentent une survie sans maladie significativement plus courte. Les gains de copie de *TERT*, mis en évidence dans 15%-25% des

cas, sont fortement corrélés à une expression augmentée de l'ARNm de *TERT* et à un pronostic péjoratif avec survie sans maladie et survie globale raccourcies. L'association gains de copie de *TERT* et surexpression de *MYC* caractérise des formes particulièrement agressives à haut risque de reprise évolutive (**Figure 4**).

Ces résultats indiquent l'existence d'un effet significatif du gain de copie de *TERT* sur son niveau d'expression et fournissent de nouvelles données sur la signification clinique de la surexpression de *TERT* et *MYC* dans les cancers du sein.

**Figure 4** : Courbes de survie sans maladie et survie globale des patientes selon la présence ou l'absence du polymorphisme T349C du promoteur de *TERT* dans la cohorte 1 (**A**), selon la présence ou l'absence de gain de *TERT* dans la cohorte 2 (**B**), selon l'expression de *MYC* en cas de gain de *TERT* dedans la cohorte 2 (**C**).



#### ***d. Paramètres télomériques et formes avancées du cancer du poumon non à petites cellules : valeur prédictive et pronostique***

FAUGERAS E, VÉRONÈSE L, JEANNIN G, JANICOT H, BAILLY S, BAY JO, PEREIRA B, CAYRE A, PENAULT-LLORCA F, CACHIN F, MERLE P, TCHIRKOV A. Telomere Status of Advanced Non-Small-Cell Lung Cancer Offers a Novel Promising Prognostic and Predictive Biomarker. *Cancers (Basel)*. 2022 Dec 31;15(1):290.

**Cf. page 104**

Participation à l'encadrement d'une étudiante en **Master 2 : E. FAUGERAS (2018)**

Le cancer du poumon est la première cause de décès par cancer dans le monde (64). De manière générale, le pronostic est défavorable, mais la réponse au traitement et la durée de survie varient selon les patients, ce qui peut être expliqué par l'hétérogénéité biologique interindividuelle des tumeurs (65).

La longueur télomérique semble corrélée à la survie dans les formes précoces de cancer du poumon non à petites cellules (CPNPC) (66,67), mais l'impact pronostique du statut télomérique reste indéterminé dans les formes avancées qui sont les plus fréquentes.

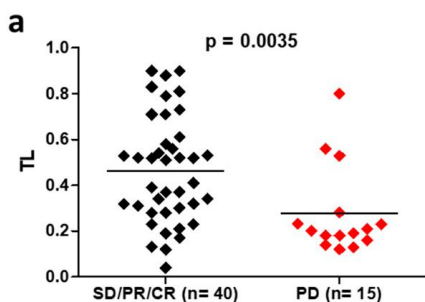
L'objectif de notre travail était d'évaluer la valeur pronostique et prédictive des paramètres télomériques dans les formes avancées de CPNPC, à partir d'une cohorte rétrospective de cancers du poumon du CHU de Clermont-Ferrand. Nous avons mesuré dans 79 biopsies obtenues avant traitement la longueur des télomères par PCR quantitative et évalué l'expression de la télomérase (*TERT*) et des gènes du complexe shelterin (*TRF1*, *TRF2*, *POT1*, *TPP1*, *RAP1*, and *TIN2*) par RT-PCR quantitative.

Les patients non-répondeurs à la chimiothérapie de 1<sup>ère</sup> ligne se caractérisent par des télomères courts et un faible niveau d'expression de *RAP1*. Le niveau d'expression de *TERT* semble également un peu plus élevé. En analyse multivariée, les télomères courts et la surexpression de *TERT* et *TRF2* sont associés à une survie sans événement et une survie globale réduites. Des faibles niveaux d'expression de *RAP1* et *TIN2* sont également corrélées à des survies sans événement et globale raccourcies. Des télomères courts sont associés à une survie raccourcie après traitement par nivolumab (**Figure 5**).

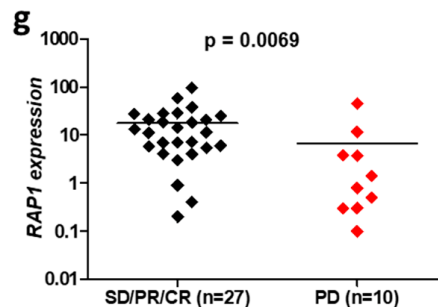
Les paramètres télomériques dans les formes avancées de CPNPC constituent des biomarqueurs associés à des sous-groupes de patients d'évolutions différentes, permettant leur identification pour un traitement personnalisé.

**Figure 5** : Comparaison de la longueur des télomères (TL) en **(A)** et de l'expression de RAP1 en **(B)** selon la réponse à la première ligne de chimiothérapie (progression (PD) versus stabilité de la maladie (SD), réponse partielle (PR) ou réponse complète (CR)). En **(C)**, analyse multivariée pour la survie sans événement et la survie globale incluant la longueur télomérique et l'expression de TERT, TRF2, RAP1 et TIN2. En **(D)**, courbe de survie globale à partir de l'initiation d'une immunothérapie par nivolumab en fonction de la longueur télomérique.

**A/**



**B/**



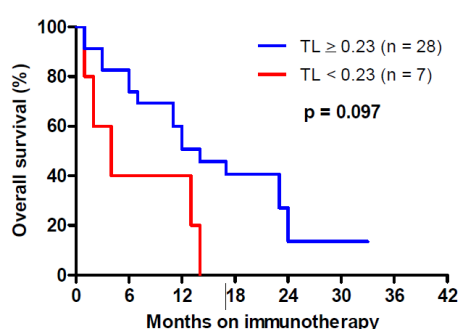
**C/**

**Table 3.** Multivariate analyses for EFS and OS in the advanced NSCLC cohort.

Variable	Multivariate Cox for EFS *		Multivariate Cox for OS *	
	HR (95% CI)	p-Value	HR (95% CI)	p-Value
Short telomeres	4.35 (1.69–11.11)	0.0023	5.26 (2.08–12.5)	0.00041
High <i>TERT</i>	4.12 (1.47–11.53)	0.0069	3.99 (1.51–10.50)	0.0051
High <i>TRF2</i>	7.31 (2.43–22.01)	0.00041	8.24 (2.43–27.93)	0.007
Low <i>RAP1</i>	9.09 (2.70–33.33)	0.00032	14.29 (3.85–50.00)	0.000051
Low <i>TIN2</i>	3.33 (1.39–8.33)	0.0069	3.03 (1.19–7.69)	0.02

\* Adjusted for age and performance status.

**D/**



# PROJETS DE RECHERCHE

---

## **1. Introduction**

Concernant mes travaux en cours et mes projets à moyen terme, ils suivent deux thèmes directeurs en lien avec les travaux précédents : l'étude de l'impact de la dysfonction télomérique d'une part et des anomalies cytogénomiques d'autre part sur le pronostic et la résistance thérapeutique des hémopathies chroniques, particulièrement la LLC.

## **2. Dysfonction télomérique au cours des hémopathies chroniques**

### **a. Etude longitudinale des télomères et instabilité génomique de la LLC**

Nous avons vu que la dysfonction des télomères est une étape clé du processus tumoral et l'étude du statut télomérique dans la LLC peut permettre de mieux comprendre la physiopathologie de la maladie et sa réponse au traitement.

Plusieurs options de traitement existent pour le traitement de la LLC, utilisant principalement des thérapies ciblées, plus rarement des combinaisons d'immunochimiothérapie, dont les indications sont principalement déterminées selon l'âge, l'état général du patient et la présence ou l'absence de certaines caractéristiques génomique, comme le statut *TP53* ou le statut mutationnel des gènes des immunoglobulines (IGHV). Néanmoins, les rechutes restent inéluctables, la maladie devenant alors de plus en plus résistante.

Le protocole ICLL001-BOMP du groupe FILO (dont le Pr Tournilhac, membre de l'EA CHELTER, est investigateur principal) a évalué entre 2012 et 2015 l'efficacité d'une combinaison d'immunochimiothérapie comprenant de la Bendamustine, de l'Ofatumumab, et de la Méthylprednisolone à forte dose, chez des patients à haut risque en rechute précoce après fludarabine ou réfractaire après 1 à 3 lignes de traitements.

Nous disposons ainsi d'une collection biologique de prélèvements de 74 patients atteints de LLC en rechute ou réfractaire avant traitement par BOMP, et pour certains à la rechute, associée aux données cliniques et aux facteurs de pronostic biologiques reconnus. Les prélèvements séquentiels disponibles à l'inclusion et à la rechute post-BOMP pour 19 patients ont permis l'étude de la dynamique télomérique longitudinale dans un essai prospectif. L'intervalle médian entre les 2 prélèvements était de 17.5 mois [4.9-37.8]. Le suivi médian était de 33.9 mois [15.7-48.5]. Six patients sur 19 avait une délétion 17p et/ou mutation *TP53*. La longueur des télomères (LT) a été évaluée par

PCR quantitative et l'expression de *hTERT* et des gènes du complexe shelterin (*TRF1*, *TRF2*, *TIN2*, *POT1*, *RAP1*, *TPP1*, *TIN2* spliced variant) par RT-PCR quantitative.

Entre l'inclusion et la rechute, les télomères raccourcissent significativement ( $p=0.0006$ ) et l'expression de l'ensemble des gènes du complexe shelterin diminue également : *TRF1* (2 fold-change,  $p<0.0001$ ), *TRF2* (2.7 fold-change,  $p<0.0001$ ), *POT1* (1.8 fold-change,  $p=0.0002$ ), *RAP1* (2 fold-change,  $p=0.0009$ ), *TPP1* (1.8 fold-change,  $p=0.0003$ ), *TIN2* (2.3 fold-change,  $p=0.0004$ ), *TIN2* spliced variant (2.2 fold-change,  $p=0.002$ ). A l'inverse, l'expression de *hTERT* ne change pas ( $p=0.64$ ).

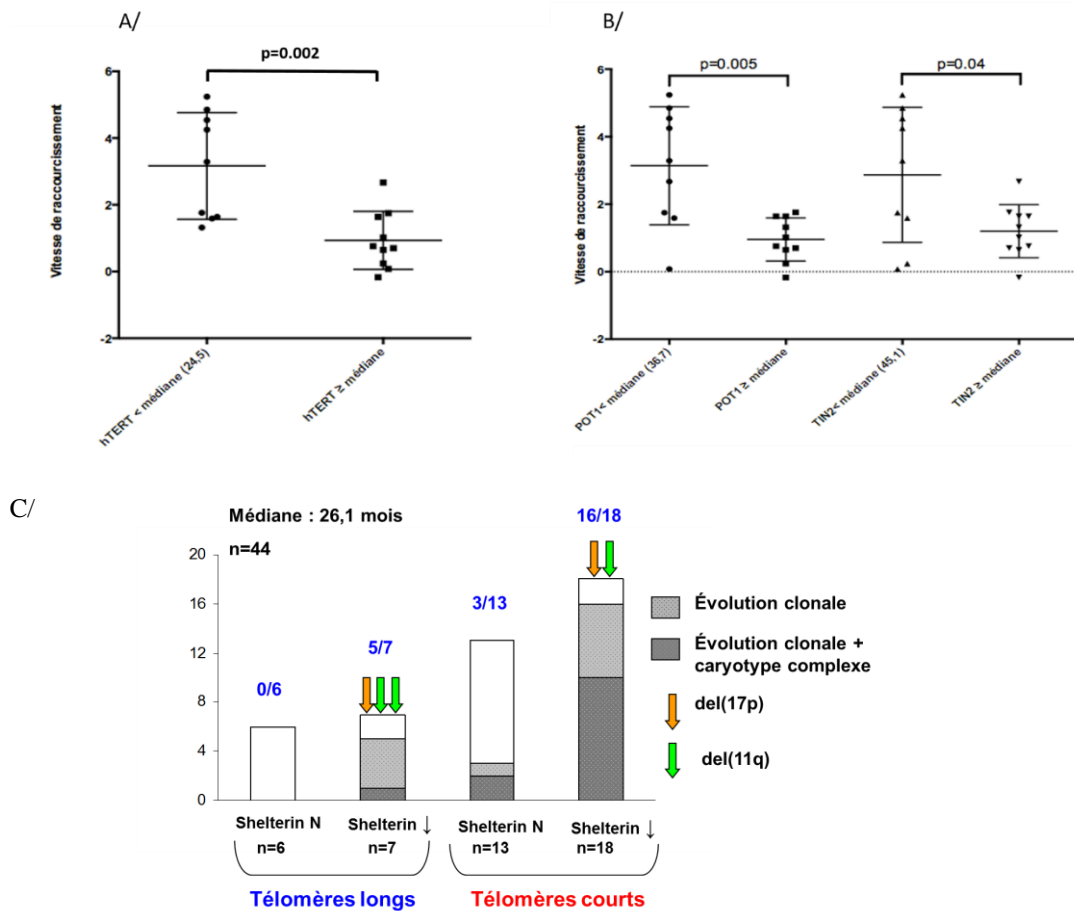
La cinétique de raccourcissement télomérique varie selon les patients. Nous avons calculé le raccourcissement télomérique relatif par mois, correspondant au pourcentage de la longueur des télomères initiale et nous avons identifié 3 groupes de patients avec raccourcissement lent ( $<0.69\%$ ,  $n=4$ ), intermédiaire ( $n=11$ ) et rapide ( $>4.24\%$ ,  $n=4$ ). Un raccourcissement significativement plus rapide survient chez les patients présentant une expression faible de *hTERT* ( $3.2\%$  vs  $0.7\%$ ,  $p=0.002$ ), *POT1* ( $3.140\%$  vs  $0.9560\%$ ,  $p=0.0054$ ) et *TIN2* ( $2.870\%$  vs  $1.199\%$ ,  $p=0.04$ ), en utilisant les valeurs médianes comme cut-off (**Figure 6**)

Nous montrons qu'après immunochimiothérapie, la longueur des télomères et l'expression des gènes du complexe shelterin chez les patients atteints de LLC montrent une évolution variable dans le temps, ce qui pourrait précéder une étape suivante d'instabilité génomique. Pour répondre à cette question, les paramètres des télomères à l'inclusion et à la rechute seront confrontés à d'autres données biologiques, notamment la cytogénétique, la FISH et le profil mutationnel. Nous avons déjà constaté dans la série de patients des travaux précédents (68) que certains patients *TP53* WT dont l'expression des gènes du complexe shelterin est diminuée présente une apparition d'anomalies cytogénétiques ou une évolution clonale, quelle que soit la longueur des télomères. En revanche, les patients dont le niveau d'expression des gènes du complexe shelterin est normal semblent être protégés de l'évolution clonale, même en cas de télomères raccourcis (**Figure 6**).

Participation à l'encadrement d'une étudiante en Master 2 : **L. BOUNAIX** (2017)

Encadrement d'étudiants en Master 1 : **M. MORARD** (2018), **C. CROIZIER** (2018), **L. LAFFOND** (2019) et **J. ANDRAL** (2019)

**Figure 6 : dynamique télomérique au cours de la LLC.** Vitesse de raccourcissement selon l'expression de *hTERT* en (A), selon l'expression de *POT1* et *TIN2* en (B). En (C), évolution clonale de 44 patients sans anomalies de *TP53*, selon les paramètres télomériques.



## b. Dynamique télomérique et leucémies chroniques

La leucémie myéloïde chronique (LMC) est une hémopathie chronique caractérisée par une hétérogénéité intra-clonale et une résistance thérapeutique, comme la LLC. On observe dans la LMC et dans la LLC une hypométhylation globale, touchant le corps des gènes et les enhancers et affectant l'épissage alternatif. L'hétérogénéité de méthylation augmente de façon importante au moment de l'aggravation de la maladie, tant au moment de la phase blastique de la LMC, qu'au cours des stades avancés de la LLC (69,70). De façon générale, des données suggèrent que la méthylation de l'ADN est impliquée dans la régulation de la longueur des télomères (71,72) et que le raccourcissement télomérique modifie la méthylation de l'ADN subtélomérique (73,74).

Le projet est d'évaluer la relation entre méthylation de l'ADN et dynamique des télomères et l'implication de cette relation dans l'hétérogénéité intra-clonale initiale et l'évolution/la réponse thérapeutique.

Dans la LMC, le travail expérimental est en cours (thèse de Sciences du **Dr Joevin Besombes**, sous la direction du Pr Tchirkov et du Pr Berger).

### 3. Anomalies cytogénomiques des hémopathies chroniques

#### a. LLC ou autres syndromes lymphoprolifératifs B et réarrangement de *BCL3*

**LAUREN VERONESE**, HEDI BENSABER, LOUIS THOMAS DANNUS, GEATAN GIANNONE, CHRISTOPHER CHOISSET, CORENTIN GRIMPET, NASSERA ABERMIL, ESTELLE BALDUCCI, AUDREY BIDET, ELISE CHAPIRO, LUCILLE COURONNE, AGNES DAUDIGNON, NATHALIE DOUET-GILBERT, VIRGINIE ECLACHE, BAPTISTE GAILLARD, JEAN-BAPTISTE GAILLARD, FATEN HSOUMI CHRISTINE LEFEBVRE, NATHALIE NADAL, MARIE-JOELLE MOZZICONAZZI, DOMINIQUE PENTHER, BENEDICTE RIBOURTOUT, STEVEN RICHEBOURG, LAUREN RIGOLLET, CHRISTINE TERRE, GWENDOLINE SOLER, OLIVIER TOURNILHAC, ROMAIN GUIEZE, FLORENCE NGUYEN-KHAC\* AND ANDREI TCHIRKOV\*. Molecular landscape of B-cell lymphoproliferative syndromes with *BCL3* translocation: a Groupe Francophone de Cytogénétique (GFCH) /French Leukemia Innovative Organization (FILO) study. **Manuscrit en cours de finalisation.**

Encadrement d'un étudiant en **Master 1 : C. CHOISSET** (2020)

Je coordonne avec le Pr Florence Nguyen-Khac (APHP Pitié-Salpêtrière) une étude du GFCH, portant sur les LLC et syndromes lymphoprolifératifs B (SLP-B) avec réarrangement du locus *BCL3* (19q13), qui sont des anomalies récurrentes rares et mal connues. Cette anomalie est décrite chez 0,9% des LLC ou lymphomes lymphocytiques non traités (75). Elle est aussi observée dans d'autres SLP-B, notamment le lymphome de la zone marginale (LZM) (76–78).

La translocation classique t(14;19)(q32;q13) implique les locus *IGH* (14q32) et *BCL3* (19q13.3). Le gène *IGH* code les gènes des chaînes lourdes des immunoglobulines ; le gène *BCL3* code une protéine nucléaire membre de la famille IκB, régulant la famille de facteurs de transcription NFκB. La translocation t(14;19) aboutit à l'activation transcriptionnelle du gène *BCL3* (76,77,79,80). Des translocations variantes sont également décrites impliquant les gènes IGK (2p12) ou IGL (22q11) codant les chaînes légères kappa et lambda des immunoglobulines, au lieu du gène *IGH*, ou très rarement d'autres partenaires non-immunoglobulines.

La présence d'une translocation *BCL3* dans un contexte de LLC ou de LZM semble associée à des spécificités cliniques et biologiques. Pour la LLC, la morphologie et l'immunophénotype des lymphocytes tumoraux sont généralement atypiques (79,80). Parmi les facteurs pronostiques reconnus de la LLC, les anomalies cytogénétiques additionnelles les plus courantes sont la trisomie 12, la délétion 6q, la délétion 17p ou les anomalies complexes (81,82) ; le statut mutationnel des gènes des chaînes lourdes des immunoglobulines (IGHV) est fréquemment non muté et le subset #8 de mauvais pronostic est sur-représenté. Les patients sont souvent jeunes et le pronostic de l'hémopathie est péjoratif avec une nécessité de traitement plus précoce et une survie globale raccourcie (75,77). Pour le LZM, il y a peu de données, mais là aussi la présentation est atypique, avec des caractéristiques proches de celles de la LLC : trisomie 12, expression de CD5, présentation leucémique. Le diagnostic est d'ailleurs parfois difficile entre LLC atypique et MZL CD5+, ce qui pose la question d'un continuum entre ces 2 entités (78).

A l'heure actuelle, le spectre mutationnel de ces entités reste mal connu, compte-tenu de la rareté de cette anomalie dans les séries rapportées. L'objectif de ce travail était donc de décrire à l'aide d'un panel NGS le spectre mutationnel des patients atteints de SLP-B avec translocation *BCL3* (*IGH*-



*BCL3* ou variantes) dans une large série rétrospective. Nous avons inclus 114 patients via le GFCH, ce qui en fera la plus grande série rapportée.

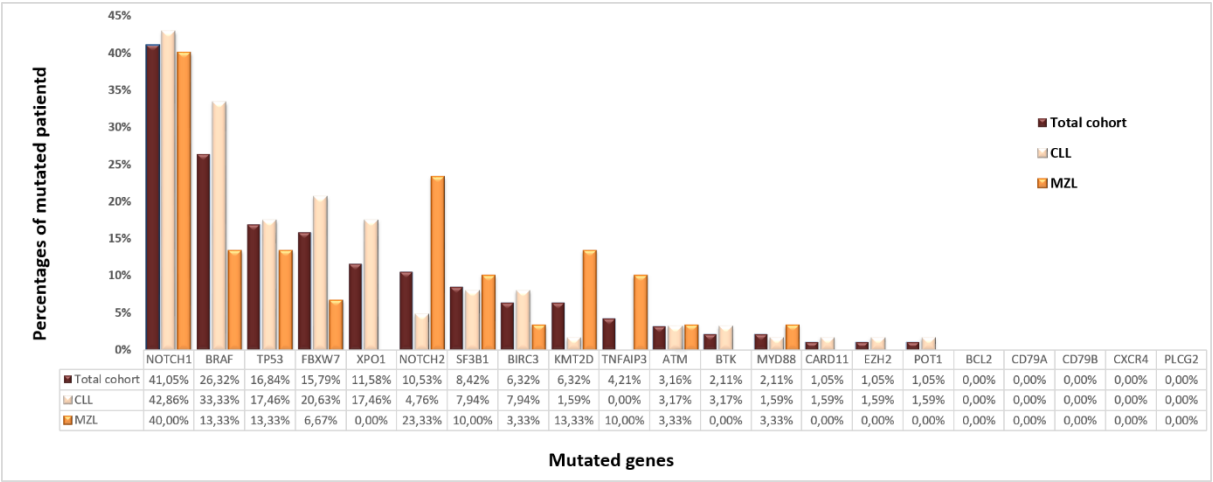
Concernant les diagnostics, les patients présentaient des LLC, LZM, lymphomes B diffus à grandes cellules et un lymphome primaire du médiastin. Les LLC atypiques et les MZL CD5+ constituaient la majorité de la cohorte (58 patients). La translocation *BCL3* est un événement clonal préférentiellement associé à une trisomie 12, un caryotype complexe, une présentation leucémique, une positivité de CD5 et à un statut IGHV non muté.

Sur le plan moléculaire, nous décrivons 182 variants pathogènes ou probablement pathogène chez 82,1 % des patients, avec une prévalence de mutations de *NOTCH1* (41 %), suivi de *BRAF* non V600 (26,3 %), *TP53* (16,8 %), *FBXW7* (15,8 %), *XPO1* (11,6 %), *NOTCH2* (10,5 %), *SF3B1* (8,4 %) et *BIRC3* (6,3 %) (**Figure 7**). Considéré individuellement, aucun paramètre cytogénétique ou mutation génétique n'est corrélé à la survie globale (OS). Les patients ont été répartis en trois groupes de complexité croissante en fonction des caractéristiques cytogénétiques et moléculaires par une approche de clustering non supervisé. Les patients présentant la complexité cytogénomique la plus élevée ont une OS médiane significativement plus courte (43 mois) par rapport aux patients présentant une complexité cytogénomique faible ou intermédiaire (114 mois et 85 mois, respectivement ;  $P = 0.037$ ) (**Figure 8**).

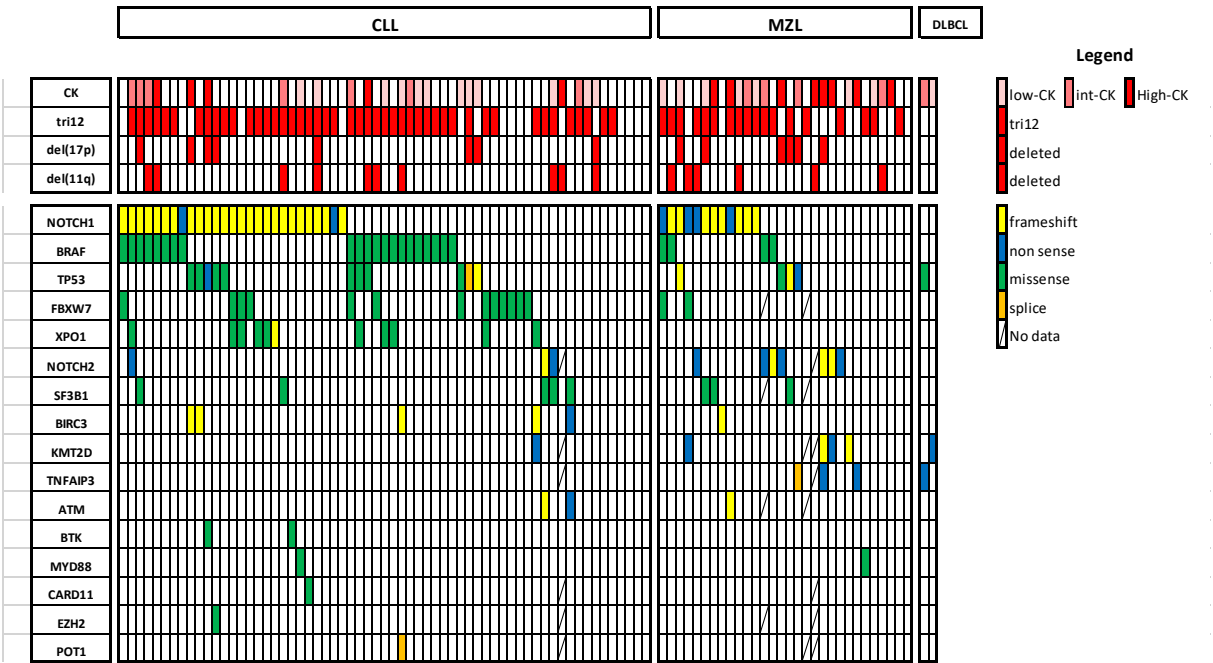
En conclusion, nous démontrons que les SLP-B *BCL3* sont des LLC et LZM fréquemment atypiques, caractérisés par une fréquence élevée de mutations de la voie NOTCH (*NOTCH1/NOTCH2/FBXW7*) et de la voie des MAPK (*BRAF* non V600). La combinaison de la cytogénétique conventionnelle et d'un panel NGS ciblé permet de diagnostiquer avec précision ces entités *BCL3* et d'évaluer leur pronostic en fonction de la complexité génomique.

**Figure 7 : profil moléculaire des SLP-B avec translocation *BCL3*.** En (A), fréquences des mutations dans la cohorte complète, les LLC et les LZM. En (B), heat-map incluant les principales anomalies cytogénétiques et les mutations.

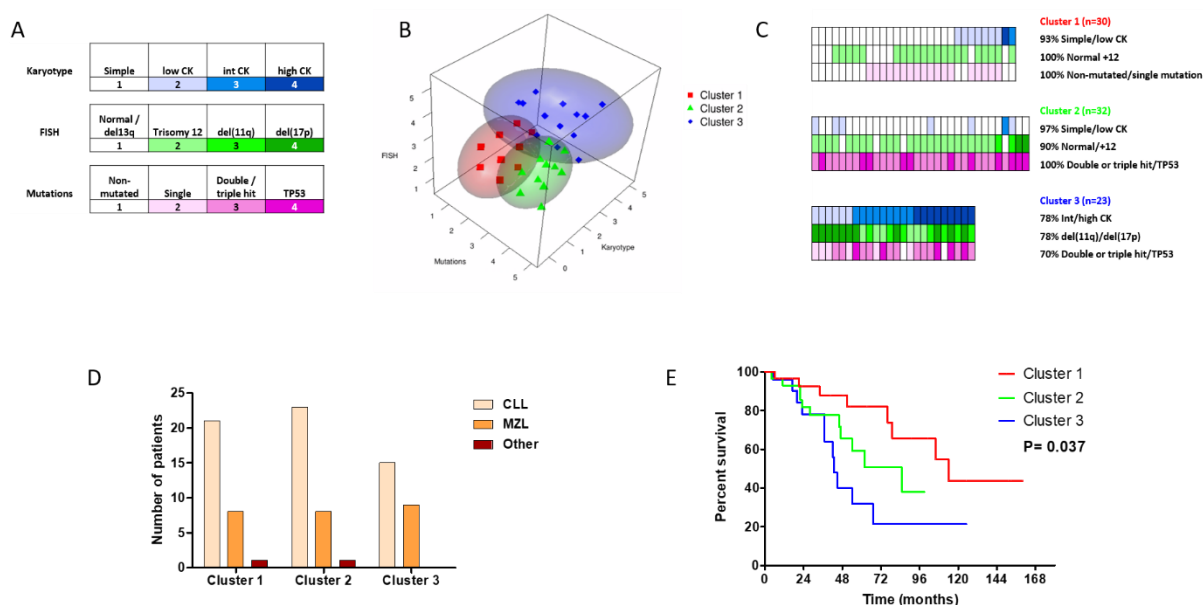
A/



B/



**Figure 8 : analyse de survie selon la complexité cytogénomique.** En (A), catégories cytogénomiques, en (B), représentation 3D et en (C), composition détaillée des 3 clusters obtenus après clustering non supervisé combinant le caryotype, la FISH et le profil mutationnel. Caryotype simple : 1 ou 2 anomalies ; caryotype faiblement complexe (low-CK) : 3 anomalies ; caryotype de complexité intermédiaire (int-CK) : 4 anomalies ; caryotype hautement complexe (high-CK) : au moins 5 anomalies. En (D), répartition des diagnostics dans les 3 clusters. En (E), analyse de Kaplan-Meier et test log-rank pour la survie globale.



## b. Caractérisation clinico-biologiques des SLP-B avec trisomies 12 et 19

Avec le Dr Rigollet (CHU Saint Etienne), le Dr Gachard (CHU Limoges) et le Pr Nguyen-Khac (APHP Pitié-Salpêtrière), nous coordonnons un autre travail collaboratif en cours au sein du GFCH, s'intéressant cette fois aux SLP-B comportant au caryotype une trisomie 12 et une trisomie 19 (+12/+19), associées ou non à d'autres anomalies.

Dans la LLC, ces deux anomalies +12/+19 coexistent dans seulement 1.5% à 2.1% des cas (83). Plusieurs publications ont rapporté que les patients avec +12/+19 au sein d'un caryotype simple ou complexe présentent des similitudes dans leur présentation clinique et biologique : présence d'un pic monoclonal, expression d'une Ig de surface de type IgG, prévalence d'expression des chaînes légères lambda, expression du CD38, statut mutationnel des gènes *IGHV* de type muté, faible incidence d'anomalies du gène *TP53* et pronostic favorable (même en cas de caryotypes complexes) (7,83–85).

L'anomalie chromosomique la plus fréquemment associée au profil +12/+19 est la trisomie 18 complète. La prévalence de la triade +12/+18/+19 est estimée à 1% dans la LLC.

Sur une série monocentrique de 2509 caryotypes de LLC (Hospices civils de Lyon, non publié), 29 patients (1.2%) présentent ces trois aneuploïdies, sans autre anomalie pour la moitié d'entre eux. Ces patients présentent de façon significative un score de Matutes proche de 3.

L'objectif de cette étude est de constituer une série multicentrique rétrospective de SLP-B avec +12/+19 associées ou non à au moins une autre anomalie afin de mieux les caractériser sur le plan clinique, morphologique, immunophénotypique, cytogénétique et moléculaire. Les inclusions sont en cours.

### ***c. Mécanismes génomiques de résistance thérapeutique de la LLC***

#### ***Facteurs génomiques de résistance au venetoclax***

JASNEET KAUR KHALSA, JUSTIN CHA, FILIPPO UTRO, AISHATH NAEEM, ISHWARYA MURALI, YANAN KUANG, KEVIN VASQUEZ, LIANG LI, SVITLANA TYEKUCHEVA, STACEY M. FERNANDES, **LAUREN VERONESE**, ROMAIN GUIEZE, BINU KANDATHILPARAMBIL SASI, ZIXU WANG, JOHN-HANSON MACHADO, HARRISON BAI1, MARYAM ALASFOUR, KAHN RHRISORRAKRAI, CHAYA LEVOVITZ, BRIAN P. DANYSH, KARA SLOWIK, RAQUEL A. JACOBS, CLOUD P. PAWELET, IGNATY LESHCHINER, LAXMI PARIDA, GAD GETZ, JENNIFER R. BROWN. Genetic events associated with venetoclax resistance in CLL identified by whole exome and RNA sequencing on longitudinal patient samples. Blood. 2023 May 5; Blood. 2022016600. doi: 10.1182/blood.2022016600. **Cf page 117**

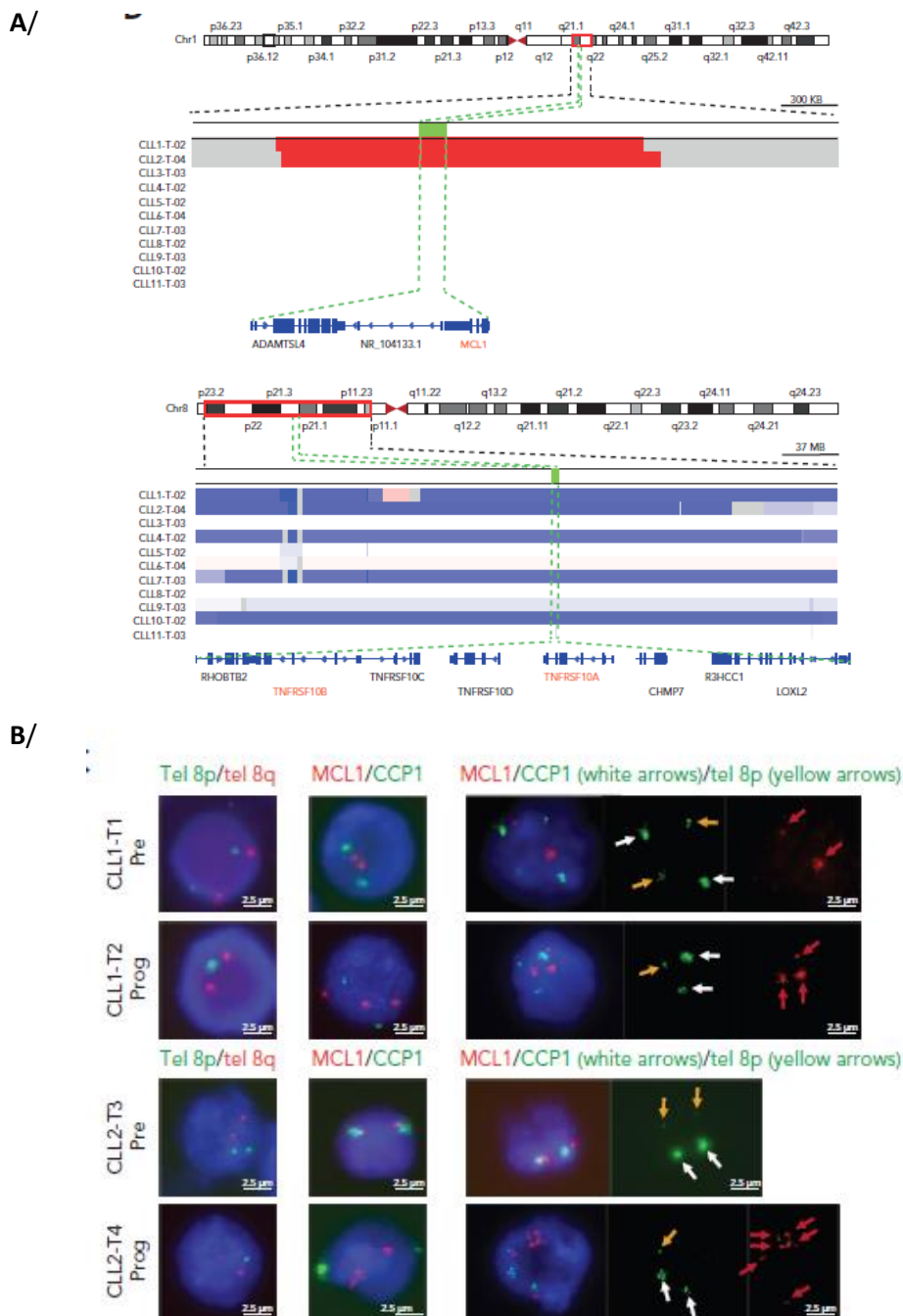
En 2023, la LLC est toujours considérée comme une maladie incurable. Pour les patients nécessitant un traitement, les phases de réponses et de reprises évolutives se succèdent, avec des rechutes de plus en plus rapprochées témoignant de l'acquisition d'une résistance thérapeutique.

Les thérapies ciblées ont remplacé les immunochimiothérapies et constituent actuellement des traitements de choix de cette maladie, en monothérapie ou combinaison, en continu ou en durée fixe. En particulier, le vénétoclax, un inhibiteur de BCL2, offre un taux de réponse globale de 80% et constitue une option en première (autorisation d'accès précoce en cours) ou deuxième ligne (AMM depuis 2018). Malgré des taux de réponse élevés, ce traitement n'est pas curatif et l'évolution clonale et la rechute sont la norme après un délai variable (16.5% de progression à 24 mois selon Seymour et al, NEJM, 2018) (21).

L'étude des premiers cas de rechute après vénétoclax a permis de décrire plusieurs mécanismes de résistance : de façon logique, des mutations de la cible BCL2 ont été rapportées (86,87), généralement sous-clonales. Des altérations du métabolisme énergétique ont été décrites (88). Les travaux du Pr Guieze au Dana Farber Cancer Institute (DFCI) ont également montré que l'hyperexpression de gènes anti-apoptotiques *BCLXL* (ou *BCL2L1*) et *MCL1* permet aux cellules de LLC de contourner l'effet du venetoclax (88). L'expression de *MCL1* est fréquemment associée à un gain de la région chromosomique 1q21 contenant le gène *MCL1*. Très récemment, l'équipe du Pr Brown (DFCI) a rapporté l'acquisition de délétions 8p, soit isolées (2 patients) soit associées au gain 1q (2 patients), parmi 11 patients exposés au venetoclax (soit 36% de la série) (89). Ces délétions 8p

détectées par WES emportent la région 8p21 et s'accompagnent d'une sous-expression des gènes *TNFRSF10A/10B* (8p21) impliqués dans l'apoptose TRAIL-dépendante. Grâce aux collaborations du Pr Guizèze, j'ai contribué à ce travail en confirmant par FISH que la délétion 8p et le gain 1q co-existent dans les cellules de ces 2 patients (**Figure 9**). In vitro, les cellules des patients avec délétion 8p sont plus résistantes au vénétoclax que les cellules sans délétion 8p. La co-existence de délétion 8p et gain 1q est associée à une meilleure sensibilité à une combinaison de vénétoclax et inhibiteur de MCL1.

**Figure 9** : profil génétique des patients sous venetoclax. En (**A**), représentation des pertes (en bleu) et gain (en rouge) pour 11 patients lors de la progression sous venetoclax. En (**B**), profil FISH des patients CLL1 et CLL2 confirmant la co-existence dans les mêmes cellules de la délétion 8p et du gain 1q lors de la progression sous venetoclax.



## Protocole RAVEN

Cependant, à l'heure actuelle, l'image d'ensemble de ces mécanismes de résistance n'est pas encore connue, les cas publiés sont peu nombreux et il n'existe pas de publications de cohortes explorées de façon exhaustive. Ce constat a motivé la mise en place du protocole RAVEN « Résistance Au VENetoclax » (IP Dr Croizier et Pr Guièze) pour collecter des cas nationaux de façon multicentrique à l'aide des centres investigateurs FILO. L'élaboration du protocole a constitué le travail de thèse de médecine du Dr Croizier ; l'ensemble du projet RAVEN s'intègre dans le cadre de son doctorat d'Université. Les échantillons obtenus permettront la recherche par FISH de gain 1q et délétion 8p et la recherche de mutation de *BCL2* par NGS et PCR digitale. Le travail de Master 1 d'Elvire Marceau et Chloé Cardon-Bardot a permis de réaliser une partie de la mise au point de la recherche de mutation *BCL2* en PCR digitale dans notre équipe, avec l'aide du Dr Garcia, ingénieure de recherche dans l'EA CHELTER. Des études d'expression seront également réalisées : une première étape a été initiée dans le cadre du travail de Master 1 de Mélanie TUROT avec la caractérisation moléculaire et cytogénétique d'une lignée cellulaire résistante au vénétoclax.

Il s'agit d'une étude multicentrique non interventionnelle. Cette étude a démarré en 2022 (promotion CHU Clermont-Ferrand) avec des inclusions possibles jusqu'en 2024 via les centres investigateurs FILO. Nous disposons déjà d'une série d'échantillons pour 21 patients, avec l'objectif d'inclure 50 patients en 2 ans, ces patients étant encore relativement rares. Les données cliniques et biologiques sont en cours de recueil. A l'heure actuelle, nous avons recherché des mutations de *BCL2* par panel NGS ciblé pour 17 d'entre eux. Nous avons réalisé la recherche de gain 1q pour 19 patients et de délétion 8p pour 16 patients.

Nous mettons en évidence des mutations de *BCL2* chez 2 (11%) patients, avec plusieurs variants dont le variant G101V récurrent rapporté par Blombery (86,87,90). Les fréquences alléliques (VAF) sont assez faibles (entre 1.2% et 3%), confirmant la nature sous-clonale de ces anomalies. La recherche de mutation G101V sera également réalisée en PCR digitale pour tous les patients afin de rechercher des clones inférieurs à 1% de VAF (seuil de détection du NGS) (**Figure 10**).

Nous mettons en évidence un gain 1q chez 2 (10.5%) patients et une délétion 8p chez 4 (25%) patients, avec co-existence des 2 anomalies chez 1 patient (le gain 1q étant clonal et la délétion 8p sous-clonale). On note aussi 2 patients présentant des remaniements d'un chromosome 1, sans gain de copie de *MCL1*, en cours d'exploration. L'un des patients mutés *BCL2* comporte un gain 1q et l'autre une délétion 8p. On remarque la forte prévalence de mutation *TP53* (50%) dans cette série de patients, en accord avec les premières publications sur la délétion 8p post venetoclax (88,89).

L'apport de la technique de cartographies optiques du génome (COG) dans l'exploration des LLC a été démontré en 2022, avec un intérêt particulier pour les caryotypes complexes (91). Cette approche constitue une technique de choix pour compléter les analyses cytogénomiques prévues dans le protocole RAVEN et fournir une vision globale des réarrangements génomiques. Nous disposerons du matériel congelé pour l'extraction des ADN de haut poids moléculaire pour la plupart des patients. Nous avons obtenu une bourse de l'ACLF qui permettra la réalisation de 12 COG : les patients d'intérêt seront sélectionnés selon leur profil mutationnel et FISH. La partie technique de la COG sera réalisée en prestation de service par la plate-forme Gentyane à Clermont-Ferrand et l'interprétation des données sera effectuée par nos soins.

Nous espérons à terme améliorer l'utilisation du vénétoclax en pratique clinique en permettant d'adapter sa stratégie de prescription aux caractéristiques cytogénomiques des patients.

Encadrement d'étudiantes en **Master 1 : E MARCEAU** (2022)

Participation à l'encadrement d'une étudiante en **thèse de sciences : Dr C. CROIZIER** (travail en cours)

**Figure 10 : heat-map** incluant les mutations *BCL2*, *TP53*, autres, le gain 1q et la délétion 8p pour 21 patients explorés dans le cadre de la cohorte RAVEN.

NOM	BCL2-mut	TP53-mut	Autre mut	Gain 1q	Del(8p)
#1					
#2			XPO1		
#3			XPO1		
#4			BIRC3 CXCR4		
#5			SF3B1 PLCG2		
#6			BIRC ATM		
#7					
#8			SF3B1 BRAF NFKBIE		
#9					
#10			NOTCH1		
#11					
#12			BTK		
#13			BTK		
#14					
#15			BTK BIRC3		
#16			NOTCH1, XPO1, SF3B1	der1	
#17					
#18			SF3B1	der1	
#19					
#20			SF3B1 XPO1		EN COURS
#21	EN COURS	EN COURS	EN COURS	EN COURS	EN COURS

## Facteurs génomiques de résistance aux inhibiteurs de BTK

A côté des inhibiteurs de BCL2, les inhibiteurs de BTK (iBTK) constituent l'autre groupe majeur de thérapies ciblées de l'arsenal thérapeutique de la LLC en 2023 (11,92–94) avec en tête de file l'ibrutinib, iBTK covalent de première génération. Les taux de réponse sont là aussi remarquables (89% de taux de réponse globale dans l'essai pivotal PCYC-1102)(95), largement supérieurs à ceux obtenus avec l'immunochimiothérapie (94,96)). Cependant, le taux de réponse varie selon les sous-groupes de patients (97) et des progressions surviennent après des durées variables de traitement (94–96).

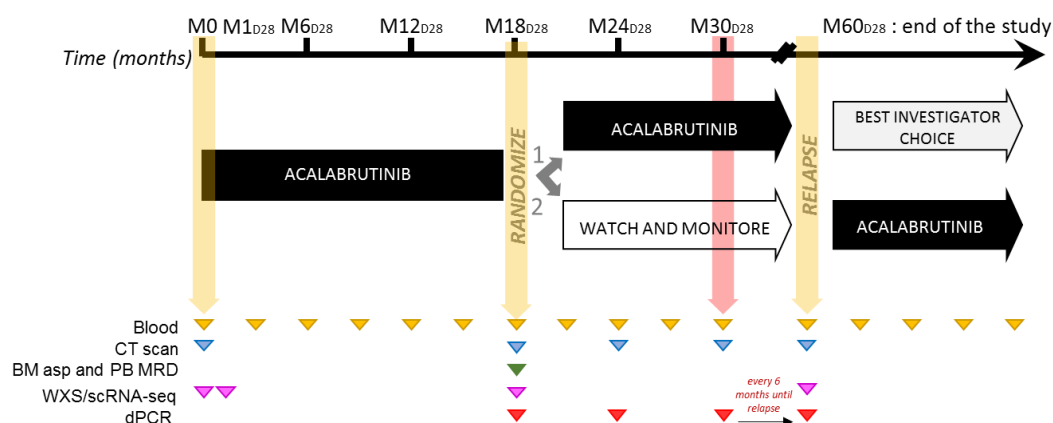
L'un des mécanismes de résistance identifié correspond à la survenue de mutation de la cible BTK ou de son effecteur d'aval, PLCG2 (98–102). Dans 70% des cas, l'émergence des mutations précède la rechute clinique de quelques mois (99). La survenue d'une délétion 8p pourrait aussi jouer un rôle dans la résistance aux iBTK par haploinsuffisance des gènes codant le récepteur de TRAIL (101).

Avec le problème de la tolérance du traitement, la survenue de ces mutations sous l'effet de la pression de sélection d'un traitement continu a participé à soulever la question de schémas thérapeutiques à durée fixe, pour des périodes plus courtes. L'essai FILOCLL14 STAIR "STop and restart Acalabrutinib In fRail patients with previously untreated CLL" (IP Pr Ysebaert et Pr Guïze) évalue l'efficacité de l'acalabrutinib en première ligne chez le sujet âgé pour une durée fixe de 18 mois et son impact sur l'évolution clonale.

Nous sommes en charge du monitoring longitudinal des mutations de *BTK* et *PLCG2* pour l'ensemble de la cohorte, tous les 6 mois à partir de la fin du traitement (**Figure 11**). A cette fin, les travaux de Master 1 de Quentin Bochart, Julier Massacrier et Auriane Papon ont permis la mise au point de la recherche des principales mutations *BTK* et *PLCG2* en PCR digitale dans notre équipe, supervisé par le Dr Garcia.

Encadrement d'une étudiante en **Master 1 : A. PAPON (2023)**

**Figure 11** : conception de l'essai STAIR





## 4. **Bilan**

J'exerce la fonction de MCU-PH en Cytogénétique-Histologie-Embryologie (sous-section 4202 du CNU) depuis 2009. Pour mon activité hospitalière, je travaille depuis ma nomination dans le secteur d'onco-hématologie du Service de Cytogénétique Médicale dirigé depuis 2022 par le Pr Tchirkov, où je prends en charge des analyses de cytogénétique conventionnelle (actuellement caryotype et FISH, complété prochainement par la mise en place de la technique de cartographie optique du génome) et de diagnostic moléculaire (PCR, RT-PCR, NGS, analyse de fragment, Sanger, habilitation Auragen) dans le cadre du diagnostic et du suivi des hémopathies malignes et de certaines tumeurs solides. J'exerce mon activité d'enseignement en majorité en 1<sup>er</sup> cycle des études médicales, mais j'interviens également dans diverses formations paramédicales ou de 3<sup>ème</sup> cycle. Parmi mes activités d'intérêt général, je peux citer le rôle de membre du bureau du GFCH depuis 2018.

Mes travaux de recherche ont tout d'abord porté sur les marqueurs du pronostic et de l'instabilité génomique au cours de la LLC dans le cadre de ma thèse de science (financement de la Ligue Contre le Cancer, 14k euros). Ces travaux ont été réalisés dans l'équipe d'accueil 4677 ERTICa « Equipe de Recherche sur le Traitement Individualisé du Cancer », dont la thématique était l'optimisation de la prise en charge thérapeutique des tumeurs avec une forte instabilité génétique, en étroite collaboration avec l'équipe d'accueil 7283 CREaT « Cancer Resistance Exploring and Targeting ». Nous avons notamment montré que l'expression du gène anti-apoptotique *MCL1* est un marqueur prédictif de la survie globale parmi l'ensemble des patients et parmi les stades précoces ; ce marqueur est également prédictif de la survie sans traitement des patients en stade A. Nous avons ensuite exploré la fonction télomérique en rapport avec les anomalies chromosomiques à valeur pronostique, reflets de l'instabilité génomique. Notre travail a contribué à démontrer la relation entre le statut télomérique et l'instabilité génomique au cours de la LLC. Nous avons également montré qu'une relation similaire existe entre dysfonction de la voie TP53 et perturbation du statut télomérique. Ces résultats démontrent que les paramètres télomériques et les aberrations chromosomiques sont affectés par la présence d'une altération de *TP53*.

Après ma thèse, j'ai participé à plusieurs travaux dans le cadre de l'équipe de recherche ERTICa qui a ensuite évolué en UMR1240 IMOST « Imagerie Moléculaire et Stratégies Théranostiques ». L'objectif principal de nos travaux était d'identifier des biomarqueurs pronostiques et prédictifs de la réponse thérapeutique dans le cancer du sein et le cancer du poumon non à petites cellules. Nous avons notamment montré que le dysfonctionnement des télomères et la réparation des dommages à l'ADN pourraient contribuer de manière synergique à la progression tumorale et à la chimiorésistance dans le cancer du sein. Ces paramètres sont prédictifs de l'évolution des patientes traitées par

chimiothérapie néoadjuvante et pourraient constituer des biomarqueurs pronostiques pour adapter la chimiothérapie adjuvante. Nous avons également montré dans les cancers du sein l'existence d'une corrélation forte entre gain de copie et niveau d'expression de *TERT*. Ces gains de copie de *TERT* sont associés à un pronostic péjoratif avec survie sans maladie et survie globale raccourcies. L'association gains de copie de *TERT* et surexpression de *MYC* caractérise des formes particulièrement agressives à haut risque de reprise évolutive. Enfin, dans les formes avancées de cancer pulmonaire non à petites cellules, nous avons montré que les paramètres télomériques constituent des biomarqueurs associés à des sous-groupes de patients d'évolutions différentes, permettant leur identification pour un traitement personnalisé.

Ces travaux mènent aux projets actuels, qui sont déclinés en deux axes : l'étude de l'impact de la dysfonction télomérique d'une part et des anomalies cytogénomiques d'autre part sur le pronostic et la résistance thérapeutique des hémopathies chroniques, particulièrement la LLC. Ces projets sont réalisés dans le cadre de l'équipe d'accueil 7453 CHELTER, « Clonal Heterogeneity, Leukemic Environment, Therapy Resistance of Chronic Leukemias » issue de l'évolution et de la restructuration de l'équipe d'accueil 7283 Creat.

Concernant la dysfonction télomérique, nous nous intéressons tout d'abord à la dynamique télomérique dans une série de patients atteints de LLC à haut risque (protocole ICLL001-BOMP du groupe FILO). Nos premiers résultats montrent qu'après immunochemiothérapie, la longueur des télomères et l'expression des gènes du complexe shelterin chez les patients atteints de LLC présentent une évolution variable dans le temps, qui pourrait dans certains cas précéder l'instabilité génomique. L'analyse du lien entre cette dynamique et la survenue d'anomalies cytogénomiques est en cours. Dans une autre hémopathie chronique, la LMC, l'évaluation de la relation entre méthylation de l'ADN et dynamique des télomères est en cours, afin de documenter l'implication de cette relation dans l'hétérogénéité intra-clonale initiale et la réponse thérapeutique.

Concernant l'étude des anomalies cytogénomiques de la LLC en lien avec le pronostic, nous avons pu décrire à l'aide d'un panel NGS ciblé le spectre mutationnel des patients atteints de SLP-B avec translocation *BCL3* dans une large série rétrospective (financement de la Ligue Contre le Cancer, 27k euros). Nous démontrons que les SLP-B *BCL3* sont caractérisés par une fréquence élevée de mutations de la voie NOTCH (*NOTCH1/NOTCH2/FBXW7*) et de la voie des MAPK (*BRAF* non V600). Un autre travail est en cours, s'intéressant cette fois aux SLP-B avec +12/+19, associées ou non à d'autres anomalies. Ces deux projets mettent en jeu grâce à des collaborations au sein du GFCH, ainsi qu'au sein du FILO pour le travail sur les SLP-B *BCL3*.

Enfin, pour le volet anomalies cytogénomiques et résistance thérapeutique, je prends part au protocole RAVEN (Résistance Au VENetoclax, IP Dr Croizier et Pr Guièze, promotion CHU Clermont-

Ferrand), qui vise à collecter des cas nationaux de façon multicentrique à l'aide des centres investigateurs FILO pour obtenir une image d'ensemble des mécanismes de résistance au vénétoclax dans la LLC. Nous disposons déjà d'une série d'échantillons pour 21 patients qui ont été explorés par panel NGS ciblé et FISH. Nous proposons également d'évaluer ces patients par cartographie optique du génome afin d'obtenir une vision globale des réarrangements génomiques (Bourse de l'ACLF, 15k euros). Pour finir, nous sommes en charge du monitoring longitudinal des mutations de *BTK* et *PLCG2* pour les patients de l'essai FILOCLL14 STAIR (IP Pr Ysebaert et Pr Guièze) pour apprécier l'impact de l'acalabrutib sur l'évolution clonale.

Au total, les travaux et projets présentés ici sur la biologie de la LLC et des télomères portent sur les thématiques historiques de nos équipes de recherche au fil des ans, actuellement réunies au sein de l'équipe d'accueil 7453 CHELTER. L'étude des télomères évolue vers un projet plus fondamental avec notamment l'évaluation du lien mécanistique entre méthylation et statut télomérique. L'étude des anomalies cytogénomiques de la LLC au diagnostic ou après traitement représente un volet translationnel, en lien fort avec l'activité hospitalière et l'expertise clinique, cytogénétique et moléculaire clermontoise de la LLC, et avec les collaborations nationales au sein du FILO. Cet axe s'appuie également sur les activités du GFCH et sur les nombreux travaux et projets collaboratifs qui animent cette communauté francophone internationale.

## REFERENCES

---

1. Juliusson G, Oscier DG, Fitchett M, Ross FM, Stockdill G, Mackie MJ, et al. Prognostic subgroups in B-cell chronic lymphocytic leukemia defined by specific chromosomal abnormalities. *N Engl J Med*. 1990 Sep 13;323(11):720–4.
2. Haferlach C, Dicker F, Schnittger S, Kern W, Haferlach T. Comprehensive genetic characterization of CLL: a study on 506 cases analysed with chromosome banding analysis, interphase FISH, IgVH status and immunophenotyping. *Leukemia*. 2007 Dec;21(12):2442–51.
3. Put N, Konings P, Rack K, Jamar M, Roy NV, Libouton JM, et al. Improved detection of chromosomal abnormalities in chronic lymphocytic leukemia by conventional cytogenetics using CpG oligonucleotide and interleukin-2 stimulation: A Belgian multicentric study. *Genes Chromosomes Cancer*. 2009 Oct;48(10):843–53.
4. Struski S, Gervais C, Helias C, Herbrecht R, Audhuy B, Mauvieux L. Stimulation of B-cell lymphoproliferations with CpG-oligonucleotide DSP30 plus IL-2 is more effective than with TPA to detect clonal abnormalities. *Leukemia*. 2009 Mar;23(3):617–9.
5. Nguyen-Khac F, Bidet A, Daudignon A, Lafage-Pochitaloff M, Ameye G, Bilhou-Nabéra C, et al. The complex karyotype in hematological malignancies: a comprehensive overview by the Francophone Group of Hematological Cytogenetics (GFCH). *Leukemia*. 2022 Jun;36(6):1451–66.
6. Jondreville L, Krzisch D, Chapiro E, Nguyen-Khac F. The complex karyotype and chronic lymphocytic leukemia: prognostic value and diagnostic recommendations. *Am J Hematol*. 2020 Nov;95(11):1361–7.
7. Baliakas P, Jeromin S, Iskas M, Puiggros A, Plevova K, Nguyen-Khac F, et al. Cytogenetic complexity in chronic lymphocytic leukemia: definitions, associations, and clinical impact. *Blood*. 2019 Mar 14;133(11):1205–16.
8. Baliakas P, Iskas M, Gardiner A, Davis Z, Plevova K, Nguyen-Khac F, et al. Chromosomal translocations and karyotype complexity in chronic lymphocytic leukemia: a systematic reappraisal of classic cytogenetic data. *Am J Hematol*. 2014 Mar;89(3):249–55.
9. Döhner H, Stilgenbauer S, Benner A, Leupolt E, Kröber A, Bullinger L, et al. Genomic Aberrations and Survival in Chronic Lymphocytic Leukemia. *N Engl J Med*. 2000 Dec 28;343(26):1910–6.
10. Hallek M, Al-Sawaf O. Chronic lymphocytic leukemia: 2022 update on diagnostic and therapeutic procedures. *Am J Hematol*. 2021 Dec 1;96(12):1679–705.
11. Quinquenel A, Aurran-Schleinitz T, Clavert A, Cymbalista F, Dartigeas C, Davi F, et al. Diagnosis and Treatment of Chronic Lymphocytic Leukemia: Recommendations of the French CLL Study Group (FILO). *HemaSphere*. 2020 Oct;4(5):e473.
12. Zenz T, Kröber A, Scherer K, Häbe S, Bühler A, Benner A, et al. Monoallelic TP53 inactivation is associated with poor prognosis in chronic lymphocytic leukemia: results from a detailed genetic characterization with long-term follow-up. *Blood*. 2008 Oct 15;112(8):3322–9.
13. Zenz T, Eichhorst B, Busch R, Denzel T, Häbe S, Winkler D, et al. TP53 mutation and survival in chronic lymphocytic leukemia. *J Clin Oncol Off J Am Soc Clin Oncol*. 2010 Oct 10;28(29):4473–9.

14. Campo E, Cymbalista F, Ghia P, Jäger U, Pospisilova S, Rosenquist R, et al. TP53 aberrations in chronic lymphocytic leukemia: an overview of the clinical implications of improved diagnostics. *Haematologica*. 2018 Dec;103(12):1956–68.
15. Stilgenbauer S, Schnaiter A, Paschka P, Zenz T, Rossi M, Döhner K, et al. Gene mutations and treatment outcome in chronic lymphocytic leukemia: results from the CLL8 trial. *Blood*. 2014 May 22;123(21):3247–54.
16. Bomben R, Rossi FM, Vit F, Bittolo T, D'Agaro T, Zucchetto A, et al. TP53 Mutations with Low Variant Allele Frequency Predict Short Survival in Chronic Lymphocytic Leukemia. *Clin Cancer Res Off J Am Assoc Cancer Res*. 2021 Oct 15;27(20):5566–75.
17. Brieghel C, Aarup K, Torp MH, Andersen MA, Yde CW, Tian X, et al. Clinical Outcomes in Patients with Multi-Hit TP53 Chronic Lymphocytic Leukemia Treated with Ibrutinib. *Clin Cancer Res Off J Am Assoc Cancer Res*. 2021 Aug 15;27(16):4531–8.
18. Morabito F, Del Poeta G, Mauro FR, Reda G, Sportoletti P, Laurenti L, et al. TP53 disruption as a risk factor in the era of targeted therapies: A multicenter retrospective study of 525 chronic lymphocytic leukemia cases. *Am J Hematol*. 2021 Aug 1;96(8):E306–10.
19. Visentin A, Mauro F, Scarfò L, Gentile M, Farina L, Reda G, et al. Continuous Venetoclax in Previously Untreated Patients with Chronic Lymphocytic Leukemia and TP53 Abnormalities. a Study of the Italian Campus CLL. *Blood*. 2022 Nov 15;140(Supplement 1):7045–6.
20. Fischer K, Al-Sawaf O, Bahlo J, Fink AM, Tandon M, Dixon M, et al. Venetoclax and Obinutuzumab in Patients with CLL and Coexisting Conditions. *N Engl J Med*. 2019 Jun 6;380(23):2225–36.
21. Seymour JF, Kipps TJ, Eichhorst B, Hillmen P, D'Rozario J, Assouline S, et al. Venetoclax–Rituximab in Relapsed or Refractory Chronic Lymphocytic Leukemia. *N Engl J Med*. 2018 Mar 22;378(12):1107–20.
22. Landau DA, Carter SL, Stojanov P, McKenna A, Stevenson K, Lawrence MS, et al. Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. *Cell*. 2013 Feb 14;152(4):714–26.
23. Landau DA, Tausch E, Taylor-Weiner AN, Stewart C, Reiter JG, Bahlo J, et al. Mutations driving CLL and their evolution in progression and relapse. *Nature*. 2015 Oct 22;526(7574):525–30.
24. Nandakumar J, Cech TR. Finding the end: recruitment of telomerase to telomeres. *Nat Rev Mol Cell Biol*. 2013 Feb;14(2):69–82.
25. de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev*. 2005 Sep 15;19(18):2100–10.
26. Martínez P, Blasco MA. Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins. *Nat Rev Cancer*. 2011 Mar;11(3):161–76.
27. Lin TT, Letsolo BT, Jones RE, Rowson J, Pratt G, Hewamana S, et al. Telomere dysfunction and fusion during the progression of chronic lymphocytic leukemia: evidence for a telomere crisis. *Blood*. 2010 Sep 16;116(11):1899–907.
28. Artandi SE, DePinho RA. Telomeres and telomerase in cancer. *Carcinogenesis*. 2010 Jan 1;31(1):9–18.
29. Artandi SE, Chang S, Lee SL, Alson S, Gottlieb GJ, Chin L, et al. Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. *Nature*. 2000 Aug 10;406(6796):641–5.

30. Chin L, Artandi SE, Shen Q, Tam A, Lee SL, Gottlieb GJ, et al. p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. *Cell*. 1999 May 14;97(4):527–38.
31. Pantic M, Zimmermann S, El Daly H, Opitz OG, Popp S, Boukamp P, et al. Telomere dysfunction and loss of p53 cooperate in defective mitotic segregation of chromosomes in cancer cells. *Oncogene*. 2006 Jul 27;25(32):4413–20.
32. Tchirkov A, Chateaux C, Magnac C, Vasconcelos Y, Davi F, Michel A, et al. hTERT expression and prognosis in B-chronic lymphocytic leukemia. *Ann Oncol*. 2004 Oct;15(10):1476–80.
33. Roos G, Kröber A, Grabowski P, Kienle D, Bühler A, Döhner H, et al. Short telomeres are associated with genetic complexity, high-risk genomic aberrations, and short survival in chronic lymphocytic leukemia. *Blood*. 2008 Feb 15;111(4):2246–52.
34. Bechter OE, Eisterer W, Pall G, Hilbe W, Kühr T, Thaler J. Telomere length and telomerase activity predict survival in patients with B cell chronic lymphocytic leukemia. *Cancer Res*. 1998 Nov 1;58(21):4918–22.
35. Grabowski P, Hultdin M, Karlsson K, Tobin G, Åleskog A, Thunberg U, et al. Telomere length as a prognostic parameter in chronic lymphocytic leukemia with special reference to VH gene mutation status. *Blood*. 2005 Jun 15;105(12):4807–12.
36. Ricca I, Rocci A, Drandi D, Francese R, Compagno M, Lobetti Bodoni C, et al. Telomere length identifies two different prognostic subgroups among VH-unmutated B-cell chronic lymphocytic leukemia patients. *Leukemia*. 2007 Apr;21(4):697–705.
37. Sellmann L, de Beer D, Bartels M, Opalka B, Nüchel H, Dührsen U, et al. Telomeres and prognosis in patients with chronic lymphocytic leukaemia. *Int J Hematol*. 2011 Jan;93(1):74–82.
38. Rossi D, Lobetti Bodoni C, Genuardi E, Monitillo L, Drandi D, Cerri M, et al. Telomere length is an independent predictor of survival, treatment requirement and Richter's syndrome transformation in chronic lymphocytic leukemia. *Leukemia*. 2009 Jun;23(6):1062–72.
39. Poncet D, Belleville A, t'Kint de Roodenbeke C, Roborel de Climens A, Ben Simon E, Merle-Beral H, et al. Changes in the expression of telomere maintenance genes suggest global telomere dysfunction in B-chronic lymphocytic leukemia. *Blood*. 2008 Feb 15;111(4):2388–91.
40. Rampazzo E, Bonaldi L, Trentin L, Visco C, Keppel S, Giunco S, et al. Telomere length and telomerase levels delineate subgroups of B-cell chronic lymphocytic leukemia with different biological characteristics and clinical outcomes. *Haematologica*. 2012 Jan 1;97(1):56–63.
41. Brugat T, Nguyen-Khac F, Grellet A, Merle-Béral H, Delic J. Telomere dysfunction-induced foci arise with the onset of telomeric deletions and complex chromosomal aberrations in resistant chronic lymphocytic leukemia cells. *Blood*. 2010 Jul 15;116(2):239–49.
42. Kammori M, Sugishita Y, Okamoto T, Kobayashi M, Yamazaki K, Yamada E, et al. Telomere shortening in breast cancer correlates with the pathological features of tumor progression. *Oncol Rep*. 2015 Aug 1;34(2):627–32.
43. Lu L, Zhang C, Zhu G, Irwin M, Risch H, Menato G, et al. Telomerase expression and telomere length in breast cancer and their associations with adjuvant treatment and disease outcome. *Breast Cancer Res*. 2011 Jun 6;13(3):R56.

44. Ennour-Idrissi K, Maunsell E, Diorio C. Telomere Length and Breast Cancer Prognosis: A Systematic Review. *Cancer Epidemiol Biomarkers Prev.* 2017 Jan 8;26(1):3–10.
45. Chin K, de Solorzano CO, Knowles D, Jones A, Chou W, Rodriguez EG, et al. In situ analyses of genome instability in breast cancer. *Nat Genet.* 2004 Sep;36(9):984–8.
46. Fordyce CA, Heaphy CM, Bisoffi M, Wyaco JL, Joste NE, Mangalik A, et al. Telomere content correlates with stage and prognosis in breast cancer. *Breast Cancer Res Treat.* 2006 Sep;99(2):193–202.
47. Martinez-Delgado B, Gallardo M, Tanic M, Yanowsky K, Inglada-Perez L, Barroso A, et al. Short telomeres are frequent in hereditary breast tumors and are associated with high tumor grade. *Breast Cancer Res Treat.* 2013 Sep;141(2):231–42.
48. Heaphy CM, Subhawong AP, Gross AL, Konishi Y, Kouprina N, Argani P, et al. Shorter telomeres in luminal B, HER-2 and triple-negative breast cancer subtypes. *Mod Pathol Off J U S Can Acad Pathol Inc.* 2011 Feb;24(2):194–200.
49. Heaphy CM, Asch-Kendrick R, Argani P, Meeker AK, Cimino-Mathews A. Telomere length alterations unique to invasive lobular carcinoma. *Hum Pathol.* 2015 Aug;46(8):1197–203.
50. Heaphy CM, Baumgartner KB, Bisoffi M, Baumgartner RN, Griffith JK. Telomere DNA content predicts breast cancer-free survival interval. *Clin Cancer Res Off J Am Assoc Cancer Res.* 2007 Dec 1;13(23):7037–43.
51. Simpson K, Jones RE, Grimstead JW, Hills R, Pepper C, Baird DM. Telomere fusion threshold identifies a poor prognostic subset of breast cancer patients. *Mol Oncol.* 2015 Jun;9(6):1186–93.
52. Kirschner K, Melton DW. Multiple roles of the ERCC1-XPF endonuclease in DNA repair and resistance to anticancer drugs. *Anticancer Res.* 2010 Sep;30(9):3223–32.
53. Zhu XD, Niedernhofer L, Kuster B, Mann M, Hoeijmakers JHJ, de Lange T. ERCC1/XPF removes the 3' overhang from uncapped telomeres and represses formation of telomeric DNA-containing double minute chromosomes. *Mol Cell.* 2003 Dec;12(6):1489–98.
54. McNeil EM, Melton DW. DNA repair endonuclease ERCC1-XPF as a novel therapeutic target to overcome chemoresistance in cancer therapy. *Nucleic Acids Res.* 2012 Nov 1;40(20):9990–10004.
55. Goyal S, Parikh RR, Green C, Schiff D, Moran MS, Yang Q, et al. Clinicopathologic significance of excision repair cross-complementation 1 expression in patients treated with breast-conserving surgery and radiation therapy. *Int J Radiat Oncol Biol Phys.* 2010 Mar 1;76(3):679–84.
56. Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. *Eur J Cancer Oxf Engl* 1990. 1997 Apr;33(5):787–91.
57. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011 Mar 4;144(5):646–74.
58. Poonepalli A, Banerjee B, Ramnarayanan K, Palanisamy N, Putti TC, Hande MP. Telomere-mediated genomic instability and the clinico-pathological parameters in breast cancer. *Genes Chromosomes Cancer.* 2008 Dec;47(12):1098–109.
59. Gay-Bellile M, Romero P, Cayre A, Véronèse L, Privat M, Singh S, et al. ERCC1 and telomere status in breast tumours treated with neoadjuvant chemotherapy and their association with patient prognosis. *J Pathol Clin Res.* 2016 Oct;2(4):234–46.

60. Cao Y, Bryan TM, Reddel RR. Increased copy number of the TERT and TERC telomerase subunit genes in cancer cells. *Cancer Sci.* 2008 Jun;99(6):1092–9.
61. Wu KJ, Grandori C, Amacker M, Simon-Vermot N, Polack A, Lingner J, et al. Direct activation of TERT transcription by c-MYC. *Nat Genet.* 1999 Feb;21(2):220–4.
62. Hahn WC, Meyerson M. Telomerase activation, cellular immortalization and cancer. *Ann Med.* 2001 Mar;33(2):123–9.
63. Zhu J, Zhao Y, Wang S. Chromatin and epigenetic regulation of the telomerase reverse transcriptase gene. *Protein Cell.* 2010 Jan;1(1):22–32.
64. Ferlay J, Colombet M, Soerjomataram I, Mathers C, Parkin DM, Piñeros M, et al. Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. *Int J Cancer.* 2019 Apr 15;144(8):1941–53.
65. Zito Marino F, Bianco R, Accardo M, Ronchi A, Cozzolino I, Morgillo F, et al. Molecular heterogeneity in lung cancer: from mechanisms of origin to clinical implications. *Int J Med Sci.* 2019 Jun 10;16(7):981–9.
66. Jeon HS, Choi YY, Choi JE, Lee WK, Lee E, Yoo SS, et al. Telomere length of tumor tissues and survival in patients with early stage non-small cell lung cancer. *Mol Carcinog.* 2014 Apr;53(4):272–9.
67. Fernández-Marcelo T, Gómez A, Pascua I, de Juan C, Head J, Hernando F, et al. Telomere length and telomerase activity in non-small cell lung cancer prognosis: clinical usefulness of a specific telomere status. *J Exp Clin Cancer Res CR.* 2015 Aug 7;34(1):78.
68. Guièze R, Pages M, Véronèse L, Combes P, Lemal R, Gay-Bellile M, et al. Telomere status in chronic lymphocytic leukemia with TP53 disruption. *Oncotarget.* 2016 Aug 30;7(35):56976–85.
69. Oakes CC, Claus R, Gu L, Assenov Y, Hüllein J, Zucknick M, et al. Evolution of DNA methylation is linked to genetic aberrations in chronic lymphocytic leukemia. *Cancer Discov.* 2014 Mar;4(3):348–61.
70. Landau DA, Clement K, Ziller MJ, Boyle P, Fan J, Gu H, et al. Locally disordered methylation forms the basis of intratumor methylome variation in chronic lymphocytic leukemia. *Cancer Cell.* 2014 Dec 8;26(6):813–25.
71. Mendelsohn AR, Larrick JW. Telomerase May Paradoxically Accelerate Aging of the DNA Methylome. *Rejuvenation Res.* 2018 Apr;21(2):168–72.
72. Lu AT, Seeboth A, Tsai PC, Sun D, Quach A, Reiner AP, et al. DNA methylation-based estimator of telomere length. *Aging.* 2019 Aug 18;11(16):5895–923.
73. Choudhury SR, Cui Y, Narayanan A, Gilley DP, Huda N, Lo CL, et al. Optogenetic regulation of site-specific subtelomeric DNA methylation. *Oncotarget.* 2016 Jul 4;7(31):50380–91.
74. Buxton JL, Suderman M, Pappas JJ, Borghol N, McArdle W, Blakemore AIF, et al. Human leukocyte telomere length is associated with DNA methylation levels in multiple subtelomeric and imprinted loci. *Sci Rep.* 2014 May 14;4:4954.
75. Fang H, Reichard KK, Rabe KG, Hanson CA, Call TG, Ding W, et al. IGH translocations in chronic lymphocytic leukemia: Clinicopathologic features and clinical outcomes. *Am J Hematol.* 2019 Mar;94(3):338–45.



76. Chapiro E, Radford-Weiss I, Bastard C, Luquet I, Lefebvre C, Callet-Bauchu E, et al. The most frequent t(14;19)(q32;q13)-positive B-cell malignancy corresponds to an aggressive subgroup of atypical chronic lymphocytic leukemia. *Leukemia*. 2008 Nov;22(11):2123–7.
77. Michaux L, Dierlamm J, Wlodarska I, Bours V, Van den Berghe H, Hagemeijer A. t(14;19)/BCL3 rearrangements in lymphoproliferative disorders: a review of 23 cases. *Cancer Genet Cytogenet*. 1997 Mar;94(1):36–43.
78. Nguyen-Khac F, Chapiro E, Radford-Weiss I, Bastard C, Dominique L, Callet-Bauchu E, et al. The t(14;19)(q32;q13) Translocation in B Lymphoproliferative Disorders: A Continuum from Chronic Lymphocytic Leukemia (CLL) to Marginal Zone Lymphoma (MZL)? *Blood*. 2006 Nov 16;108(11):4944.
79. Huh YO, Schweighofer CD, Ketterling RP, Knudson RA, Vega F, Kim JE, et al. Chronic lymphocytic leukemia with t(14;19)(q32;q13) is characterized by atypical morphologic and immunophenotypic features and distinctive genetic features. *Am J Clin Pathol*. 2011 May;135(5):686–96.
80. Huh YO, Abruzzo LV, Rassidakis GZ, Parry-Jones N, Schlette E, Brito-Bapabulle V, et al. The t(14;19)(q32;q13)-positive small B-cell leukaemia: a clinicopathologic and cytogenetic study of seven cases. *Br J Haematol*. 2007;136(2):220–8.
81. Nguyen-Khac F, Chapiro E, Lesty C, Grelier A, Luquet I, Radford-Weiss I, et al. Specific chromosomal IG translocations have different prognoses in chronic lymphocytic leukemia. *Am J Blood Res*. 2011;1(1):13–21.
82. Martín-Subero JI, Ibbotson R, Klapper W, Michaux L, Callet-Bauchu E, Berger F, et al. A comprehensive genetic and histopathologic analysis identifies two subgroups of B-cell malignancies carrying a t(14;19)(q32;q13) or variant BCL3-translocation. *Leukemia*. 2007 Jul;21(7):1532–44.
83. Sellmann L, Gesk S, Walter C, Ritgen M, Harder L, Martín-Subero JI, et al. Trisomy 19 is associated with trisomy 12 and mutated IGHV genes in B-chronic lymphocytic leukaemia. *Br J Haematol*. 2007 Jul;138(2):217–20.
84. Baliakas P, Puiggros A, Xochelli A, Sutton LA, Nguyen-Khac F, Gardiner A, et al. Additional trisomies amongst patients with chronic lymphocytic leukemia carrying trisomy 12: the accompanying chromosome makes a difference. *Haematologica*. 2016 Jul;101(7):e299-302.
85. Ibbotson R, Athanasiadou A, Sutton LA, Davis Z, Gardiner A, Baliakas P, et al. Coexistence of trisomies of chromosomes 12 and 19 in chronic lymphocytic leukemia occurs exclusively in the rare IgG-positive variant. *Leukemia*. 2012 Jan;26(1):170–2.
86. Blombery P, Anderson MA, Gong J nan, Thijssen R, Birkinshaw RW, Thompson ER, et al. Acquisition of the Recurrent Gly101Val Mutation in BCL2 Confers Resistance to Venetoclax in Patients with Progressive Chronic Lymphocytic Leukemia. *Cancer Discov*. 2019 Mar 1;9(3):342–53.
87. Blombery P, Thompson ER, Nguyen T, Birkinshaw RW, Gong JN, Chen X, et al. Multiple BCL2 mutations cooccurring with Gly101Val emerge in chronic lymphocytic leukemia progression on venetoclax. *Blood*. 2020 Mar 5;135(10):773–7.
88. Guièze R, Liu VM, Rosebrock D, Jourdain AA, Hernández-Sánchez M, Martínez Zurita A, et al. Mitochondrial Reprogramming Underlies Resistance to BCL-2 Inhibition in Lymphoid Malignancies. *Cancer Cell*. 2019 Oct 14;36(4):369-384.e13.

89. Khalsa JK, Cha J, Utro F, Naeem A, Murali I, Kuang Y, et al. Genetic events associated with venetoclax resistance in CLL identified by whole exome sequencing of patient samples. *Blood*. 2023 May 5;blood.2022016600.
90. Lucas F, Larkin K, Gregory CT, Orwick S, Doong TJ, Lozanski A, et al. Novel BCL2 mutations in venetoclax-resistant, ibrutinib-resistant CLL patients with BTK/PLCG2 mutations. *Blood*. 2020 Jun 11;135(24):2192–5.
91. Puiggros A, Ramos-Campoy S, Kamaso J, de la Rosa M, Salido M, Melero C, et al. Optical Genome Mapping: A Promising New Tool to Assess Genomic Complexity in Chronic Lymphocytic Leukemia (CLL). *Cancers*. 2022 Jul 11;14(14):3376.
92. Ahn IE, Tian X, Wiestner A. Ibrutinib for Chronic Lymphocytic Leukemia with TP53 Alterations. *N Engl J Med*. 2020 Jul 30;383(5):498–500.
93. Burger JA, Barr PM, Robak T, Owen C, Ghia P, Tedeschi A, et al. Long-term efficacy and safety of first-line ibrutinib treatment for patients with CLL/SLL: 5 years of follow-up from the phase 3 RESONATE-2 study. *Leukemia*. 2020 Mar;34(3):787–98.
94. Byrd JC, Hillmen P, O'Brien S, Barrientos JC, Reddy NM, Coutre S, et al. Long-term follow-up of the RESONATE phase 3 trial of ibrutinib vs ofatumumab. *Blood*. 2019 May 9;133(19):2031–42.
95. Byrd JC, Furman RR, Coutre SE, Flinn IW, Burger JA, Blum K, et al. Ibrutinib Treatment for First-Line and Relapsed/Refractory Chronic Lymphocytic Leukemia: Final Analysis of the Pivotal Phase Ib/II PCYC-1102 Study. *Clin Cancer Res Off J Am Assoc Cancer Res*. 2020 Aug 1;26(15):3918–27.
96. Moreno C, Greil R, Demirkan F, Tedeschi A, Anz B, Larratt L, et al. First-line treatment of chronic lymphocytic leukemia with ibrutinib plus obinutuzumab versus chlorambucil plus obinutuzumab: final analysis of the randomized, phase III iLLUMINATE trial. *Haematologica*. 2022 Sep 1;107(9):2108–20.
97. Ahn IE, Tian X, Ipe D, Cheng M, Albitar M, Tsao LC, et al. Prediction of Outcome in Patients With Chronic Lymphocytic Leukemia Treated With Ibrutinib: Development and Validation of a Four-Factor Prognostic Model. *J Clin Oncol Off J Am Soc Clin Oncol*. 2021 Feb 20;39(6):576–85.
98. Quinquenel A, Fornecker LM, Letestu R, Ysebaert L, Fleury C, Lazarian G, et al. Prevalence of BTK and PLCG2 mutations in a real-life CLL cohort still on ibrutinib after 3 years: a FILO group study. *Blood*. 2019 Aug 15;134(7):641–4.
99. Bődör C, Kotmayer L, László T, Takács F, Barna G, Kiss R, et al. Screening and monitoring of the BTKC481S mutation in a real-world cohort of patients with relapsed/refractory chronic lymphocytic leukaemia during ibrutinib therapy. *Br J Haematol*. 2021;194(2):355–64.
100. Tatarczuch M, Waltham M, Shortt J, Polekhina G, Hawkes EA, Ho SJ, et al. Molecular associations of response to the new generation BTK inhibitor zanubrutinib in marginal zone lymphoma. *Blood Adv*. 2023 Mar 22;bloodadvances.2022009412.
101. Burger JA, Landau DA, Taylor-Weiner A, Bozic I, Zhang H, Sarosiek K, et al. Clonal evolution in patients with chronic lymphocytic leukaemia developing resistance to BTK inhibition. *Nat Commun*. 2016 May 20;7:11589.
102. Woyach JA, Furman RR, Liu TM, Ozer HG, Zapatka M, Ruppert AS, et al. Resistance mechanisms for the Bruton's tyrosine kinase inhibitor ibrutinib. *N Engl J Med*. 2014 Jun 12;370(24):2286–94.

## PUBLICATIONS SELECTIONNEES

---

- VERONESE L, TOURNILHAC O, VERRELLE P, et al. Low *MCL-1* mRNA expression correlates with prolonged survival in B-cell chronic lymphocytic leukemia. *Leukemia*. 2008; 22(6): 1291-3.....**p.63**
- L. VERONESE, O. TOURNILHAC, M. CALLANAN, et al. Telomeres and chromosomal instability in chronic lymphocytic leukemia. *Leukemia* 2013 Feb;27(2):490-3.....**p.65**
- GUIEZE R\*, PAGES M\*, VERONESE L, et al. Telomere status in chronic lymphocytic leukemia with *TP53* disruption. *Oncotarget* 2016 Aug 30;7(35):56976-56985.....**p.69**
- GAY-BELLILE M, ROMERO P, CAYRE A, et al. *ERCC1* and telomere status in breast tumours treated with neoadjuvant chemotherapy and their association with patient prognosis. *J Pathol Clin Res* 2016 Jul 13;2(4):234-246.....**p.79**
- GAY-BELLILE M, VÉRONÈSE L, COMBES P, et al. *TERT* promoter status and gene copy number gains: effect on *TERT* expression and association with prognosis in breast cancer. *Oncotarget* 2017 Aug 24;8(44):77540-77551.....**p.92**
- FAUGERAS E, VÉRONÈSE L, JEANNIN G, et al. Telomere Status of Advanced Non-Small-Cell Lung Cancer Offers a Novel Promising Prognostic and Predictive Biomarker. *Cancers (Basel)*. 2022 Dec 31;15(1):290.....**p.104**
- KHALSA JASNEET KAUR, CHA JUSTIN, UTRO FILIPPO, et al. Genetic events associated with venetoclax resistance in CLL identified by whole exome and RNA sequencing on longitudinal patient samples. *Blood*. 2023 May 5; *Blood*. 2022016600. ....**p.117**

## LETTER TO THE EDITOR

### Low *MCL-1* mRNA expression correlates with prolonged survival in B-cell chronic lymphocytic leukemia

Leukemia advance online publication, 29 November 2007;  
 doi:10.1038/sj.leu.2405052

B-cell chronic lymphocytic leukemia (B-CLL) is a heterogeneous malignant disease that involves deregulated apoptosis. Overexpression of anti-apoptotic proteins of the *BCL-2* (B-cell lymphoma gene-2) family such as *BCL-2* and *MCL-1* (myeloid cell leukemia-1) is commonly detected in B-CLL. Higher levels of *MCL-1* have been correlated with a poor treatment response.<sup>1</sup> *MCL-1* gene silencing by RNA interference was reported to induce apoptosis in B-CLL cells, suggesting that the *MCL-1* gene might be a therapeutic target.<sup>2</sup> Still, the impact of different *MCL-1* gene expression levels on the prognosis of B-CLL patients remains unknown.

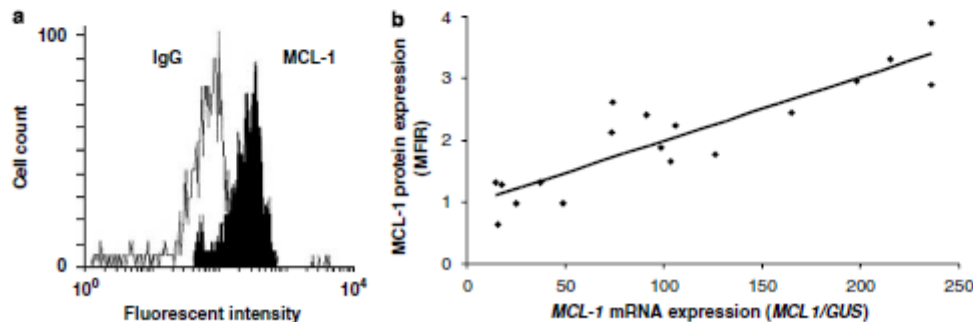
To address this issue, we quantified the expression of *MCL-1* gene in 119 B-CLL patients and correlated the results with patient survival and known prognostic markers.

This study was retrospective. Seventy-nine men and 40 women (median age of 62 years) were diagnosed with B-CLL between January 1980 and March 2006 at the Clermont-Ferrand Hospital, Pasteur Institute and Pitié-Salpêtrière Hospital (France). Peripheral blood samples were obtained after informed consent. Mononuclear cells were isolated and cryopreserved. At the time of sampling, 69 patients were in Binet stage A, 31 in Binet stage B and 19 in Binet stage C. The median interval from diagnosis to sampling was 2 months. Treatment decisions and modalities in all centers were based on the French Cooperative Group guidelines. Stage A patients remained untreated until disease progression. Of note, all samples from stage A patients were obtained before any therapy. Stage B/C patients received early treatment, mainly chlorambucil or fludarabine as a first-line therapy. Median follow-up for the whole population was 9 years.

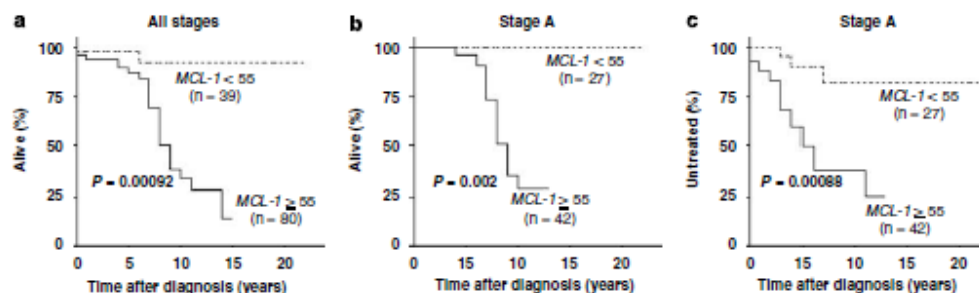
The amount of the full-length *MCL-1* transcript encoding the anti-apoptotic isoform of *MCL-1* protein (*MCL-1L*) was assessed using real-time quantitative reverse transcriptase-PCR (RQ-PCR) and normalized for  $\beta$ -glucuronidase (*GUS*) gene expression, using previously reported primers.<sup>3,4</sup> The median percentage of tumor lymphocytes in patient samples was 74% (37–99%, range) as assessed with immunophenotype analysis ( $n=69$ ). Residual normal cells did not significantly affect the determination of *MCL-1* mRNA, since there was no difference in *MCL-1* levels between the samples with >74% tumor purity and those with <74% purity (Kruskal–Wallis test,  $P=0.41$ , NS). In 18 samples, we quantified *MCL-1* protein expression using flow cytometry and found that the mRNA and protein levels were significantly correlated (Figure 1), suggesting that the amount of *MCL-1* mRNA was predictive of the *MCL-1* protein expression.

The *MCL-1/GUS* ratio was highly variable within a range of 10–776, with a median value of 85. We scored the relative level of *MCL-1* mRNA as low (first tertile, <55), intermediate (second tertile, 55–133) and high (third tertile, >133) and compared the survival in these groups. The survival times were not different in patients with intermediate and high *MCL-1* expression (log-rank test,  $P=0.78$ ). However, patients expressing *MCL-1* at intermediate/high levels ( $\geq 55$ ) have significantly shorter survival than patients expressing *MCL-1* at low levels (<55). This relationship was significant within the whole patient population ( $P=0.00092$ ; Figure 2a) and among stage A patients ( $P=0.002$ ; Figure 2b). Moreover, the time to first therapy was significantly shorter in previously untreated stage A patients with intermediate/high levels of *MCL-1* mRNA compared with those with low levels of *MCL-1* mRNA ( $P=0.00088$ ; Figure 2c).

In a multivariate Cox analysis with the major prognostic indicators of B-CLL,<sup>5</sup> Binet stage ( $P=0.013$ ) and immunoglobulin V gene (IgV) mutational status ( $P=0.0014$ ), *MCL-1* mRNA expression was an independent, significant predictor of overall



**Figure 1** (a) The lymphocyte population from B-cell chronic lymphocytic leukemia (B-CLL) samples was examined for the myeloid cell leukemia-1 (*MCL-1*) protein expression by intracellular staining and flow cytometry. Cells were incubated with the *MCL-1L* antibody and control IgG antibody, and *MCL-1* and IgG were quantified by the mean fluorescence intensity (MFI). The *MCL-1* value was normalized by dividing the *MCL-1* value by the control IgG value, obtaining the MFI ratio (MFI ratio) for each sample. The graph demonstrates the *MCL-1* and control IgG staining patterns in a B-CLL sample (MFI ratio of 3.9). (b) Correlation between *MCL-1* mRNA and *MCL-1* protein expression levels in primary B-CLL cells. The *MCL-1* mRNA level was measured using real-time quantitative reverse transcriptase-PCR and expressed as an *MCL-1/GUS* ratio multiplied by 100.



**Figure 2** Overall survival in the whole B-cell chronic lymphocytic leukemia (B-CLL) patient population (a), in stage A patients (b) and treatment-free survival in stage A patients (c) as a function of myeloid cell leukemia-1 (MCL-1) mRNA expression. Patients expressing MCL-1 at low levels (<55) have significantly longer overall and treatment-free survival when compared with those expressing MCL-1 at intermediate/high levels (≥55).

survival ( $P=0.0093$ ). The hazard ratio for higher MCL-1 expression (≥55) was 7.0 (95% confidence interval 1.6–30.6).

In addition, MCL-1 mRNA levels did not correlate with clinical stage and known biological prognostic markers of B-CLL<sup>5</sup> such as IgV mutational status ( $n=87$ ), CD38 expression ( $n=69$ ), serum thymidine kinase ( $n=62$ ) and  $\beta 2$ -microglobulin ( $n=63$ ) levels, lymphocyte doubling time ( $n=64$ ) and human telomerase reverse-transcriptase mRNA expression ( $n=113$ ) (data not shown).

We also determined the levels of BCL-2 gene expression using RQ-PCR<sup>6</sup> in the whole B-CLL cohort. The BCL-2/GUS ratio varied between 15 and 1780 with a median of 385. The level of BCL-2 expression was on average fivefold higher than the level of MCL-1 mRNA (Kruskal–Wallis test,  $P<10^{-7}$ ). The expression of BCL-2 was less variable (82%, coefficient of variation) than the expression of MCL-1 (105%, coefficient of variation). Of note, the levels of BCL-2 and MCL-1 transcripts were significantly correlated (Spearman's rank test,  $r=0.42$ ,  $P=0.00016$ ). To assess the relative prognostic importance of BCL-2 and MCL-1 transcript levels, we included these two parameters in a Cox model for overall survival. MCL-1 ( $P=0.0092$ ) but not BCL-2 gene expression ( $P=0.52$ ) had a significant impact on prognosis. In a previous study, BCL-2 mRNA was found to be overexpressed in most of the B-CLL samples and alone was not predictive of patient outcome.<sup>7</sup>

The current study shows that the level of MCL-1 mRNA expression may be an independent prognostic marker in B-CLL, which is not correlated to traditional indicators of prognosis such as IgV gene mutation profile. In particular, a low MCL-1 gene expression identifies stage A patients with indolent and stable disease and a longer time interval between diagnosis and need of therapy. Lower levels of MCL-1 protein expression have been previously correlated with the ability of patients with B-CLL to achieve a complete remission after treatment.<sup>1</sup> Of note, our data suggest that the MCL-1 mRNA and protein levels in B-CLL are correlated. Taken together, these observations show that low MCL-1 expression levels characterize B-CLL patients with a low risk of disease progression who also are likely to respond to treatment, which may be useful for the therapeutic management of cases with early B-CLL.

In the future prospective studies, it would be important to validate these results and explore if the MCL-1 transcript level is predictive of a treatment response. It would also be essential to investigate in individual patients whether the MCL-1 expression level remains stable during the course of the disease or increases during progression or development of drug resistance.

The prognostic role of MCL-1 gene expression further implicates this pro-survival molecule in the pathogenesis of B-CLL. Given that most B-CLL cells contain high levels of BCL-2, it was suggested that the combination of MCL-1 and BCL-2 might provide a superior protection against apoptosis.<sup>8</sup> Of note, BCL-2 and MCL-1 can inhibit apoptosis by sequestering pro-death proteins. Del Gaizo Moore *et al.*<sup>8</sup> have recently observed that in B-CLL cells almost all pro-apoptotic BIM appeared to be present in complex with BCL-2. They suggested that in B-CLL cells with abundant BCL-2, this protein is so highly occupied by BIM molecules that the cells have very poor anti-apoptotic reserve, which may potentially explain the notable chemosensitivity of B-CLL. Upregulation of the MCL-1 gene might provide additional survival capacities to B-CLL cells during physiological stress or drug treatment.

Specific downregulation of MCL-1 gene expression by short interfering RNA has been recently shown to reduce the MCL-1 protein level and promote apoptosis and death of primary B-CLL cells.<sup>2</sup> In this regard, our finding that low MCL-1 gene expression is related to a good prognosis may be relevant, supporting MCL-1 as a potential therapeutic target. However, it should be noted that the downregulation of MCL-1 influences only some of the death pathways and eliminates only a proportion of B-CLL cells. Targeting MCL-1 expression in B-CLL could be effective in combination with other antitumor agents such as rituximab.<sup>2</sup>

L. Veronèse<sup>1,2</sup>, O. Tournilhac<sup>3</sup>, P. Verrelle<sup>2,4</sup>, F. Davi<sup>5</sup>, G. Dighiero<sup>6</sup>, E. Chautard<sup>2</sup>, R. Veyrat-Masson<sup>7</sup>, F. Kwiatkowski<sup>8</sup>, C. Goumy<sup>1</sup>, P. Vago<sup>1</sup>, P. Travade<sup>1</sup> and A. Tchirkov<sup>1,2,4</sup>

<sup>1</sup>Université Clermont 1, UFR Médecine, CHU Clermont-Ferrand, Service de Cytogénétique Médicale, Clermont-Ferrand, France;

<sup>2</sup>EA 3846, Université d'Auvergne, Clermont-Ferrand, France;

<sup>3</sup>Service d'Hématologie Clinique, CHU Clermont-Ferrand, Clermont-Ferrand, France;

<sup>4</sup>Département de Radiothérapie, Centre Jean Perrin, Clermont-Ferrand, France;

<sup>5</sup>Service d'Hématologie Biologique & Université Pierre et Marie Curie, Hôpital Pitié-Salpêtrière, Paris, France;

<sup>6</sup>Institut Pasteur de Montevideo, Montevideo, Uruguay;

<sup>7</sup>Service d'Hématologie Biologique, CHU Clermont-Ferrand, Clermont-Ferrand, France and

<sup>8</sup>Service Statistiques et Communications Médicales, Centre Jean Perrin, Clermont-Ferrand, France

E-mail: andrei.tchirkov@cjp.fr



## LETTER TO THE EDITOR

# Telomeres and chromosomal instability in chronic lymphocytic leukemia

Leukemia advance online publication, 10 August 2012;  
doi:10.1038/leu.2012.194

Telomeres are protective chromosomal end structures composed of G-rich nucleotide repeats and an associated protein complex termed shelterin. Because of incomplete replication, telomeric repeats are lost with every cell division, and this telomere attrition is involved in cell senescence and cancer (reviewed in Artandi and DePinho<sup>1</sup>). Short, unprotected telomeres are erroneously fused by the DNA-repair system, which gives rise to aneuploidies or structural chromosome rearrangements through chromosome fusion-bridge-breakage cycles. Thus, telomere dysfunction can drive genomic instability during tumorigenesis.<sup>1</sup>

In chronic lymphocytic leukemia (CLL), short telomeres have been associated with poor-prognosis cytogenetics.<sup>2,3</sup> Besides telomere length, the functional state of telomeres depends on several molecular factors. Telomeres can be regenerated by telomerase and are protected from fusions by shelterin proteins. Detailed investigations assessing all together the expression of telomerase and shelterin genes, telomere length, recurrent aberrations and overall karyotype instability have not yet been reported in CLL. Here, we studied the telomere status and chromosomal aberrations in a series of CLL patients. A clustering analysis of the data identified groups with distinct cytogenetic and telomere profiles.

This prospective study included 77 patients (57 males and 20 females, median age of 67 years) diagnosed with CLL at the Clermont-Ferrand University Hospital. Blood samples were obtained after informed consent. At the time of sampling, 34 patients were in Binet stage A, 17 in stage B and 26 in stage C. Samples were obtained before any treatment in 60 (77.9%) patients. Among the remaining 17 cases, 16 received no treatment within 6 months prior to the date of sampling.

Karyotype was analyzed after immunostimulation of cell cultures with the CpG-oligonucleotide DSP30 and interleukin 2. Interphase fluorescence *in situ* hybridization (FISH) was performed with a panel of commercially available probes (Abbott Molecular, Rungis, France) for the detection of trisomy 12 and deletions of 13q14, 11q22.3 (ATM) and 17p13 (p53).

Telomere studies were performed on mononuclear cells (MNCs). Average telomere length was evaluated with quantitative real-time DNA PCR.<sup>2</sup> The method measures the difference between a sample and a reference normal DNA in the ratio of telomere repeat copy number to single-gene copy number (relative T/S ratio). This T/S ratio is proportional to the average telomere length assessed with a classical telomere restriction fragment analysis ( $r=0.89$ ; Supplementary Figure S1).

The expression of *hTERT* (human telomerase reverse transcriptase) and core members of the shelterin complex, *TRF1* and *TRF2* (telomeric repeat binding factors 1 and 2) and *POT1* (protection of telomeres 1) genes were quantified using real-time quantitative PCR.<sup>4,5</sup> The normalized copy numbers (NCNs) were expressed as the ratio between transcript copy numbers of the target and control (*GUS*) genes multiplied by 100. The mean percentage of CLL cells determined in MNC samples as the CD19+ CD5+ population was

74 ± 19% (s.d.). To evaluate the possible impact of normal cell contamination, we analyzed several samples ( $n=10$ ) before and after selection of CD19+ cells. The mean values of CD19+ cells were 75.6 ± 13.9% before and 98.2 ± 2.4% after selection. We did not find any statistically significant difference between the values of telomere length and gene expression obtained before and after selection (data not shown).

Unsupervised hierarchical clustering analyses on the basis of either a combination of cytogenetic and telomeric characteristics or cytogenetic and telomeric parameters alone were performed to identify subclasses with distinct chromosome aberration and telomeric profiles (Figures 1a–c). Karyotype and FISH data were qualitative. Karyotypes were characterized by the presence or absence of aneuploidies, translocations or deletions. FISH patterns were as follows: normal, del(13q), trisomy 12, del(17p) or del(11q). Single FISH aberrations were found in 48 cases and multiple aberrations in 10 cases. In nine cases, deletions of 17p or 11q were accompanied either by a del(13q) or a trisomy 12. One case showed the presence of a trisomy 12 and a del(13q). Telomere characteristics were included as quantitative values: relative T/S ratios for telomere length and NCN of *hTERT*, *TRF1*, *TRF2* and *POT1* transcripts. The differences in the incidence of chromosomal aberrations and in the values of telomere parameters across the clusters were analyzed statistically (Table 1).

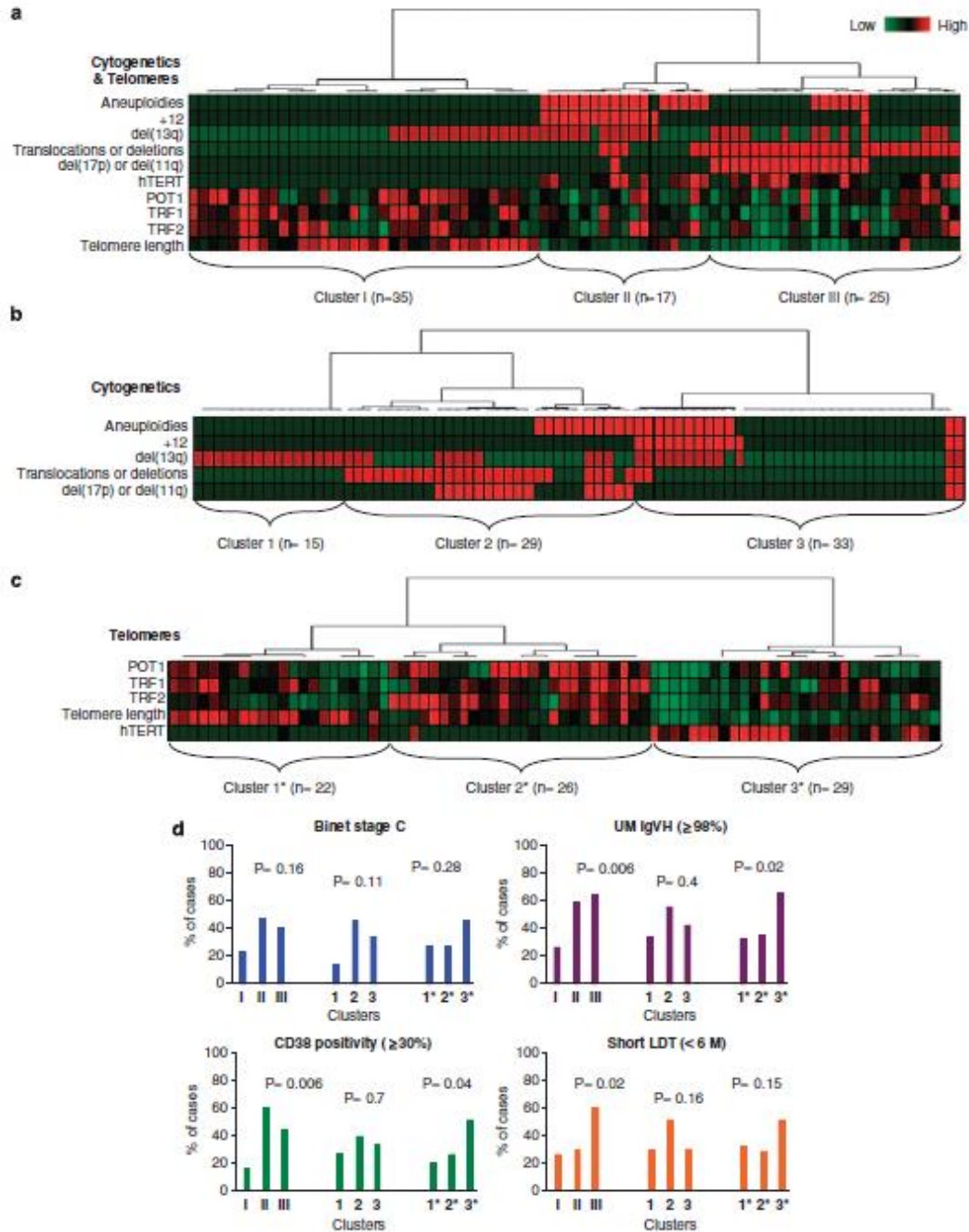
Clustering of the combined data set highlighted three groups. Cluster I demonstrated good-prognosis cytogenetics (isolated del(13q) or normal cytogenetics), long telomeres, negative or low *hTERT* and a high expression of the shelterin complex genes (*TRF1*, *TRF2* and *POT1* values were similar to those of normal B cells, data not shown). In contrast, clusters II and III displayed multiple chromosome aberrations, mostly aneuploidies in cluster II and structural chromosomal changes in cluster III. All poor-prognosis del(17p) and del(11q) cases were in cluster III, which also showed multiple karyotypic abnormalities (50% of cases) and translocations (72% of cases), the majority of translocations (86%) being unbalanced. Clusters II and III were characterized by a striking decrease in telomere length and shelterin gene-expression levels. Thus, rising chromosomal instability was associated with telomeric deficiency. The *hTERT* gene was activated in clusters II and III, consistently with its role in stabilization of rearranged tumor genomes.<sup>1</sup> The results also highlight the relationship of 17p and 11q deletions with more severe telomere and chromosome instability. These deletions lead to the loss of *p53* and *ATM* genes, respectively, which are involved in monitoring telomere and DNA integrity. An impact of *p53* and *ATM* deficiency on telomere length was previously reported in CLL cells.<sup>2,6</sup>

Separate clustering of cytogenetic (Figure 1b) and telomeric data (Figure 1c) also showed the relationship between the telomere status and chromosomal aberrations. In particular, cytogenetic cluster 1 without any chromosomal aberration was normal in terms of telomere characteristics (Table 1). Cytogenetic cluster 2 displays structural chromosomal changes, including 17p or 11q deletions, and telomere shortening, high *hTERT* and decreased shelterin gene-expression levels. Clustering of telomere parameters alone revealed an interesting group (cluster 3\*) with deficient telomeres with the highest *hTERT* level, presenting high incidences of various

aberrations, such as aneuploidies (52%), translocations or deletions (79%), del(17p) and del(11q) (5%) and a trisomy 12 (24%).

Unlike telomere length, the level of shelterin complex genes in CLL was not previously analyzed in relation to chromosomal

instability. The total expression of shelterin genes was found to be lower in clusters harboring an increased rate of aberrations (clusters II, 2 and 3\*, Table 1). This reduced expression was also associated with shorter telomere length. Shelterin downregulation





may either be secondary to telomere shortening or, in contrast, may drive telomere dysfunction, thus compromising chromosomal integrity.<sup>7</sup> The present data do not allow us to examine this issue, although some evidence exists. In particular, cluster 1\* characterized by long telomeres and low level of chromosome aberrations contains a small subcluster with significantly decreased shelterin gene expression (seven last patients of cluster 1\*, Figure 1). Interestingly, this subcluster did not harbor more chromosome aberrations than the second subcluster (first 15 patients) characterized by higher shelterin levels (data not shown). Given the small patient number, this result should be taken with caution, but it suggests that the shelterin reduction may be insufficient for the acquisition of substantial chromosomal instability when telomeres are (still) long. Follow-up studies in such patients could help to investigate if a sustained shelterin downregulation would lead to more rapid telomere erosion and/or accumulation of aberrations. In addition, interactions with other shelterin complex members could have a role in chromosomal instability. Studies of *RAP1*, *TPP1* and *TIN2* gene expression are needed to clarify the involvement of shelterin alterations in chromosomal instability in CLL.

We also evaluated well-established factors predictive of CLL progression in patients from different clusters to determine if the obtained classification may be clinically significant. Figure 1d

shows the incidence of negative prognostic features, such as Binet C stage, unmutated (UM) immunoglobulin variable region genes (IgVH), CD38 positivity ( $\geq 30\%$ ) and short (<6 months) lymphocyte doubling time (LDT), in different clusters. No significant relationship was found with Binet C stage. In contrast, clusters with enhanced telomere and chromosome instability showed higher incidences of classical biological factors of poor prognosis. Of note, the strongest associations with UM IgVH and CD38 positivity were found for clusters obtained by combining telomeric and cytogenetic parameters, whereas the links with telomeric clusters were weaker but still significant and, finally, the associations with cytogenetic clusters were not significant. Only the combined model showed a significant relationship with short LDT. Cytogenetics and telomeres are known to represent valuable prognostic factors in CLL,<sup>2</sup> and perhaps the combinations of both characteristics could provide a more accurate prediction of disease progression. Our study was prospective, and so far we were not able to compare survival of patients from different clusters. This issue can be addressed in a large cohort of CLL patients with adequate follow-up.

In conclusion, the identification of groups with distinct telomeric and cytogenetic profiles supports the link between telomere status and genomic instability in CLL. Telomere shortening, changes in shelterin and *hTERT* gene expression coincided

**Table 1.** Frequencies of chromosomal aberrations and values of quantitative telomere characteristics in different clusters

	Cytogenetics and telomeres				Cytogenetics				Telomeres			
	Cluster I, n = 35	Cluster II, n = 17	Cluster III, n = 25	P <sup>a</sup>	Cluster 1, n = 15	Cluster 2, n = 29	Cluster 3, n = 33	P <sup>b</sup>	Cluster 1*, n = 22	Cluster 2*, n = 26	Cluster 3*, n = 29	P <sup>b</sup>
<b>Chromosomal aberrations</b>												
Aneuploidies	0.0%	94.1%	24.0%	<10 <sup>-6</sup>	0.0%	34.5%	36.4%	0.02	18.2%	11.5%	51.7%	<10 <sup>-2</sup>
Translocations or deletions	0.0%	29.4%	96.0%	<10 <sup>-6</sup>	0.0%	86.2%	12.1	<10 <sup>-6</sup>	13.6%	11.5%	79.3%	<10 <sup>-5</sup>
Del(17p) or del(11q)	0.0%	5.9%	64.0%	<10 <sup>-6</sup>	0.0%	51.7%	6.1%	<10 <sup>-4</sup>	0.0%	3.8%	55.2%	<10 <sup>-4</sup>
+12	0.0%	70.6%	4.0%	<10 <sup>-6</sup>	0.0%	0.0%	39.4%	<10 <sup>-4</sup>	9.1%	15.4%	24.1%	0.35
Del(13q)	57.1%	35.3%	52.0%	0.33	0.0%	62.0%	63.6%	<10 <sup>-2</sup>	50.0%	57.7%	44.8%	0.64
<b>Telomere parameters<sup>b</sup></b>												
Telomere length (T/S ratio)	1.6 ± 0.1	0.9 ± 0.1	0.6 ± 0.1	<10 <sup>-6</sup>	1.7 ± 0.1	0.7 ± 0.1	1.2 ± 0.1	<10 <sup>-6</sup>	1.6 ± 0.1	1.2 ± 0.1	0.6 ± 0.04	<10 <sup>-6</sup>
<i>hTERT</i> (NCN)	4.4 ± 3.1	90 ± 23	88 ± 17	<10 <sup>-5</sup>	0.0	96 ± 17	56 ± 9	<10 <sup>-4</sup>	9.0 ± 4.8	9.7 ± 5.8	118 ± 16	<10 <sup>-6</sup>
<i>TRF1</i> (NCN)	145 ± 9	97 ± 9	84 ± 9	<10 <sup>-3</sup>	142 ± 10	83 ± 8	130 ± 10	<10 <sup>-3</sup>	114 ± 9	145 ± 12	88 ± 8	<10 <sup>-3</sup>
<i>TRF2</i> (NCN)	193 ± 12	183 ± 21	153 ± 16	0.11	192 ± 14	155 ± 15	192 ± 14	0.15	133 ± 8	235 ± 14	161 ± 15	<10 <sup>-4</sup>
<i>POT1</i> (NCN)	195 ± 12	131 ± 14	132 ± 14	<10 <sup>-2</sup>	208 ± 16	130 ± 12	165 ± 13	<10 <sup>-2</sup>	141 ± 12	216 ± 14	125 ± 12	<10 <sup>-4</sup>
Total shelterin <sup>c</sup> (NCN)	534 ± 26	413 ± 36	369 ± 31	<10 <sup>-3</sup>	542 ± 34	369 ± 27	488 ± 31	<10 <sup>-3</sup>	389 ± 26	596 ± 27	376 ± 27	<10 <sup>-6</sup>

Abbreviations: *hTERT*, human telomerase reverse transcriptase; NCN, normalized copy number; *POT1*, protection of telomeres 1; *TRF*, telomeric repeat binding factor. <sup>a</sup>Aberration frequencies were compared using the Chi-square test and quantitative telomere characteristics using the Kruskal-Wallis H-test. <sup>b</sup>Mean values with s.e. <sup>c</sup>Total shelterin gene expression (*TRF1* + *TRF2* + *POT1*).

**Figure 1.** (a–c) Hierarchical clustering of 77 CLL patients according to the pattern of chromosomal aberrations and telomere characteristics. Clustering was performed on the combined telomeres and cytogenetics data set (a) and separately: cytogenetics alone (b) and telomeres alone (c). Qualitative and quantitative values were normalized (centered and reduced) before clusterization. Aneuploidies, translocations or deletions, del(17p) or del(11q), and trisomy 12 are presented in green (absence of abnormality) and red (presence of abnormality) squares. Presence of the low-risk del(13q) is shown in green, the absence in dark red. The different color intensities of dichotomic parameters reflect normalized values and not original binary (0/1) values. High normalized quantitative values of telomere length and expression of telomere-related genes are shown in red, intermediate values in black and lower values in green (intensity scale is shown). These normalized values, which are not logarithmic, are necessary to calculate the distances between subjects and items. Distances between clusters were calculated using 1-Pearson's correlation coefficient values and the dendrogram was constructed according to Ward's algorithm. Of note, the average proportion of tumor cells in patient samples belonging to different clusters was essentially the same. For instance, the mean tumor cell content was 75% in cluster I, 75% in cluster II and 72% in cluster III (Kruskal-Wallis H-test,  $P = 0.68$ , not significant). (d) The proportions of cases with negative prognostic factors in different clusters.  $P$ -values were obtained with the Chi-square test showing the overall difference between the clusters.



with increasing levels of chromosome aberrations, and this was particularly affected by *p53* or *ATM* gene deletions. These CLL groups are associated with established prognostic factors and may be clinically significant.

# CONFLICT OF INTEREST

The authors declare no conflict of interest.

L. Véronèse<sup>1,2,3</sup>, O. Tournilhac<sup>4,5</sup>, M. Callanan<sup>6,7</sup>, N. Prie<sup>1,2,3</sup>,  
F. Kwiatkowski<sup>3,8</sup>, P. Combes<sup>1,2,3</sup>, M. Chauvet<sup>6,7</sup>, F. Davi<sup>9</sup>,  
L. Gouas<sup>1,2,3</sup>, P. Verrelle<sup>5,8</sup>, R. Guèze<sup>4,5</sup>, P. Vago<sup>1,2,3</sup>,  
J.O. Bay<sup>4,5</sup> and A. Tchirkov<sup>1,2,3,8</sup>  
<sup>1</sup>CHU Clermont-Ferrand, Cytogénétique Médicale,  
Clermont-Ferrand, France;  
<sup>2</sup>Clermont Université, Université d'Auvergne,  
Faculté de Médecine, Histologie Embryologie Cytogénétique,  
Clermont-Ferrand, France;  
<sup>3</sup>EA 4677 ERTiCa, Université d'Auvergne,  
Clermont-Ferrand, France;  
<sup>4</sup>CHU Clermont-Ferrand, Hématologie Clinique Adulte,  
Clermont-Ferrand, France;  
<sup>5</sup>EA 7283 CREaT, Université d'Auvergne,  
Clermont-Ferrand, France;  
<sup>6</sup>Inserm U823, Institut Albert Bonniot,  
Université Joseph Fourier, Grenoble, France;

<sup>7</sup>CHU Grenoble, Laboratoire de Génétique  
Onco-hématologique, Grenoble, France;

<sup>8</sup>Centre Jean Perrin, Clermont-Ferrand, France and

<sup>9</sup>Service d'Hématologie Biologique, Hôpital Pitié-Salpêtrière,  
Paris, France

E-mail: atchirkov@chu-clermontferrand.fr

# REFERENCES

- Artandi SE, DePinho RA. Telomeres and telomerase in cancer. *Carcinogenesis* 2010; **31**: 9–18.
- Roos G, Kröber A, Grabowski P, Klein D, Bühler A, Döhner H et al. Short telomeres are associated with genetic complexity, high-risk genomic aberrations, and short survival in chronic lymphocytic leukemia. *Blood* 2008; **111**: 2246–2252.
- Brugat T, Nguyen-Khac F, Grellet A, Meste-Béral H, Delic J. Telomere dysfunction-induced foci arise with the onset of telomeric deletions and complex chromosomal aberrations in resistant chronic lymphocytic leukemia cells. *Blood* 2010; **116**: 239–249.
- Tchirkov A, Chaletex C, Magnac C, Vasconcelos Y, Davi F, Michel A et al. hTERT expression and prognosis in B-chronic lymphocytic leukemia. *Ann Oncol* 2004; **15**: 1476–1480.
- Poncet D, Belleville A, Tink de Roodenbeke C, Roborel de Climens A, Ben Simon E, Meste-Béral H et al. Changes in the expression of telomere maintenance genes suggest global telomere dysfunction in B-chronic lymphocytic leukemia. *Blood* 2008; **111**: 2388–2391.
- Britt-Compton B, Lin TT, Ahmed G, Weston V, Jones RE, Fegan C et al. Extreme telomere erosion in ATM-mutated and 11q-deleted CLL patients is independent of disease stage. *Leukemia* 2012; **26**: 826–830.
- Coolson JC, Laughton CA. The levels of telomere-binding proteins in human tumours and therapeutic implications. *Eur J Cancer* 2009; **45**: 536–550.

Supplementary Information accompanies the paper on the Leukemia website (<http://www.nature.com/leu>)

## Telomere status in chronic lymphocytic leukemia with *TP53* disruption

Romain Guièze<sup>1,\*</sup>, Mélanie Pages<sup>2,\*</sup>, Lauren Véronèse<sup>3</sup>, Patricia Combes<sup>3</sup>, Richard Lemal<sup>1</sup>, Mathilde Gay-bellile<sup>3</sup>, Martine Chauvet<sup>4</sup>, Mary Callanan<sup>4</sup>, Fabrice Kwiatkowski<sup>5</sup>, Bruno Pereira<sup>6</sup>, Philippe Vago<sup>3</sup>, Jacques-Olivier Bay<sup>1</sup>, Olivier Tournilhac<sup>1</sup>, Andreï Tchirkov<sup>3</sup>

<sup>1</sup>CHU Clermont-Ferrand, Hématologie Clinique, Clermont-Ferrand, F-63003; EA 7283 CREaT, Université d'Auvergne Clermont-Ferrand, France

<sup>2</sup>Department de Neuropathologie, Hôpital Sainte-Anne, Paris, France; Université Paris Descartes, Paris, France

<sup>3</sup>Univ Clermont 1, UFR Médecine, Cytologie Histologie Embryologie Cytogénétique, Clermont-Ferrand, F-63001; CHU Clermont-Ferrand, Cytogénétique Médicale, Clermont-Ferrand, F-63003; EA 4677 ERTICa, Université d'Auvergne, Clermont-Ferrand, France

<sup>4</sup>Inserm U823, Institut Albert Bonniot & Université Joseph Fourier, Grenoble, France; CHU Grenoble, Laboratoire de Génétique Onco-hématologique, Grenoble, France

<sup>5</sup>Centre Jean Perrin, Clermont-Ferrand, France; EA 4677 ERTICa, Université d'Auvergne, Clermont-Ferrand, France

<sup>6</sup>Direction de la Recherche Clinique et de l'Innovation, Département de Biostatistiques, CHU Clermont-Ferrand, Clermont-Ferrand, France

\*These authors have contributed equally to this work

Correspondence to: Andreï Tchirkov, email: atchirkov@chu-clermontferrand.fr

Keywords: chronic lymphocytic leukemia, *TP53*, telomere, *hTERT*, shelterin

Received: April 13, 2016

Accepted: July 10, 2016

Published: July 29, 2016

### ABSTRACT

In chronic lymphocytic leukemia (CLL), telomere dysfunction is associated with poor outcomes. *TP53* is involved in cellular responses to dysfunctional telomeres, and its inactivation is the strongest adverse prognostic factor for CLL. Given the biological relationship between *TP53* and telomeres, and their prognostic value, it is important to improve our understanding of the impact of *TP53* alterations on telomeres. We performed a comprehensive study of the deletions and mutations of the *TP53* gene and telomere parameters, including *hTERT* and the shelterin complex, in 115 CLL patients. We found that any type of *TP53* alteration was associated with very short telomeres and high *hTERT* expression, independently of other biological CLL features. Patients with disrupted *TP53* showed telomere deletions and chromosomal end-to-end fusions in cells with complex karyotypes. *TP53* disruption was characterized by downregulation of shelterin genes. Interestingly, low expression of *POT1*, *TPP1* and *TIN2* was also found in some patients with wild-type *TP53* and had an adverse impact on progression-free survival after standard genotoxic therapy. In conclusion, we have demonstrated that patients with disrupted *TP53* have severe telomere dysfunction and high genomic instability. Thus, the telomeric profile could be tested as a biomarker in CLL patients treated with new therapeutic agents.

### INTRODUCTION

Telomeres protect chromosomal ends and are comprised of tracts of G-rich nucleotide repeats bound by a protein complex, shelterin, containing TRF1 and TRF2 (telomeric repeat binding factors 1 and 2), POT1 (protection of telomeres 1), TIN2 (TRF1-interacting

protein 2), TPP1 (POT1-TIN2 organizing protein, also known as ACD) and RAP1 (repressor-activator protein 1) [1, 2].

The protective function of telomeres requires a sufficient amount of telomeric repeats and integrity of the shelterin complex. Telomere shortening due to incomplete replication of telomeric DNA, as well as

shelterin defects, result in telomere dysfunction, which can lead to chromosomal and genomic instability. However, cells with dysfunctional telomeres are removed via senescence or apoptosis, and the p53 protein, a guardian of genomic integrity, regulates this physiological response. *hTERT* (human telomerase reverse transcriptase) is a key component of telomerase, which can regenerate and stabilize shortened telomeres, enabling unlimited cell proliferation. Telomere dysfunction, p53 deficiency and *hTERT* activation cooperate in oncogenesis, promoting genetically unstable and immortal tumor cell clones [3–5].

In chronic lymphocytic leukemia (CLL), telomere shortening and *hTERT* expression are generally observed in patients with advanced disease and/or high-risk biological features, such as unmutated immunoglobulin heavy chain variable (*IGHV*) genes, but some early-stage patients can also display short telomeres and high *hTERT*. Telomere shortening and *hTERT* expression have been also related to poor outcomes [6–10]. The shelterin complex is globally dysregulated at the transcriptional level [11, 12] and the presence of dysfunctional telomeres has been correlated with a downregulation of shelterin genes [11, 13], but the relationship between shelterin status and the clinico-biological parameters and outcomes of CLL remain unknown.

Previous reports suggest that CLL patients with *TP53* loss, due to *del*(17p), may show excessive telomere shortening compared to other cytogenetic subgroups [12, 14–16]. Deletion of 17p and also *TP53* mutations in the absence of *del*(17p) are found in 10–15% of untreated CLL patients, and represent the strongest adverse prognostic factor to predict early resistance to standard genotoxic agents and a poor outcome [17, 18]. Given the high prognostic significance of *TP53* and telomere status and their cooperation in oncogenesis, we investigated the potential link between *TP53* gene alterations and impaired telomeres in CLL. We performed a comprehensive study of deletions and mutations of the *TP53* gene and of telomere parameters, including assessment of telomeres as well as *hTERT* and shelterin gene expression in 115 CLL patients.

## RESULTS

### *TP53* mutations and deletions are associated with short telomeres and *hTERT* overexpression

*TP53* mutations were found in 20 patients (17.4%); of these, 10 also had a *del*(17p). Four additional cases had a *del*(17p) only. We found that telomere length and *hTERT* expression did not differ between patients with a *del*(17p) and those carrying a *TP53* mutation in the absence of a 17p deletion (Figure 1A–1B). In contrast, telomere length in these cases was considerably shorter and *hTERT* expression was higher compared to that in patients with wild-type (wt) *TP53*. In further analyses, we

considered *TP53* mutated and/or deleted cases as a single *TP53*-disrupted subgroup ( $n=24$ ).

We then investigated the impact of *TP53* status on telomeres in relation to other adverse biological features of CLL, which are known to correlate with telomere length and *hTERT* expression [14, 19]. In our cohort, short telomere length was, as expected, associated with unmutated *IGHV* genes ( $P=0.00096$ ) and short lymphocyte doubling time (LDT;  $P=0.00083$ ). *hTERT* overexpression was found in patients with unmutated *IGHV* ( $P=0.0032$ ) and short LDTs ( $P=0.0016$ ). Although all these characteristics increased the risk of having short telomere length and high *hTERT* expression, *TP53* disruption had the strongest impact in the multiple logistic regression analyses (Figure 1C). This is illustrated in Figure 1D, showing that telomere length was significantly shorter in patients with a *TP53*-disrupted status compared to a *TP53* wild-type status, irrespective of the *IGHV* mutational status. Similarly, *hTERT* expression was significantly higher in patients with *TP53* disruption compared to those with a wild-type *TP53* status in the subgroups that had either a long or a short LDT (Figure 1E).

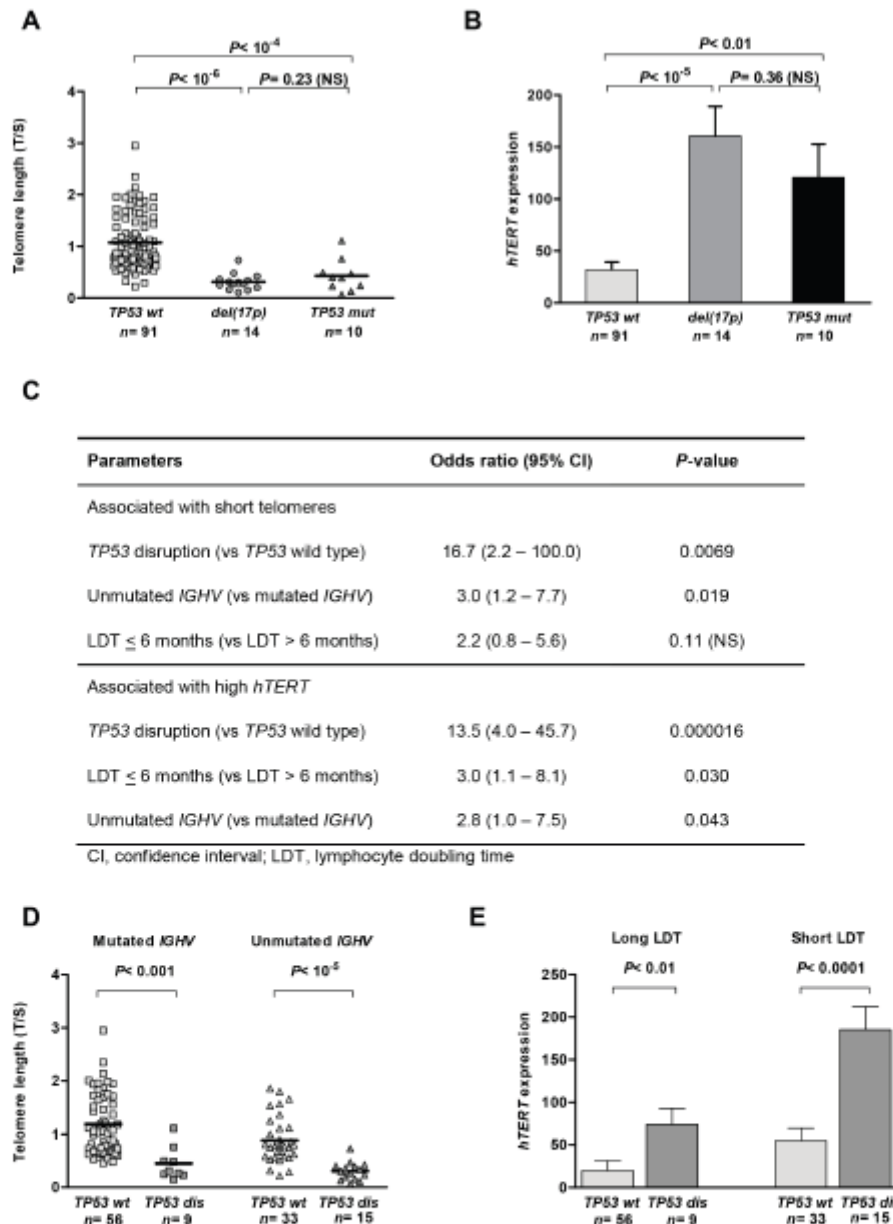
We also compared telomere lengths and *hTERT*-expression levels between cytogenetic CLL subgroups. There was gradual and significant telomere shortening, and an increase in *hTERT* from low-risk subgroups to a *TP53*-disrupted subgroup (Supplementary Figure S1A–S1B). In addition, the mean number of karyotype aberrations increased gradually and was greatest in patients with disrupted *TP53* (Supplementary Figure S1C).

### Telomere abnormalities in metaphase from *TP53*-disrupted cases

We performed pantelomeric FISH analysis on six *TP53*-disrupted cases and six *TP53* wild-type cases (Figure 2). The mean number of telomere signal losses, representative of severe telomere erosion (Figure 2B), was significantly higher in *TP53*-disrupted than in wild-type *TP53* cases (5.6 vs 1.1, respectively,  $t$ -test,  $P<0.01$ ). In contrast, no difference was observed in the frequency of multiple telomeric signals, which are markers of telomere replication defects: 0.7 in *TP53*-disrupted cases vs 0.65 in wild-type *TP53* cases, NS. In addition, nine out of 24 *TP53*-disrupted cases had complex karyotypes, and four of these showed telomeric fusions (Figure 2B), which are characteristic of extreme telomere erosion, thus highlighting the association between telomeric and chromosomal instability.

### Low shelterin gene expression in patients with *TP53* disruption

We first performed unsupervised hierarchical clustering analysis that included *TP53* status and telomere characteristics: i.e. shelterin and *hTERT* gene-expression



**Figure 1: *TP53* loss and mutation are equally associated with telomere shortening and *hTERT* overexpression in chronic lymphocytic leukaemia (CLL).** Mean telomere length **A.** and *hTERT* expression **B.** in patients with wild-type (wt) *TP53* and in patients with either *del(17p)* or *TP53* mutations (mut) in the absence of *del(17p)* (Kruskal–Wallis H test). The impact of *TP53* disruption (dis) was independent of *IGHV* status and lymphocyte doubling time (LDT), as shown in the multiple logistic regression, with an association between shorter (<0.925, mean) telomeres and higher (>55.5, mean) *hTERT* **C.** This is illustrated by comparing telomere lengths in patients with mutated and unmutated *IGHV* **D.** and *hTERT* expression to patients with long and short LDTs **E.** Bars correspond to the standard error of the mean.



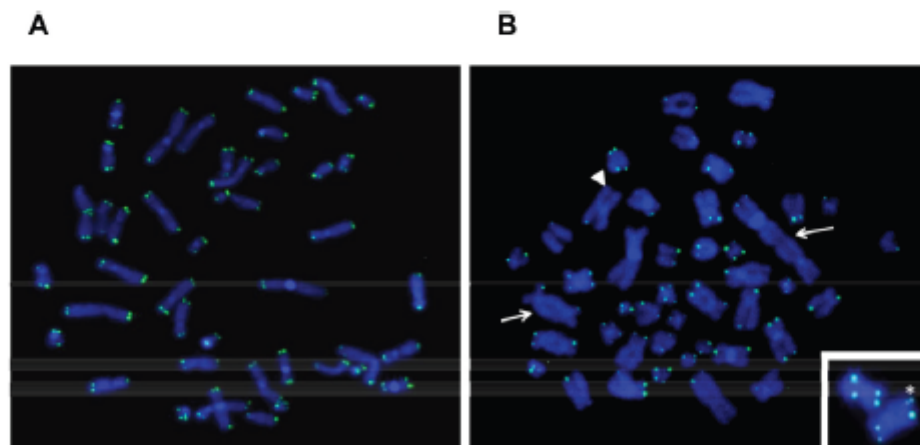
levels and relative telomere length (Figure 3A). This approach identified three distinct groups. Cluster #1 ( $n=34$ ) was characterized by shortened telomeres, high *hTERT* and low shelterin gene expression. This cluster included 22 of the 24 patients with *TP53* disruption. Interestingly, we also observed a smaller group of patients with wild-type *TP53* and without *hTERT* overexpression and telomere shortening, but with downregulated shelterin genes (cluster #2,  $n=24$ ). Finally, there was a large group (cluster #3,  $n=55$ ) that had heterogeneous telomere lengths, low *hTERT* and relatively high levels of shelterin gene expression. Overall, *TP53* disruption was clearly associated with low shelterin gene expression, but a similar downregulation was also found in a proportion of wild-type *TP53* cases.

The expression of shelterin genes has not yet been correlated with established CLL prognostic factors; we performed this analysis in our series (Figure 3B). Significant downregulation of several shelterin genes were found in patients in Binet C stage (*TRF1*, *TRF2* and *TIN2*). A short LDT was associated with significant downregulation of all the shelterin genes except for *TRF2* and *POT1*. Expression of *TRF1*, *TPP1* and *TIN2* was lower in cases with unmutated *IGHV*. *TP53*-disruption was also significantly associated with lower expression of *TRF1*, *POT1*, *TPP1* and *TIN2*. This was independent of the type of *TP53* alteration (data not shown).

Regarding the correlation with cytogenetic subgroups, the changes to shelterin gene expression were not consistent, except from *TIN2*, which showed highly significant gradual reduction from a normal/del(13q) pattern to a *TP53*-disruption subgroup, in parallel with telomere shortening (Supplementary Figure S2).

## Prognostic value of telomere length and shelterin gene expression in treated patients with wild-type *TP53*

As expected, survival analyses in treated patients confirmed the significantly shorter progression-free survival (PFS) of patients with disrupted *TP53* compared to patients with wild-type *TP53* ( $P=0.0033$ ; Figure 4A). In addition, telomere length, *hTERT* and three shelterin-gene (*POT1*, *TPP1*, *TIN2*) transcript levels were also significant predictors of PFS (data not shown), but these parameters were strongly correlated with *TP53* status. We therefore investigated whether these telomere characteristics had an additional prognostic value beyond classifying patients into *TP53*-disrupted or *TP53*-wild-type subgroups. We performed survival analysis among patients with wild-type *TP53* and found that short telomeres ( $P=0.00047$ ; Figure 4B), low *POT1* ( $P=0.0039$ ; Figure 4C), low *TPP1* ( $P=0.0047$ ; Figure 4D) and low *TIN2* ( $P=0.0051$ ; Figure 4E) identified subsets of patients with significantly shorter survival times. These parameters were also associated with a higher risk of progression in the univariate Cox's analysis (Figure 4F). A multivariate Cox's analysis of known poor prognostic CLL features (*IGHV* status, LDT, CD38, Binet stage) showed that unmutated *IGHV* genes significantly and independently predicted PFS in this patient subgroup with the hazard ratio of 2.2 (95% CI: 1.0 to 4.3,  $P=0.022$ ). When adjusted for *IGHV* mutation status and telomere length, shelterin transcript levels remained significant predictors of PFS in patients with wild-type *TP53* (Figure 4F).



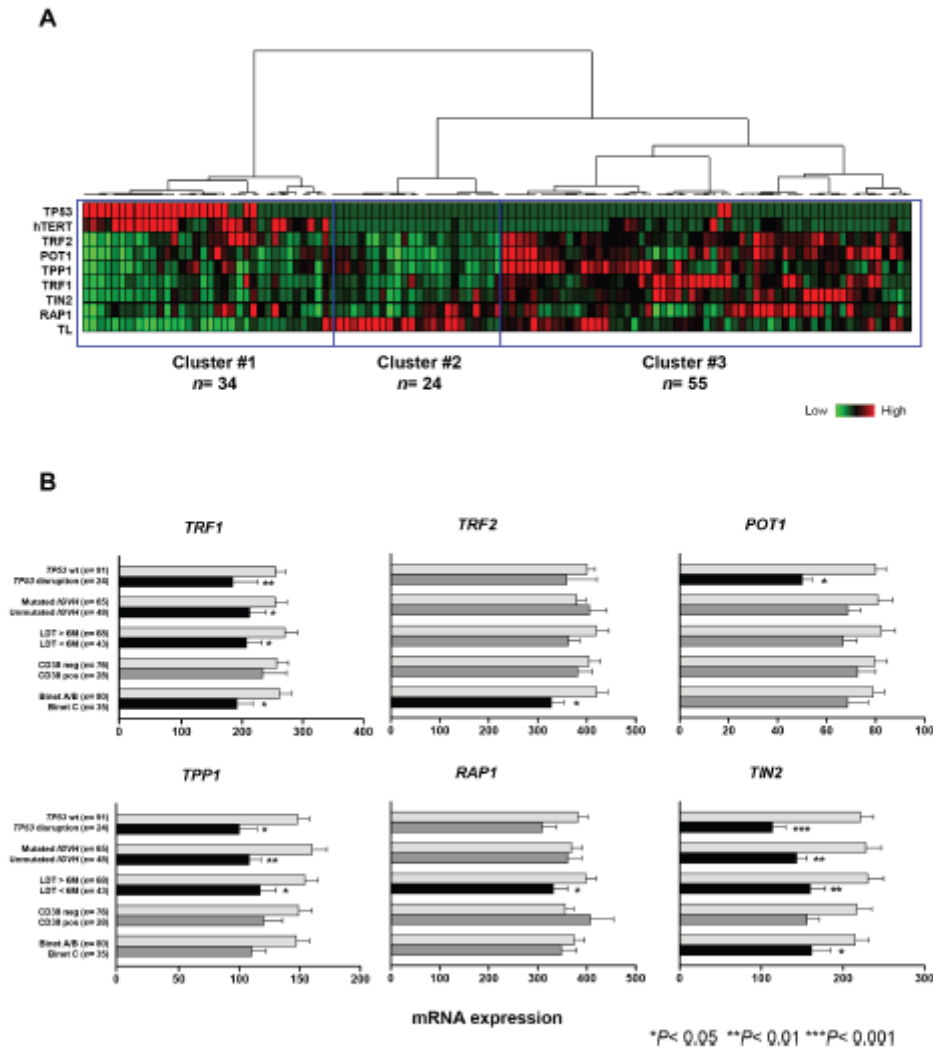
**Figure 2: Pantelomeric FISH of representative metaphases from wild-type (wt) *TP53* A. and disrupted *TP53* B.** *TP53* disruption was associated with telomeric fusions that led to dicentric chromosomes (arrows) and frequent loss of telomeric signals (one of them is shown with an arrowhead). A duplication of a telomeric signal is shown with an asterisk.

## DISCUSSION

We have shown that CLL patients with disrupted *TP53* have severe telomere shortening associated with *hTERT* overexpression and downregulation of shelterin genes. This telomere profile was found in patients with *TP53* mutations in the absence of del(17p) and in patients with concomitant *TP53* mutations and del(17p) or del(17p)

alone: this suggests that any alteration to *TP53* leads to important telomere dysfunction in CLL.

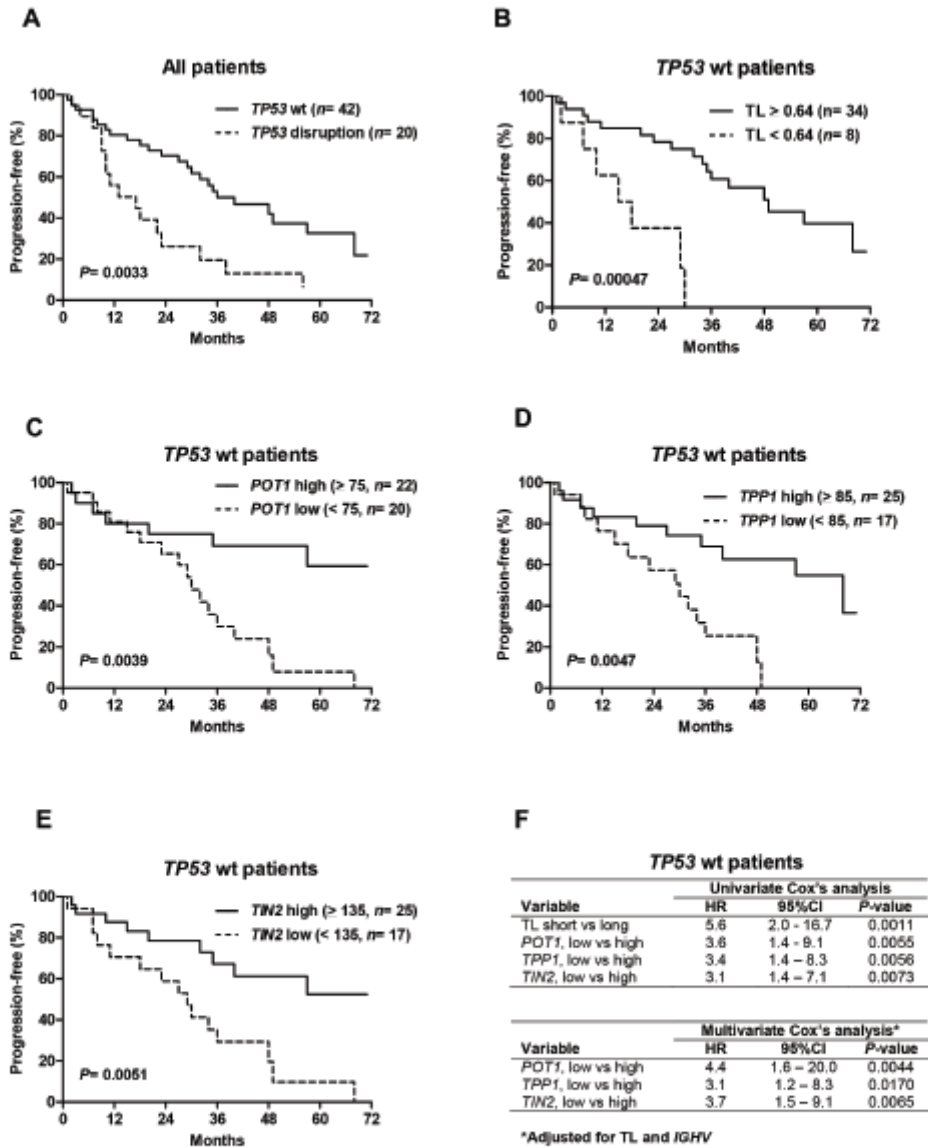
Both *TP53* alteration and short telomeres have been previously correlated with progressive CLL, and with unfavorable biological factors such as unmutated *IGHV* and a high proliferation rate [14, 19, 20]. We have demonstrated that the association of *TP53* disruption with short telomeres and high *hTERT* was an independent



**Figure 3: Low shelterin-gene expression in CLL patients with disrupted *TP53* and in patients with adverse prognostic factors.** A. Hierarchical clustering of 115 CLL patients according to *TP53* status, telomere length and expression levels of *hTERT* and shelterin genes (*TRF1*, *TRF2*, *POT1*, *TPP1*, *RAP1*, *TIN2*). Patients with disrupted *TP53* (red) are clustered into a distinct group with short telomeres, high *hTERT* and low levels of shelterin genes. B. Mean expression levels of shelterin genes in different CLL subgroups according to *TP53* status, *IGHV* mutation profile, CD38 expression, lymphocyte doubling time (LDT) and Binet stage (H test). Bars correspond to the standard error of the mean.

factor in this adverse biological profile, and that *TP53* was the strongest determinant of telomere status and *hTERT* expression. We have also shown that patients with disrupted *TP53* had the shortest telomeres among the cytogenetic subgroups; this association has been

previously described for a 17p deletion [16], but has not yet been reported for mutated *TP53*. Patients with disrupted *TP53* also had the highest levels of *hTERT* among the subgroups.



**Figure 4: Telomere length and shelterin expression predict progression-free survival (PFS) in patients with wild-type (wt) *TP53*.** A. Kaplan-Meier estimates of PFS in the whole patient population as a function of *TP53* status and the wild-type *TP53* cohort according to telomere length B. *POT1* C. *TPP1* D. and *TIN2* E. levels. The optimal cut-off values were determined using recursive partitioning. CLL patients with short telomeres and low *POT1*, *TPP1* and *TIN2* levels had significantly shorter PFSs. F. Corresponding hazard ratios (HR) and 95%-confidence intervals (95%CI) are presented. When adjusted for telomere length and *JGHV* mutation profile, low *POT1*, *TPP1* and *TIN2* expression remained significant predictors for a shorter PFS.

Using pantelomeric FISH, we found that *TP53* disruption was associated with increased numbers of telomeric deletions, and we also observed chromosomal end-to-end fusions in patients with disrupted *TP53* and complex karyotypes. These findings illustrate that impaired p53 pathways can allow continuous cell division despite telomere shortening, and the accumulation of cells with critically eroded telomeres, thus leading to chromosomal fusions and genomic instability. In addition, wild-type p53 is a powerful inhibitor of *hTERT*, and loss of this function can also enhance expression of *hTERT*, which is essential for survival and replication of tumor cells [21]. Telomere attrition and *TP53* disruption may thus represent two important cooperating events of tumor progression in CLL, as has been previously shown in epithelial tumor models [4, 22].

Telomere shortening in CLL patients with disrupted *TP53* was also associated with low expression of *TRF1*, *POT1*, *TPP1* and *TIN2* shelterin genes. Using clustering analysis, we found that almost all cases of disrupted *TP53* were within a distinct subset that had short telomeres and downregulated shelterin genes. The abnormal expression of shelterin genes, in the context of p53-deficiency and short telomeres, may have contributed to the higher levels of genomic instability in this CLL subset.

Clustering analyses indicated that some patients with wild-type *TP53* also showed shelterin downregulation, but this low expression was independent of telomere shortening. Moreover, we also found significantly lower shelterin gene expression in cases with adverse clinical and biological parameters, such as advanced Binet stage, unmutated *IGHV* and short LDTs. Interestingly, shelterin status was not consistently correlated with the cytogenetic subgroups, with the exception of *TIN2*. These results highlight the heterogeneity of shelterin status in CLL and suggest the complexity that underlies these mechanisms.

The factors regulating shelterin genes remain unknown. Nevertheless, some events that could affect their function and expression in cancer, such as mutation and DNA methylation, have been described. Somatic mutations of *POT1* have been reported in CLL, and this represents the first description of a shelterin complex-gene mutation in human cancer [23]. CLL cells with *POT1* mutations display multiple telomeric and chromosomal aberrations, suggesting that these mutations and the subsequent alterations to shelterin can favor the acquisition of the malignant features seen in CLL cells.

In breast-cancer cell lines, a significant reduction in *POT1* mRNA level was associated with *POT1* promoter methylation [24]. Interestingly, in the clonal evolution of CLL, a *POT1* mutation seems to precede the appearance of alterations to *TP53* [26]. In addition, downregulation of shelterin genes can occur prior to telomere shortening in early CLL and so contribute to the reduction in telomere protection [13]. Sequential analyses of patients with low levels of gene expression

of shelterin would be of interest to investigate the evolution of telomere status and the timing of *TP53* appearance and other alterations, as well as the onset of chromosomal instability.

Finally, we found a significant adverse impact of short telomeres and low *POT1*, *TPP1* and *TIN2* on PFS of the CLL patients in our cohort, who had been treated mainly with standard genotoxic agents. This impact was found in the whole population and also in patients with wild-type *TP53*, emphasizing the additional prognostic value of these parameters. In particular, the significant impact of shelterin gene expression on survival in CLL has not been described previously. Of note, these shelterin components regulate telomerase recruitment via *POT1*, and *TIN2* recruits the *TPP1*-*POT1* complex, constituting a bridge between the different shelterin components [2]. Unlike *POT1*, mutated *TIN2* and *TPP1* have not been observed in tumors, but germline mutations of these genes have been reported in human genetic diseases leading to bone-marrow failure [26, 27]. Taken together, these observations suggest that *POT1*, *TIN2* and *TPP1* could be key-components in the shelterin complex implicated in telomere dysfunction and adverse outcomes in CLL.

Further studies of patients with downregulated shelterin genes are warranted for mechanistic understanding of the role of telomere abnormalities in CLL. In addition to *TP53* disruption, these investigations have to take into account other recurrent CLL mutations with an emphasis on telomeres, such as altered *ATM* and *SF3B1* genes and, as stated above, the *POT1* gene. Mutations in the *ATM* and *SF3B1* genes are known to be associated with shorter telomeres in CLL cells [28, 16]. *ATM*, a major DNA damage sensor, appears to play a complex role in telomere protection and length regulation through interactions with the shelterin complex [29]. A possible telomeric role of the *SF3B1* spliceosome protein was recently revealed by the transcriptomic study of *SF3B1*-mutated CLL cells, showing an overexpression of the *TERC* gene encoding telomerase RNA template, which could lead to aberrant telomerase regulation [30].

In conclusion, we have demonstrated that CLL patients with disrupted *TP53* show severe dysfunction of telomeres, involving telomeric DNA, *hTERT* and shelterin. This telomeric dysfunction can contribute to enhanced genomic instability and, consequently, to treatment resistance in CLL. Further studies are required to evaluate the impact of *TP53* in the context of other molecular alterations, in particular 'multiple hit' events described recently in resistant CLL [31]. Finally, this telomeric profile could be tested as a predictive marker in CLL patients treated with new non-genotoxic therapies.



## MATERIALS AND METHODS

### Patients and samples

After informed consent according to the Declaration of Helsinki, peripheral blood samples were collected from 115 CLL patients at the Clermont-Ferrand University Hospital (France) between 2007 and 2012. The patients' characteristics at the time of sampling are shown in Supplementary Table S1. Diagnosis and treatment requirements were based on the guidelines of the International Workshop on Chronic Lymphocytic Leukemia [32]. A total of 62 patients required treatment, consisting of immunochemotherapy (fludarabine, cyclophosphamide, rituximab  $n=44$ , other regimens  $n=6$ ), alemtuzumab ( $n=9$ ) or alkylating agent-based regimens ( $n=3$ ). Median follow-up time from the start of treatment was 41 months. Peripheral blood samples were obtained before treatment. Peripheral blood lymphocytes were isolated by Lymphoprep™. The proportion of tumor cells obtained was  $>85\%$ .

### Cytogenetic analyses

Analyses were performed at the time of sampling. Karyotype was performed after immuno-stimulation of cell cultures with CpG-oligonucleotide and interleukin 2. Chromosome preparation and staining were done according to standard protocols. Twenty metaphases were analyzed. Complex karyotype was defined as  $\geq 3$  abnormalities. Locus-specific fluorescence *in situ* hybridization (FISH) was performed on interphase nuclei and/or metaphases, following standard procedures and using specific probes (co-hybridized with a control probe) to detect deletions of 13q14.3, 11q22.3 and 17p13 (LSI D13S319, LSI ATM and LSI TP53). Trisomy 12 was studied using the centromeric probe D12Z3. All probes were from Vysis (Abbott Molecular, Rungis, France) and used in accordance with the manufacturer's recommended procedures. At least 200 interphase nuclei were examined. The cut-off levels for the presence of aberrations were 7% of positive cells for deletions and 5% for trisomy 12. Patients were classified according to previously reported hierarchical patterns [31]. Pantelomic FISH was performed with a Telomere PNA FISH Kit (DAKO, Glostrup, Denmark), used in accordance with the manufacturer's recommended procedures. Results were recorded using an Axioplan2 imaging fluorescence microscope (ZEISS, Göttingen, Germany) fitted with appropriate filters, a CCD camera, and the digital-imaging software, Isis, v3.8.8 (Metasystems, Althussheim, Germany).

### TP53 mutational analyses

Genomic DNA was extracted using a Nucleospin Blood L (Macherey-Nagel) kit. Mutation analyses of *TP53* exons 2–11 were done by DNA direct sequencing using

a 3130XL sequencer (Applied Biosystems, Villebon-sur-Yvette, France) and analyzed with Seqscape software. The genomic reference sequence used was IARC NC\_000017 v9. The primers were designed to cover all coding exons and intron–exon boundaries. The characteristics and distributions of mutations were assessed using the IARC *TP53* Mutation Database and the Ensembl Database. *TP53* gene mutations found in our CLL cohort are detailed in Supplementary Table S2. Most were deleterious missense mutations located in the DNA-binding domain and have been previously observed in other cancer types.

### Assessment of telomere length using quantitative PCR

Average telomere length was evaluated with quantitative real-time DNA-PCR in a LightCycler 480 System (Roche Diagnostics, Meylan, France) using SYBR Green I technology (SYBR Green Kit, Roche Diagnostics), as described elsewhere [15]. This method measures the relative telomere length as the T/S ratio between the template amounts of telomere repeat (T) and a reference single-copy gene (S) [33]. We used *GAPDH* as a reference gene. Using this approach, we have previously shown that the T/S ratio in tumor cells was proportional to average telomere length as assessed by classical telomere restriction-fragment analyses [15].

### Quantitative RT-PCR for *hTERT* and shelterin complex gene expression

Total RNA was extracted using TRIzol (Fisher Scientific, Illkirch, France) according to standard procedures. Total RNA was converted to cDNA by reverse transcription using Superscript II reverse transcriptase (Invitrogen, Cergy-pontoise, France), according to the manufacturer's instructions. The expression of *hTERT* and the shelterin complex genes *TRF1*, *TRF2*, *POT1*, *TPP1*, *RAP1* and *TIN2* were quantified using real-time RT-PCR in a LightCycler 480 System (Roche Diagnostics, Meylan, France), as described previously [11, 15]. The normalized copy numbers were expressed as the ratio between the numbers of transcript copies of the target and control (*GUSB*) genes, multiplied by 100.

### Statistical analyses

Comparisons of quantitative variables were performed using standard tests. As is usual in exploratory studies, we chose not to adjust probabilities by the Bonferroni method. While decreasing the rate of false positives, this method also increases in a similar proportion the rate of false negatives. Unsupervised hierarchical clustering analysis was performed to identify subclasses with distinct expression of shelterin genes and telomeric-length profiles. Progression-free survival (PFS)

was calculated from the date of starting treatment until a relapse, progression or death from any cause. Survival curves were established using the Kaplan–Meier method and compared with the log-rank test. The univariate and multivariate analyses was performed according Cox's regression model. The data were analyzed using SEM software [34].

## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

## REFERENCES

- de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev.* 2005; 19:2100-2110.
- Martinez P, Blasco MA. Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins. *Nat Rev Cancer.* 2011; 11:161-176.
- Chin L, Artandi SE, Shen Q, Tam A, Lee SL, Gottlieb GJ, Greider CW, DePinho RA. p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. *Cell.* 1999; 97:527-538.
- Artandi SE, Chang S, Lee SL, Alson S, Gottlieb GJ, Chin L, DePinho RA. Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. *Nature.* 2000; 406:641-645.
- Pantic M, Zimmermann S, El Daly H, Opitz OG, Popp S, Boukamp P, Martens UM. Telomere dysfunction and loss of p53 cooperate in defective mitotic segregation of chromosomes in cancer cells. *Oncogene.* 2006; 25:4413-4420.
- Damle RN, Batiwalla FM, Ghitto F, Valetto A, Albesiano E, Sison C, Allen SL, Kolitz J, Vinciguerra VP, Kudalkar P, Wasil T, Rai KR, Ferrarini M et al. Telomere length and telomerase activity delineate distinctive replicative features of the B-CLL subgroups defined by immunoglobulin V gene mutations. *Blood.* 2004; 103:375-382.
- Tchirkov A, Chateaux C, Magnac C, Vasconcelos Y, Davi F, Michel A, Kwiatkowski F, Tournilhac O, Dighiero G, Travade P. hTERT expression and prognosis in B-chronic lymphocytic leukemia. *Ann Oncol.* 2004; 15:1476-1480.
- Terrin L, Trentin L, Degan M, Corradini I, Bertorelle R, Carli P, Maschio N, Bo MD, Noventa F, Gattei V, Semenzato G, De Rossi A. Telomerase expression in B-cell chronic lymphocytic leukemia predicts survival and delineates subgroups of patients with the same igVH mutation status and different outcome. *Leukemia.* 2007; 21:965-972.
- Lin TT, Norris K, Heppel NH, Pratt G, Allan JM, Allsup DJ, Bailey J, Cawthell L, Hills R, Grimstead JW, Jones RE, Britt-Compton B, Fegan C et al. Telomere dysfunction accurately predicts clinical outcome in chronic lymphocytic leukaemia, even in patients with early stage disease. *Br J Haematol.* 2014; 167:214-223.
- Strefford JC, Kadalayil L, Forster J, Rose-Zerilli MJ, Parker A, Lin TT, Heppel N, Norris K, Gardiner A, Davies Z, Gonzalez de Castro D, Else M, Steele AJ et al. Telomere length predicts progression and overall survival in chronic lymphocytic leukemia: data from the UK LRF CLL4 trial. *Leukemia.* 2015; 29:2411-2414.
- Poncet D, Belleville A, T'kint de Roodenbeke C, Roborel de Climens A, Ben Simon E, Merle-Beral H, Callet-Bauchu E, Salles G, Sabatier L, Delic J, Gilson E. Changes in the expression of telomere maintenance genes suggest global telomere dysfunction in B-chronic lymphocytic leukemia. *Blood.* 2008; 111:2388-2391.
- Hoxha M, Fabris S, Agnelli L, Bollati V, Cutrona G, Matis S, Recchia AG, Gentile M, Cortezzi A, Morabito F, Bertazzi PA, Ferrarini M, Neri A. Relevance of telomere/telomerase system impairment in early stage chronic lymphocytic leukemia. *Genes Chromosomes Cancer.* 2014; 53:612-621.
- Augereau A, T'kint de Roodenbeke C, Simonet T, Bauwens S, Horard B, Callanan M, Leroux D, Jallades L, Salles G, Gilson E, Poncet D. Telomeric damage in early stage of chronic lymphocytic leukemia correlates with shelterin dysregulation. *Blood.* 2011; 118:1316-1322.
- Roos G, Kröber A, Grabowski P, Kienle D, Bühler A, Döhner H, Rosenquist R, Stilgenbauer S. Short telomeres are associated with genetic complexity, high-risk genomic aberrations, and short survival in chronic lymphocytic leukemia. *Blood.* 2008; 111:2246-2252.
- Véronèse L, Tournilhac O, Callanan M, Prie N, Kwiatkowski F, Combes P, Chauvet M, Davi F, Gouas L, Verrelle P, Guéze R, Vago P, Bay JO et al. Telomeres and chromosomal instability in chronic lymphocytic leukemia. *Leukemia.* 2013; 27:490-493.
- Mansouri L, Grabowski P, Degerman S, Svenson U, Gunnarsson R, Cahill N, Smedby KE, Geisler C, Juliusson G, Roos G, Rosenquist R. Short telomere length is associated with NOTCH1/SF3B1/TP53 aberrations and poor outcome in newly diagnosed chronic lymphocytic leukemia patients. *Am J Hematol.* 2013; 88:647-651.
- Zenz T, Eichhorst B, Busch R, Denzel T, Häbe S, Winkler D, Bühler A, Edelmann J, Bergmann M, Hopfinger G, Hensel M, Hallek M, Döhner H et al. TP53 mutation and survival in chronic lymphocytic leukemia. *J Clin Oncol.* 2010; 28:4473-4479.
- Gonzalez D, Martinez P, Wade R, Hockley S, Oscier D, Matutes E, Dearden CE, Richards SM, Catovsky D, Morgan GJ. Mutational status of the TP53 gene as a predictor of response and survival in patients with chronic lymphocytic leukemia: results from the LRF CLL4 trial. *J Clin Oncol.* 2011; 29:2223-2229.
- Rampazzo E, Bonaldi L, Trentin L, Visco C, Keppel S, Giunco S, Prezzato F, Facco M, Novella E, Giaretta I,

- Del Bianco P, Semenzato G, De Rossi A. Telomere length and telomerase levels delineate subgroups of B-cell chronic lymphocytic leukemia with different biological characteristics and clinical outcomes. *Haematologica*. 2012; 97:56-63.
20. Rossi D, Cerri M, Deambrogi C, Sozzi E, Cresta S, Rasi S, De Paoli L, Spina V, Gattei V, Capello D, Forconi F, Lauria F, Gaidano G. The prognostic value of TP53 mutations in chronic lymphocytic leukemia is independent of Del17p13: implications for overall survival and chemorefractoriness. *Clin Cancer Res*. 2009; 15:995-1004.
21. Xu D, Wang Q, Gruber A, Björkholm M, Chen Z, Zaid A, Selivanova G, Peterson C, Wiman KG, Pisa P. Downregulation of telomerase reverse transcriptase mRNA expression by wild type p53 in human tumor cells. *Oncogene*. 2000; 19:5123-5133.
22. Artandi SE, DePinho RA. Telomeres and telomerase in cancer. *Carcinogenesis*. 2010; 31:9-18.
23. Ramsay AJ, Quesada V, Foronda M, Conde L, Martínez-Trillos A, Villamor N, Rodríguez D, Kwarciak A, Garabaya C, Gallardo M, López-Guerra M, López-Guillermo A, Puente XS, et al. POT1 mutations cause telomere dysfunction in chronic lymphocytic leukemia. *Nat Genet*. 2013; 45:526-530.
24. Motevalli A, Yasaei H, Virmouni SA, Slijepcevic P, Roberts T. The effect of chemotherapeutic agents on telomere length maintenance in breast cancer cell lines. *Breast Cancer Res Treat*. 2014; 145:581-591.
25. Landau DA, Tausch E, Taylor-Weiner AN, Stewart C, Reiter JG, Bahlo J, Kluth S, Bozic I, Lawrence M, Böttcher S, Carter SL, Cibulskis K, Mertens D et al. Mutations driving CLL and their evolution in progression and relapse. *Nature*. 2015; 526:525-530.
26. Dokal I. Dyskeratosis congenita. *Hematol Educ Program Am Soc Hematol Educ Program*. 2011; 2011:480-486.
27. Guo Y, Kartawinata M, Li J, Pickett HA, Teo J, Kilo T, Barbaro PM, Keating B, Chen Y, Tian L, Al-Odaib A, Reddel RR, Christodoulou J et al. Inherited bone marrow failure associated with germline mutation of ACD, the gene encoding telomere protein TPP1. *Blood*. 2014; 124:2767-2774.
28. Britt-Compton B, Lin TT, Ahmed G, Weston V, Jones RE, Fegan C, Oscier DG, Stankovic T, Pepper C, Baird DM. Extreme telomere erosion in ATM-mutated and 11q-deleted CLL patients is independent of disease stage. *Leukemia*. 2012; 26:826-830.
29. Diotti R, Loayza D. Shelterin complex and associated factors at human telomeres. *Nucleus*. 2011; 2:119-35.
30. Fan J, Wang L, Brooks AN, Wan Y, Neuberg DS, Rassenti LZ, Ghia EM, Kipps TJ, Brown JR, Li S, Livak KJ, Meyerson MM, Kharchenko PV, Wu CJ. Comprehensive bulk and single cell transcriptomic characterization of SF3B1 mutation reveals its pleiotropic effects in chronic lymphocytic leukemia. *Blood*. 2015; 126:2906-2906.
31. Guizé R, Robbe P, Clifford R, Guibert S de, Pereira B, Timbs A, Dillhuydy M-S, Cabes M, Ysebaert L, Burns A, Nguyen-Khac F, Davi F, Véronèse L et al. Presence of multiple recurrent mutations confers poor trial outcome of relapsed/refractory CLL. *Blood*. 2015; 126:2110-2117.
32. Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Döhner H, Hillmen P, Keating MJ, Montserrat E, Rai KR, Kipps TJ. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood*. 2008; 111:5446-5456.
33. Cawthon RM. Telomere measurement by quantitative PCR. *Nucleic Acids Res*. 2002; 30:e47.
34. Kwiatkowski F, Girard M, Hacene K, Berlie J. [Sem: a suitable statistical software adapted for research in oncology]. *Bull Cancer*. 2000; 87:715-721.



## ERCC1 and telomere status in breast tumours treated with neoadjuvant chemotherapy and their association with patient prognosis

Mathilde Gay-Bellile,<sup>1,2†</sup> Pierre Romero,<sup>1,3†</sup> Anne Cayre,<sup>1,3</sup> Lauren Véronèse,<sup>1,2</sup> Maud Privat,<sup>1,4</sup> Shalini Singh,<sup>5</sup> Patricia Combes,<sup>1,2</sup> Fabrice Kwiatkowski,<sup>1,6</sup> Catherine Abrial,<sup>1,6</sup> Yves-Jean Bignon,<sup>1,4,7</sup> Philippe Vago,<sup>1,2</sup> Frédérique Penault-Llorca<sup>1,3‡</sup> and Andreï Tchirkov<sup>1,3\*</sup>

<sup>1</sup>ERTICA EA4677 Research Team, University of Auvergne, Faculty of Medicine, 28 place Henri Dunant, F-63001 Clermont-Ferrand, France

<sup>2</sup>Department of Medical Cytogenetics, CHU Estaing, 1 place Lucie et Raymond Aubrac, F-63003 Clermont-Ferrand, France

<sup>3</sup>Department of Pathology, Jean Perrin Comprehensive Cancer Center, 58 rue Montalembert, F-63011 Clermont-Ferrand, France

<sup>4</sup>Department of Oncogenetics, Jean Perrin Comprehensive Cancer Center, 58 rue Montalembert, F-63011 Clermont-Ferrand, France

<sup>5</sup>Medical and Scientific Affairs, Office of Pathology, Ventana Medical Systems, Inc, Roche Group, 1910 E. Innovation Park Drive, Tucson, AZ, 85755, USA

<sup>6</sup>Clinical and Translational Research Division, Jean Perrin Comprehensive Cancer Center, 58 rue Montalembert, F-63011 Clermont-Ferrand, France

<sup>7</sup>Biological Resource Center BB-0033-00075, Jean Perrin Comprehensive Cancer Center, 58 rue Montalembert, F-63011 Clermont-Ferrand, France

\*Correspondence to: Prof. Andreï Tchirkov, Cytogénétique Médicale, CHU Estaing, ERTICA EA4677 Université d'Auvergne, 1 place Lucie et Raymond Aubrac, 63003 Clermont-Ferrand Cedex I, France. e-mail: atchirkov@chu-clermontferrand.fr

### Abstract

Dysfunctional telomeres and DNA damage repair (DDR) play important roles in cancer progression. Studies have reported correlations between these factors and tumour aggressiveness and clinical outcome in breast cancer. We studied the characteristics of telomeres and expression of ERCC1, a protein involved in a number of DNA repair pathways and in telomere homeostasis, to assess their prognostic value, alone or in combination, in 90 residual breast tumours after treatment with neoadjuvant chemotherapy (NCT). ERCC1 status was investigated at different molecular levels (protein and gene expression and gene copy-number variations) by immunohistochemistry, qRT-PCR and quantitative multiplex fluorescent-PCR (QMF-PCR). A comprehensive analysis of telomere characteristics was performed using qPCR for telomere length and qRT-PCR for telomerase (*hTERT*), tankyrase 1 (*TNKS*) and shelterin complex (*TRF1*, *TRF2*, *POT1*, *TPP1*, *RAP1* and *TIN2*) gene expression. Short telomeres, high *hTERT* and *TNKS* expression and low ERCC1 protein expression were independently associated with worse survival outcome. Interestingly, ERCC1 gains and losses correlated with worse disease-free ( $p = 0.026$ ) and overall ( $p = 0.043$ ) survival as compared to survival of patients with normal gene copy-numbers. Unsupervised hierarchical clustering of all ERCC1 and telomere parameters identified four subgroups with distinct prognosis. In particular, a cluster combining low ERCC1, ERCC1 gene alterations, dysfunctional telomeres and high *hTERT* and a cluster with high *TNKS* and shelterin expression correlated with poor disease-free ( $HR = 5.41$ ,  $p = 0.0044$ ) and overall survival ( $HR = 6.01$ ,  $p = 0.0023$ ) irrespective of tumour stage and grade. This comprehensive study demonstrates that telomere dysfunction and DDR can contribute synergistically to tumour progression and chemoresistance. These parameters are predictors of clinical outcome in breast cancer patients treated with NCT and could be useful clinically as prognostic biomarkers to tailor adjuvant chemotherapy post-NCT.

**Keywords:** Telomeres; telomerase; tankyrase; shelterin; breast cancer; neoadjuvant chemotherapy; ERCC1; prognosis; residual disease

Received 24 February 2016; Accepted 28 May 2016

†Co-first authors.

‡Co-senior authors.

Conflict of interest statement: The authors declare no conflict of interest.

## Introduction

Neoadjuvant chemotherapy (NCT) has been used in breast cancer to facilitate conserving surgery and allows early evaluation of response [1]. Patients achieving pathological complete response (pCR) have better disease-free survival [2]. Some characteristics of post-NCT tumours, such as tumour size or grade, are closely associated with the outcome of patients without pCR [3,4]. These criteria, however, may be misleading. Hence, studying biological prognostic markers for patients without pCR is crucial. Here, we chose to combine DNA damage repair (DDR) and telomeric parameters to assess prognosis in post-NCT breast cancer patients.

The ERCC1/XPF (excision repair cross complementation group 1/xeroderma pigmentosum factor) heterodimer is a structure-specific endonuclease mainly known for its involvement in nucleotide excision repair (NER) and a number of other DDR pathways [5]. ERCC1 regulates DNA and protein interactions and XPF provides the endonuclease activity [6]. ERCC1 has been extensively studied as a putative biomarker of response to treatments [7]. Immunohistochemical studies of ERCC1 status conducted in breast cancer patients showed correlations between high expression and good prognosis factors [8]. The exact mechanisms underlying this phenomenon remain unclear.

Telomeres are nucleoprotein structures that protect chromosomal ends from exonucleolytic degradation and inappropriate activation of DDR pathways. The shelterin complex comprises six core proteins (TRF1, TRF2, RAP1, TIN2, TPP1 and POT1) and has crucial functions in telomere protection and length regulation. Human telomerase reverse transcriptase (*hTERT*), a component of telomerase, increases the length of the telomere by adding telomeric repeats to the end of chromosomes [9]. Tankyrase 1 (TNKS) is a poly-ADP-ribose polymerase that assembles chains of poly-ADP-ribose on the target substrate, particularly TRF1. TNKS is a positive regulator of telomere length by removing TRF1 from telomeres and allowing access to telomerase [10]. Telomere shortening due to incomplete DNA synthesis occurs at initial steps of oncogenesis and leads to genomic instability. In contrast, *hTERT* increases during tumour progression, preventing cancer cells with genomic instability from entering senescence or apoptosis [11]. Telomere shortening and telomerase activation have been studied in breast cancer and were correlated with poor prognosis. However, their clinical significance in post-NCT residual tumours is unknown. In addition, the significance of changes in shelterin and tankyrase gene expression is under-investigated.

ERCC1-XPF is also involved in telomere maintenance. Mice null for ERCC1 have a single telomere phenotype characterized by telomeric DNA-containing double minute chromosomes [12]. In cell lines, the overexpression of XPF induces telomere shortening, suggesting that XPF could function as a negative regulator of telomere length via both TRF2-dependent and independent mechanisms [12,13]. In mouse keratinocytes, XPF mediates telomere degradation induced by TRF2 overexpression [14]. Finally, a partial loss of telomeres in TRF2 deficient cells was dependent on ERCC1/XPF expression [12]. Interactions between ERCC1 and telomeres have not been studied in the context of tumour cells.

In this study, ERCC1 status and telomeric parameters were investigated at different molecular levels and correlated with clinicopathological data in a cohort of invasive breast cancer patients treated with NCT without pCR. We suggest that post-NCT tumours with low ERCC1 expression and dysfunctional telomeres are of poor prognosis, whereas high ERCC1 and functional telomeres are good prognostic markers.

## Patients and methods

### Patients and samples

We retrieved archived tumour samples from 90 women diagnosed with invasive breast cancer and treated with NCT protocols [15] between 1996 and 2010 at the Jean Perrin Cancer Center, Clermont-Ferrand, France. Clinicopathological data are summarized in supplementary material, Table S1. After NCT, patients underwent appropriate surgery, and radiotherapy. Patients with important residual disease received adjuvant chemotherapy. Trastuzumab was used for the adjuvant treatment and the treatment of recurrent metastatic disease in HER2+ cases. Menopausal patients with hormone receptor-positive tumours received tamoxifen for 5 years. During follow-up (median of 9 years), 34 patients (38.9%) relapsed locally and/or presented contralateral or distant events, and 26 (28.9%) died from breast cancer.

Protein studies were performed on paraffin-embedded tissue sections of 90 post-NCT tumours and 59 matched pre-NCT biopsies. Cryopreserved post-NCT samples were available for molecular investigations for 45 out of 90 patients (Biological Resources Center BB-0033-00075). The institutional review board approved the study.

### ERCC1 immunohistochemistry (IHC)

A control haematoxylin and eosin-stained slide was analyzed for each tissue block. ERCC1 IHC staining



was operated on a Benchmark ULTRA IHC/ISH staining module (Ventana, USA) as described in Supplementary methods. Semi-quantitative assessment of ERCC1 expression was performed by two independent readers (FPL, PR) according to the following algorithm: staining intensity on tumour cells was graded on a scale from 0 to 3, with score 3 representing high intensity and endothelial cells in tonsil control tissue used as a reference and assigned an intensity of 2. The percentage of positive tumour cells calculated for each specimen was multiplied by the staining intensity to obtain an H-score ranging from 0 to 300. Only nuclear staining was scored, but the readers recorded unexpected patterns (eg cytoplasmic). Discordant cases were reviewed. Entire sections were assessed in both pre- and post-NCT samples.

#### DNA and RNA extraction

DNA and RNA were extracted from snap-frozen post-NCT tumour samples with the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions.

#### ERCC1 gene copy-number quantification

The *ERCC1* gene copy number was quantified by quantitative multiplex fluorescent polymerase chain reaction (QMF-PCR). Primers were designed for three fragments of the *ERCC1* gene (5'UTR, Exon 6 and 3'UTR). Seven other genes (*PVRL1*, *BODIL*, *RET*, *ZNF638*, *AGBL2*, *CFTR* and *POR*) were amplified as controls. QMF-PCR was performed according to published protocols [16,17]. Primers and technical details are given in Supplementary methods. Fluorescence intensities of PCR products were correlated with the copy number of the relevant exons. Fourteen control DNAs were included in each experiment: 11 normal DNAs and 3 DNAs with a known *ERCC1* copy number (deletion, normal and gain). A dosage quotient was calculated relative to all the other amplified exons in patients and controls. The mean ratios  $\pm 3$  standard deviations of controls were used as cut-off points to detect copy number variations (CNVs): ratios  $> 1.2$  were considered as gains and ratios  $< 0.8$  as losses.

#### Telomere length assessment

Average telomere length was measured by quantitative real-time DNA-PCR as described elsewhere [18]. This method, developed by Cawthon [19], measures the ratio between the telomere repeat (*T*) and reference single-copy gene (*S*) template amounts (relative *T/S* ratio). We previously showed that the *T/S* ratio in tumour cells was proportional to the average telo-

mere length assessed by a classical telomere restriction fragment analysis [18].

#### Gene expression analysis

The gene expression of *hTERT* and shelterin (*TRF1*, *TRF2*, *POT1*, *TPP1*, *RAP1* and *TIN2*) was quantified by real-time RT-PCR as described previously [18,20]. *TNKS* (tankyrase 1) and *ERCC1* expression was quantified with known primers [21,22]. The normalized copy number (NCN) for each gene was expressed as the ratio between transcript copy-numbers of the target and control (*B2M*) genes multiplied by 100.

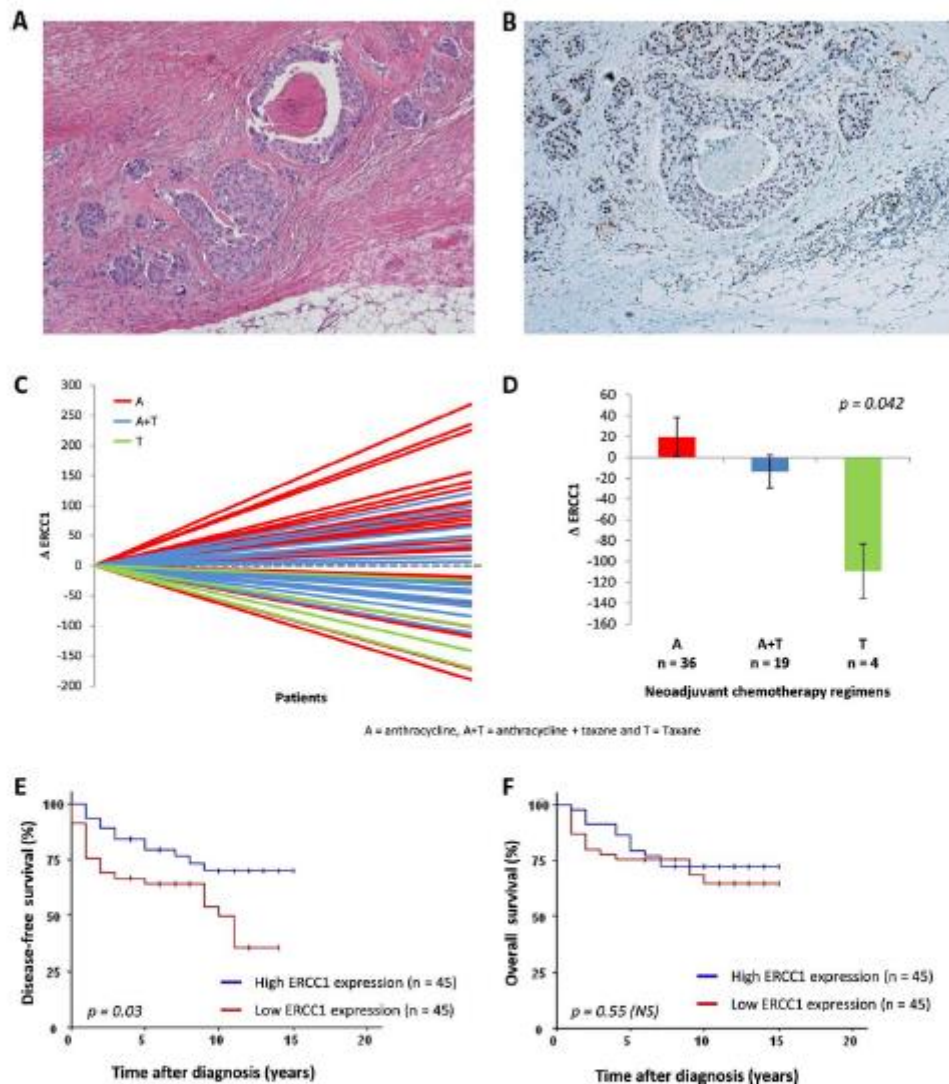
#### Statistical analysis

Standard tests (Kruskal-Wallis test, ANOVA, Student's *t*-test, chi-squared test, Spearman correlation) were used to study the relationship between characteristics. As is usual in exploratory studies, we elected not to adjust probabilities by the Bonferroni method, which, while decreasing the rate of false positives, also increases in a similar proportion the rate of false negatives. Disease-free survival (DFS) refers to the period between diagnosis and the first relapse (local or distant). Overall survival (OS) was measured from the start of NCT until the last follow-up report. Survival curves were established by the Kaplan-Meier method and compared with the log-rank test. An unsupervised hierarchical clustering was performed with SEM software [23]. Distances between clusters were calculated by 1-Pearson's correlation coefficient values and the dendrogram was constructed according to Ward's algorithm.

## Results

#### ERCC1 protein and gene expression level

In post-NCT tumours, the median H-score was 102 (0–285). Positive ERCC1 staining was nuclear (Figure 1A,B). Two patients had negative staining and three cytoplasmic staining. Correlations between clinicopathological data and ERCC1 H-scores in post-NCT tumours are shown in Table 1. There was no association between ERCC1 expression and tumour size, grade, stage, hormone-receptor expression and molecular classification. Interestingly, patients treated with anthracyclines had higher H-scores than patients receiving anthracyclines + taxanes or taxanes alone ( $p = 0.004$ ). Comparison of ERCC1 expression in pre-NCT and post-NCT samples ( $\Delta$ ERCC1) indicated an increase in ERCC1 levels after treatment with anthracyclines as compared to other regimes



**Figure 1.** Detection of pre- and post-NCT ERCC1 expression and associations with NCT regimen and survival. (A–B): Representative images of ERCC1-positive invasive carcinoma: HE staining (A) and corresponding IHC staining (B), showing nuclear positivity in tumour cells. (C–D): Variations in ERCC1 expression between pre-NCT and post-NCT samples (C) are associated with NCT regimens: tumours treated with anthracyclines had an increase only in ERCC1 levels as compared to anthracyclines + taxanes and taxanes groups (D). (E–F): Survival analysis. Patients whose tumours had low ERCC1 expression (< median, 102) had significantly shortened DFS, whereas those with high ERCC1 levels had better prognosis (E). No significant difference in OS was observed between the groups with low and high ERCC1 levels (F).

( $p = 0.042$ ), suggesting that anthracyclines induced ERCC1 expression (Figure 1C,D). After NCT, Chevaller's class 4 patients had lower H-scores than class 3 patients ( $p = 0.029$ ). There was a reduction in

ERCC1 expression in class 4 patients ( $\Delta$ ERCC1 =  $-72.4$ ) but an increase in class 3 patients ( $\Delta$ ERCC1 =  $14.66$ ,  $p = 0.010$ ). The reduction in ERCC1 levels was correlated with larger residual



Table 1. ERCC1 protein expression in association with pre- or post-NCT clinical and pathological features of breast cancer patients\*

Variable	ERCC1 H score	
	Mean ( $\pm$ SEM)	p-value
Tumour size		0.08
T1-T2 (n = 66)	115.9 ( $\pm$ 10.5)	
T3-T4 (n = 24)	78.9 ( $\pm$ 13.1)	
Nodal status		0.53
Negative (n = 46)	102.1 ( $\pm$ 12.5)	
Positive (n = 44)	110.1 ( $\pm$ 11.8)	
AJCC clinical stage		0.53
I-II (n = 78)	108.3 ( $\pm$ 9.4)	
III (n = 12)	91.5 ( $\pm$ 20.6)	
Tumour grade (SBR)		0.19
I (n = 9)	160.4 ( $\pm$ 32)	
II (n = 46)	101.1 ( $\pm$ 10.7)	
III (n = 22)	101.2 ( $\pm$ 16.9)	
HR status		0.34
Negative (n = 27)	93.2 ( $\pm$ 15.6)	
Positive (n = 59)	110.8 ( $\pm$ 10.8)	
Molecular classification		0.57
Luminal A (n = 23)	104 ( $\pm$ 16.9)	
Luminal B (n = 25)	114 ( $\pm$ 16.4)	
HER2 (n = 4)	84 ( $\pm$ 37.5)	
Triple negative (n = 13)	77 ( $\pm$ 20.2)	
Neoadjuvant chemotherapy		0.0042
Anthracycline based (n = 47)	131.2 ( $\pm$ 12.7)	
Anthracycline and Taxane based (n = 35)	85.4 ( $\pm$ 10.3)	
Taxane based (n = 8)	48.5 ( $\pm$ 26.5)	
Chevallier's pathological response		0.029
Class 3 (n = 70)	117.6 ( $\pm$ 14.1)	
Class 4 (n = 14)	72.6 ( $\pm$ 22.3)	
Tumour grade (SBR) in residual tumour		0.19
I (n = 13)	131.4 ( $\pm$ 24.7)	
II (n = 36)	109.6 ( $\pm$ 10.1)	
III (n = 19)	82.2 ( $\pm$ 19.8)	
HR status in residual tumour		0.28
Negative (n = 25)	97.4 ( $\pm$ 15.9)	
Positive (n = 60)	117.8 ( $\pm$ 10.4)	

\*Missing values are due to block exhaustion.

AJCC, American Joint Committee on Cancer; SBR, Scarff-Bloom-Richardson grading system; HR, hormone receptor; SEM: standard error of the mean.

tumour ( $p = 0.01$ ). Increased ERCC1 expression after treatment was seen in pre-NCT Scarff-Bloom-Richardson (SBR) grade I tumours ( $\Delta$ ERCC1 = 95.5), but not in grade II-III tumours, which had stable or decreased ERCC1 levels (grade II = -17.88; grade III = 3.2,  $p = 0.044$ ).

To evaluate the prognostic impact of ERCC1 expression in residual tumours, we compared survival in patients with low and high H-scores, using the median value as a cut-off. Patients with low ERCC1 levels had shorter DFS ( $p = 0.03$ ). There was no association between ERCC1 expression and OS (Figure 1E,F). Of note, pre-NCT ERCC1 and  $\Delta$ ERCC1 were not correlated with DFS and OS.

The levels of ERCC1 gene and protein expression evaluated in post-NCT tumour samples were not correlated. ERCC1 gene expression was not associated with clinicopathological characteristics (data not shown).

#### ERCC1 CNVs

Of the 45 post-NCT tumour samples, six had losses and five had gains (Figure 2A-C). These alterations involved the entire ERCC1 gene. In addition, two tumours had partial gains involving the 5'UTR primer only.

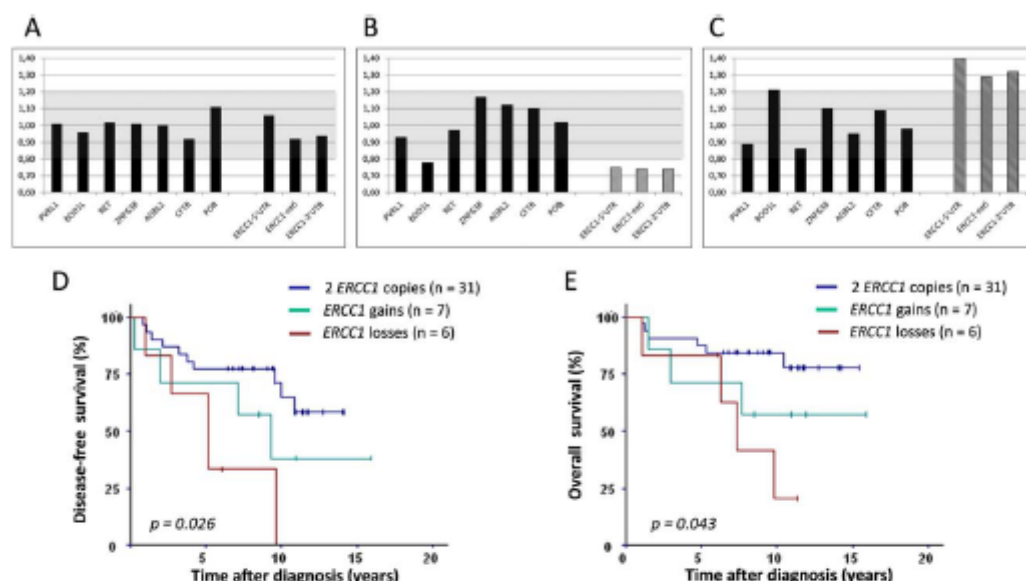
Patients with gains showed an average increase in ERCC1 gene (75%) and protein (40%) expression, whereas in those with losses ERCC1 gene and protein expression decreased by 20%. However, these changes were not significant.

We compared survival according to ERCC1 gene status: (i) losses, (ii) gains (partial or total) and (iii) normal copy-number (Figure 2D,E), and found significant differences for DFS and OS. In particular, the subgroup with ERCC1 losses had lower DFS (5.1 years,  $p = 0.0065$ ) and OS (7.2 years,  $p = 0.014$ ) than the normal copy-number subgroup (median not reached). The subgroup with ERCC1 gains had intermediate OS and DFS.

#### Telomere length and hTERT expression

The median value of hTERT expression was 3.5 (0.0-147.6) and that of telomere length 1.03 (0.22-2.71). Of note, shorter telomeres were associated with higher hTERT expression ( $p = 0.00075$ ). Correlations between telomere length and hTERT expression with clinicopathological data are summarized in Table 2. Telomere shortening was associated with advanced disease stage ( $p = 0.030$ ), positive nodal status ( $p = 0.031$ ) and higher tumour grades in pre- and post-NCT tumours ( $p = 0.0057$  and  $p = 0.05$ ). hTERT expression tended to increase in advanced stages and higher post-NCT tumour grades and correlated with hormone-receptor (HR) negativity in post-NCT tumours ( $p = 0.039$ ). No association was observed between telomere parameters and molecular classification. However, patients with triple negative breast cancer (TNBC) tended to have shorter telomeres and higher hTERT. Telomeres were shorter and hTERT higher in cases with the largest residual disease (Chevallier's class 4 versus class 3,  $p < 0.05$ ). The impact of telomere length and hTERT level on outcome was analyzed using the median values as cut-points (Figure 3A,B). Patients with short telomeres had lower DFS ( $p = 0.0076$ ) and OS





**Figure 2.** Post-NCT ERCC1 copy-numbers: detection and correlation with survival. (A–C): Determination of ERCC1 gene copy numbers. ERCC1 copy numbers were evaluated by QMF-PCR with three distinct probes on the ERCC1 gene (localized in 5'UTR, Exon 6 and 3'UTR) and seven other genes serving as controls (*PVRL1*, *BOD1L*, *RET*, *ZNF638*, *AGBL2*, *CFTR* and *POR*). The gray zone corresponds to normal gene-copy number, which was defined as the mean  $\pm 3$  standard deviations of controls. Three examples of ERCC1 profiles are presented: two copies (A), loss (B) and gain (C). (D–E) Survival analysis. ERCC1 losses and gains were associated with significantly shorter DFS (D) and OS (E) than in the group without CNVs. ERCC1 losses are associated with the worst survival and gains with an intermediate survival.

( $p = 0.050$ ). High hTERT expression was also related to decreased DFS ( $p = 0.0015$ ) and OS ( $p = 0.025$ ).

#### Shelterin and tankyrase 1 gene expression levels

Shelterin gene expression did not correlate with disease stage, tumour size, nodal status, SBR Grade (pre- and post-NCT), HR status (pre and post-NCT), pathological response, NCT regimen or survival (data not shown). High expression of the *TNKS* gene was observed in high SBR grade pre-NCT tumours ( $p = 0.026$ ). Higher tankyrase expression (fourth quartile, *TNKS* > 442) was strongly associated with worse DFS ( $p = 0.00061$ ) and OS ( $p = 0.0096$ ) (Figure 3C). Prognostic impact of *TNKS* was independent of hTERT expression and telomere length (multivariate Cox analysis, adjusted  $p = 0.013$  and  $p = 0.05$ , respectively).

#### Clustering analysis of ERCC1 and telomere parameters

We performed an unsupervised hierarchical clustering analysis to identify prognostic subgroups among 45

patients, combining telomeric and ERCC1 data in post-NCT tumours. Four balanced groups with different prognosis were defined on the basis of their molecular similarities (Figure 4A–C).

Cluster 1 ( $n = 10$ ) had good prognostic parameters (long telomere, no or very low hTERT expression) and high ERCC1 protein expression levels. Shelterin, *TNKS* and ERCC1 gene expression levels were low. The ERCC1 gene had few CNVs. These patients had good DFS and OS (9.5 and 9.6 years, means).

Cluster 2 ( $n = 11$ ) had bad prognostic parameters (short telomere, high expression of hTERT), low ERCC1 levels, low shelterin, *TNKS* and ERCC1 gene expression. Cluster 2 patients had the highest level of ERCC1 CNVs (both losses and gains) and the lowest DFS and OS (6.2 and 7.8 years, respectively).

In cluster 3 ( $n = 13$ ), the telomeres were short but hTERT expression low. Shelterin and ERCC1 gene expression levels were the highest. Of note, these patients also had *TNKS* overexpression. There were a few ERCC1 CNVs, and ERCC1 expression was intermediate. Survival was short in this subgroup (DFS of 6.7 and OS of 8 years).

Table 2. Telomere length, *hTERT* and *TNKS* expression in association with pre- or post-NCT clinical and pathological features of breast cancer patients\*

Variable	Telomere length		<i>hTERT</i> expression		<i>TNKS</i> expression	
	Mean ( $\pm$ SEM)	p-value	Mean ( $\pm$ SEM)	p-value	Mean ( $\pm$ SEM)	p-value
Tumour size		0.84		0.91		0.42
T1-T2 (n = 30)	1.080 ( $\pm$ 0.087)		15.69 ( $\pm$ 6)		345.9 ( $\pm$ 49.4)	
T3-T4 (n = 15)	1.049 ( $\pm$ 0.130)		12.55 ( $\pm$ 5.28)		389.5 ( $\pm$ 86.1)	
Nodal status		0.031		0.32		0.18
Negative (n = 21)	1.181 ( $\pm$ 0.087)		7.87 ( $\pm$ 2.79)		409 ( $\pm$ 70.7)	
Positive (n = 24)	0.972 ( $\pm$ 0.108)		20.58 ( $\pm$ 7.62)		317.9 ( $\pm$ 52.6)	
AJCC clinical stage		0.030		0.10		0.68
I-II (n = 38)	1.128 ( $\pm$ 0.078)		10.53 ( $\pm$ 3.38)		367.8 ( $\pm$ 51.3)	
III (n = 7)	0.751 ( $\pm$ 0.135)		36.99 ( $\pm$ 19.2)		320.3 ( $\pm$ 40.9)	
Tumour grade (SBR)		0.0057		0.20		0.026
I (n = 5)	1.744 ( $\pm$ 0.337)		1.68 ( $\pm$ 19.56)		326.4 ( $\pm$ 147.2)	
II (n = 22)	0.943 ( $\pm$ 0.082)		17.67 ( $\pm$ 5.87)		303.3 ( $\pm$ 54.9)	
III (n = 10)	1.107 ( $\pm$ 0.113)		23.28 ( $\pm$ 0.67)		540.4 ( $\pm$ 116.3)	
HR status		0.079		0.086		0.38
Negative (n = 11)	0.841 ( $\pm$ 0.102)		23.82 ( $\pm$ 9.9)		438.9 ( $\pm$ 116.8)	
Positive (n = 31)	1.161 ( $\pm$ 0.093)		11.47 ( $\pm$ 5.06)		335.6 ( $\pm$ 47.1)	
Molecular classification		0.078		0.23		0.60
Luminal A (n = 17)	1.17 ( $\pm$ 0.122)		8.68 ( $\pm$ 4.14)		343 ( $\pm$ 63.6)	
Luminal B (n = 15)	1.264 ( $\pm$ 0.137)		5.39 ( $\pm$ 1.56)		321.1 ( $\pm$ 70.5)	
HER2 (n = 3)	1.135 ( $\pm$ 0.155)		13.03 ( $\pm$ 7.98)		411.7 ( $\pm$ 139.8)	
Triple negative (n = 5)	0.653 ( $\pm$ 0.136)		36.36 ( $\pm$ 18.98)		531 ( $\pm$ 229.5)	
Neoadjuvant chemotherapy		0.20		0.59		0.80
Anthracycline based (n = 23)	0.987 ( $\pm$ 0.093)		19.85 ( $\pm$ 7.75)		339.9 ( $\pm$ 48.8)	
Anthracycline and Taxane based (n = 22)	1.155 ( $\pm$ 0.108)		9.20 ( $\pm$ 3.43)		381.9 ( $\pm$ 73.4)	
Chevallier's pathological response		0.045		0.041		0.50
Class 3 (n = 38)	1.115 ( $\pm$ 0.083)		12.07 ( $\pm$ 3.77)		363.1 ( $\pm$ 51)	
Class 4 (n = 5)	0.740 ( $\pm$ 0.041)		37.12 ( $\pm$ 24.76)		358.2 ( $\pm$ 69.7)	
Tumour grade (SBR) in residual tumour		0.05		0.027		0.20
I (n = 8)	1.454 ( $\pm$ 0.264)		10.34 ( $\pm$ 8.13)		340.4 ( $\pm$ 92.9)	
II (n = 17)	0.973 ( $\pm$ 0.091)		12.07 ( $\pm$ 6.56)		328.9 ( $\pm$ 68.6)	
III (n = 10)	0.928 ( $\pm$ 0.083)		32.22 ( $\pm$ 13.22)		496 ( $\pm$ 122.7)	
HR status in residual tumour		0.15		0.039		0.54
Negative (n = 12)	0.869 ( $\pm$ 0.098)		24.81 ( $\pm$ 9.21)		343.3 ( $\pm$ 46.8)	
Positive (n = 32)	1.148 ( $\pm$ 0.091)		10.9 ( $\pm$ 4.92)		370.4 ( $\pm$ 58.9)	

\*Missing values are due to block exhaustion.

AJCC, American Joint Committee on Cancer; SBR, Scarff-Bloom-Richardson grading system; HR, hormone receptors; SEM, standard error of the mean.

Cluster 4 (n = 11) had 'normal' telomeric parameters, moderate to high shelterin gene expression (in particular *TPPI* overexpression) and low *TNKS* expression levels. The level of *ERCC1* gene expression was moderate, that of *ERCC1* protein expression low, and no *ERCC1* CNVs were observed. Survival data were rather favorable in this cluster (DFS of 9 and OS of 9.5 years).

Survival analysis showed that patients from clusters 1 and 4 had better DFS and OS than patients from clusters 2 and 3 (Figure 4D). Furthermore, patients from clusters 2 and 3 had early relapses with DFS at 5 years of 62.5% compared to DFS at 5 years of 90.5% in clusters 1 and 4 ( $p = 0.014$ ). A multivariate Cox-model analysis with pre- and post-NCT tumour stages and grades showed that clusters 2 and

3 correlated with shorter DFS and OS irrespective of tumour stage or grade (Figure 4E). Prognostic profiles of clusters were significant when adjusted for pathological response with hazard ratios of 4.64 for DFS ( $p = 0.0072$ ) and 5.81 for OS ( $p = 0.023$ ).

## Discussion

There is emerging evidence that pathobiological features of post-NCT disease can determine outcome in breast cancer [24]. The adjuvant treatment decision-making process, currently based on pre-NCT biopsy criteria, is beginning to switch toward the use of post-NCT disease factors. In this study, we

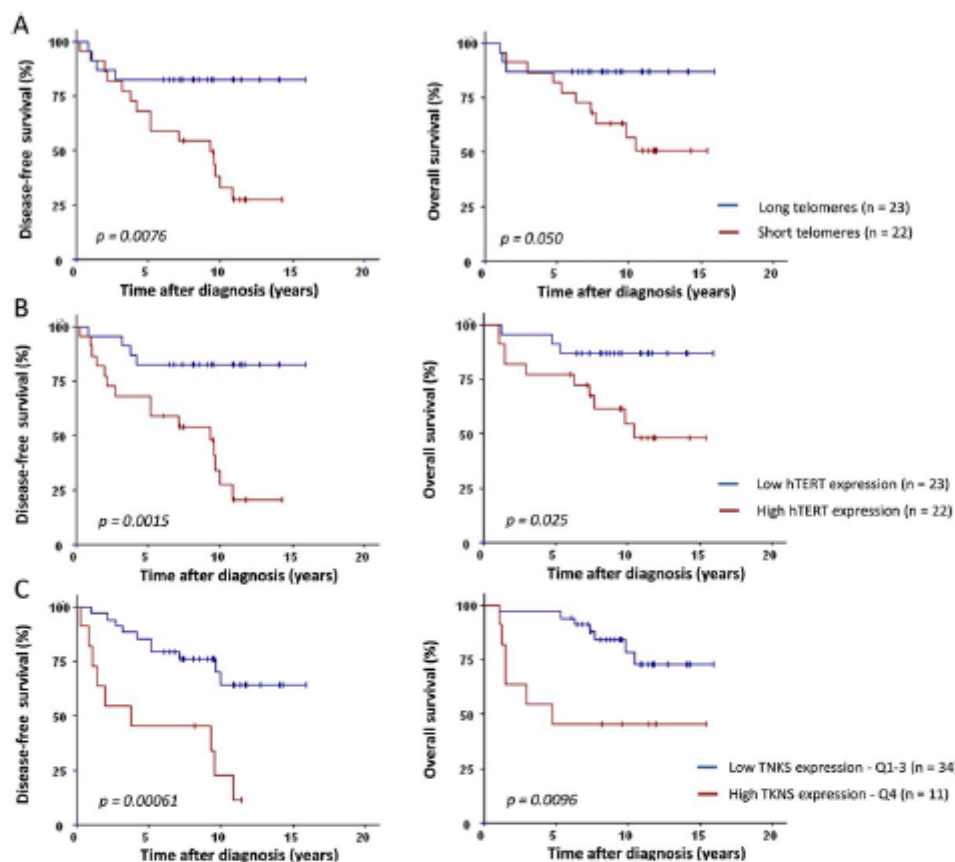


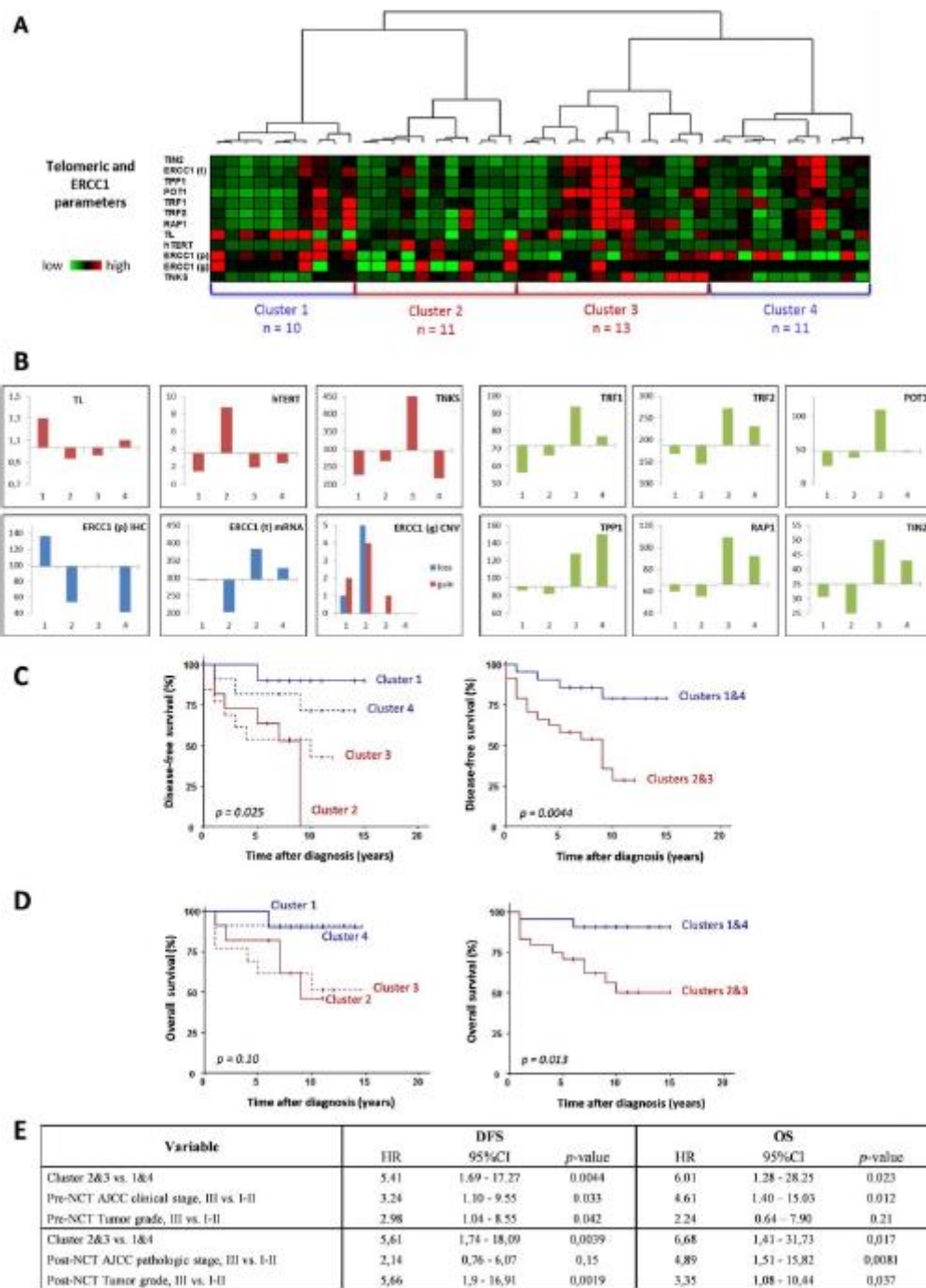
Figure 3. Post-NCT telomere length, *hTERT* and *TNKS* expression levels are predictive of disease-free and overall survival. (A) Median telomere length (T/S ratio of 1.03) was used to separate tumours into two groups: long (blue line) versus short (red line) telomeres. Short telomeres were significantly associated with worse DFS ( $p = 0.0076$ ) and OS ( $p = 0.050$ ). (B) Tumours were divided into low *hTERT* (blue line) versus high *hTERT* (red line) expression according to the median value (NCN of 3.5). High *hTERT* expression was significantly correlated with worse DFS ( $p = 0.0015$ ) and OS ( $p = 0.025$ ). (C) Higher *TNKS* expression (fourth quartile,  $TNKS \geq 443$ , red line) was significantly associated with worse DFS ( $p = 0.00061$ ) and OS ( $p = 0.0096$ ).

investigated ERCC1 expression at different molecular levels and telomere characteristics in post-NCT breast tumours and correlated the results with patient outcome.

Associations between ERCC1 protein expression and clinicopathological data have been previously explored in breast cancer. Despite their heterogeneous design, most studies suggested that high ERCC1 expression was correlated with criteria of good prognosis, such as estrogen-receptor positivity and low T/N stages [8,25]. Low ERCC1 levels were found in poor-prognosis TNBC [26,27]. Our results show better survival in cases of high ERCC1 in post-NCT

tumours, but not in pre-NCT tumours. We may infer that ERCC1 expression in residual tumours is a robust prognostic marker in breast cancer patients treated with NCT.

NCT protocols influence DDR mechanisms and pathological response. pCR rate increases after platinum-based CT in tumours underexpressing ERCC1 [28]. This is in line with the paradigm that negative/low ERCC1 tumours respond better to platinum-based CT because of insufficient NER activation [29,30]. In addition, anthracycline-based CT may induce an increase in *ERCC1* gene expression levels in breast tumours because of the activation of





ERCC1-dependent DDR mechanisms other than NER [31]. Our study showed increasing ERCC1 expression in tumours treated with anthracyclines compared with tumours treated with taxanes. Post-NCT ERCC1 status could be taken into account in future tailoring of platinum-based adjuvant therapy.

ERCC1 expression was greater in post-NCT Chevallier class 3 tumours than in class 4 cases. Better response is generally observed in tumours with higher genomic instability [32] while hormone-receptor positive tumours, which have low genomic instability, are usually poor responders to NCT [33]. Class 3 and 4 patients represent heterogeneous groups in terms of genomic instability and other biological characteristics. It is possible that ERCC1 expression levels could help to identify better responders among HR-positive cancer patients like those with luminal B tumours, which have higher levels of genomic instability and are more chemosensitive than luminal A tumours [34].

In agreement with other reports, we did not observe any prognostic significance of *ERCC1* mRNA expression at the gene level [35]. In addition, copy-number alterations of *ERCC1* gene were previously shown to be highly frequent in non-small cell lung cancer but not correlated with protein expression and survival [36]. We found that *ERCC1* CNVs tended to be associated with parallel variations in protein and gene expression levels and that both gains and losses were correlated with worse outcome. We suggest that *ERCC1* protein functionality could be affected by CNVs.

In naïve breast cancer, telomere shortening was associated with tumour aggressiveness according to TNM stage or SBR grade [22,37–39]. Short telomeres were found in aggressive HER2+ and TNBC cases [40,41]. In our series of post-NCT tumours, we also observed telomere shortening in patients with advanced stage and grade.

A relationship between short telomeres and poor clinical outcomes has already been reported [37,42,43] but

not confirmed in a study with longer median follow-up time (7.2 years) [44]. Furthermore, the impact of short telomeres on DFS was recently observed in TNBC but not in HR-positive tumours [45]. Our analysis of a cohort with a long follow-up (9 years) and a majority of HR-positive tumours strongly supports the overall link between short telomeres and poor survival in breast cancer. In order to maintain short telomeres, *hTERT* is activated in tumour cells, and high *hTERT* correlates with clinical aggressiveness in many human malignancies [18,46–48]. In agreement with previous findings in breast cancer patients [22,49,50], we observed that increased *hTERT* expression correlated with higher SBR grades and shorter survival. Thus, we suggest that telomere length and *hTERT* expression in post-NCT tumours are significant predictors of survival and could be used to tailor adjuvant chemotherapy.

Overexpression of *TNKS* has been described in breast cancer in comparison to normal tissue [51] and has been associated with higher SBR grades [22]. We found that *TNKS* overexpression appeared mainly in high-grade SBR III tumours, suggesting that this overexpression represents a relevant event in tumour progression. We also demonstrated that high *TNKS* expression was associated with shorter survival. Because of its involvement in telomere maintenance, tankyrase was recently considered as a potential target for cancer therapy [52,53]. The correlation of *TNKS* expression with survival underlines the validity of this target.

Down-regulation of shelterin genes *TRF1*, *TRF2* and *POT1* has been found in breast cancer compared with normal breast tissue [22,50,54,55]. Low expression of *POT1*, *TRF1* and *TRF2* tended to be associated with high tumour grade and poor clinical outcome, but this relationship was not significant [22,50]. We observed no correlation between shelterin gene expression and clinicopathological parameters or survival.

Clustering analysis combining telomeric and ERCC1 parameters identified four subgroups with different prognosis and with possibly different

**Figure 4.** Post-NCT telomere and ERCC1 characteristics, combined together, correlate statistically with disease-free and overall survival. (A) Unsupervised hierarchical clustering was performed on the combined telomere length (TL), *hTERT*, *TNKS*, shelterin (*TRF1*, *TRF2*, *TPP1*, *POT1*, *RAP1* and *TIN2*) and *ERCC1* gene expression, ERCC1 protein expression and *ERCC1* gene CNV. The three levels of analysis for *ERCC1* are annotated (p) for protein expression, (t) for gene expression and (g) for copy number. The different color intensities of dichotomic parameters reflect normalized values and not original binary (0/1) values. High normalized quantitative values of telomere length and expression of ERCC1 and telomere-related genes are shown in red, intermediate values in black and lower values in green (intensity scale is shown). (B) Distribution of the different parameters among the four clusters. (C) Patients from Clusters 2 and 3 (red lines) had shorter DFS than those from clusters 1 and 4 (blue lines) ( $p = 0.025$ ). A highly significant difference was observed between clusters 2 and 3 versus clusters 1 and 4. (D) Clusters 2 and 3 (red lines) tended to be associated with worse OS than clusters 1 and 4 (blue lines). Patients from grouped clusters 1 and 4 had significantly worse OS than patients from clusters 2 and 3 ( $p = 0.013$ ). (E) Prognostic value of these clusters in a multivariate Cox-model with tumour stage and tumour grade. Patients from clusters 2 and 3 had significantly shorter DFS and OS, irrespective of tumour pre- and post-NCT stage or grade.

biological significance. In particular, the analysis of poor-prognosis cluster 2 patients showed that telomere-dysfunction parameters (short telomere length, high *hTERT*, low shelterin) were associated with decreased ERCC1 expression and ERCC1 copy-number alterations suggestive of a certain deficiency in DDR mechanisms. Both telomere and DDR defects can contribute to a higher degree of genomic instability and, given the possible involvement of ERCC1 in telomere maintenance, amplify the deleterious impact of telomere dysfunction, resulting in resistant tumour phenotypes. The *hTERT* overexpression observed in this cluster could have contributed to tumour cell proliferation by stabilizing short telomeres and by conferring survival advantages owing to the telomere-independent function of *hTERT* as transcriptional modulator of the NF- $\kappa$ B and Wnt/ $\beta$ -catenin pathways [56].

Another poor-prognosis association, including high *TNKS*, *ERCC1* and shelterin gene expression, was found in cluster 3 patients. Telomere length and *hTERT* were less affected, but this subgroup had worse prognosis than clusters 1 and 4 with no marked telomere changes. *TNKS* acts as a positive regulator of telomere elongation in telomerase-positive cells and also has telomere-independent functions that contribute to correct bipolar spindle assembly during mitosis [57] and the maintenance of Wnt-signalling [58]. All these functions of tankyrase may increase the survival capacities of tumour cells. In addition, up-regulation of *ERCC1* and shelterin genes may have an adverse effect on telomeres. Shelterin interacts with proteins involved in DDR, such as ERCC1-XPF. Overexpression of XPF may negatively regulate telomere length maintenance and interfere with shelterin binding to telomeres [13].

The association of long telomeres, low *hTERT* expression, low shelterin and high ERCC1 protein expression that we observed in cluster 1 patients, irrespective of tumour stage or grade, confirms that together functional telomere and DDR protein recruitment determine the best prognosis.

In conclusion, we report new findings on the clinical significance of ERCC1, a DDR protein, and telomere parameters in breast cancer treated with NCT. The combination of these characteristics identifies subgroups with different prognosis. These results emphasize the interest in studying DDR proteins and their role in telomere maintenance in translational studies.

### Acknowledgements

This work was supported in part by grants from the French National Cancer Institute and the League

Against Cancer. PR was recipient of a scholarship from Roche Ventana in Tucson AZ, USA. Ventana provided the ERCC1 Antibody. Esteban Roberts, Staff Scientist at Roche Ventana in Tucson AZ, USA, contributed to the elaboration of the ERCC1 IHC procedure.

### Author Contributions

MGB and PR performed experiments, generated and analyzed data and wrote the manuscript draft. AC, LV, MP, SS, PC, FK, CA, YJB and PV contributed to experiments, data collection and revision of the manuscript for content, analysis and interpretation of data. AT and FPL designed the study and wrote the final manuscript.

### Abbreviations

POT1, protection of telomere 1; RAP1, Repressor/Activator Protein 1; TIN2, TRF1- and TRF2-Interacting Nuclear Protein 2; TPP1, TIN2 and POT1 interacting protein; TRF1, telomeric repeat-binding factor 1; TRF2, telomeric repeat-binding factor 2.

### References

1. Buchholz TA, Hunt KK, Whitman GJ, et al. Neoadjuvant chemotherapy for breast carcinoma: multidisciplinary considerations of benefits and risks. *Cancer* 2003; **98**: 1150–1160.
2. Fisher B, Bryant J, Wolmark N, et al. Effect of preoperative chemotherapy on the outcome of women with operable breast cancer. *J Clin Oncol* 1998; **16**: 2672–2685.
3. Penault-Llorca F, Abrial C, Raoult I, et al. Changes and predictive and prognostic value of the mitotic index, Ki-67, cyclin D1, and cyclo-oxygenase-2 in 710 operable breast cancer patients treated with neoadjuvant chemotherapy. *Oncologist* 2008; **13**: 1235–1245.
4. Pierga JY, Mouret E, Diems V, et al. Prognostic value of persistent node involvement after neoadjuvant chemotherapy in patients with operable breast cancer. *Br J Cancer* 2000; **83**: 1480–1487.
5. Kirschner K, Melton DW. Multiple roles of the ERCC1-XPF endonuclease in DNA repair and resistance to anticancer drugs. *Anticancer Res* 2010; **30**: 3223–3232.
6. Tripsianes K, Folkers G, Ah E, et al. The structure of the human ERCC1/XPF interaction domains reveals a complementary role for the two proteins in nucleotide excision repair. *Structure* 2005; **13**: 1849–1858.
7. McNeil EM, Melton DW. DNA repair endonuclease ERCC1-XPF as a novel therapeutic target to overcome chemoresistance in cancer therapy. *Nucleic Acids Res* 2012; **40**: 9990–10004.
8. Goyal S, Parikh RR, Green C, et al. Clinicopathologic significance of excision repair cross-complementation 1 expression in patients treated with breast-conserving surgery and radiation therapy. *Int J Radiat Oncol Biol Phys* 2010; **76**: 679–684.



9. Diotti R, Louzya D. Shelterin complex and associated factors at human telomeres. *Nucleus* 2011; **2**: 119–135.
10. Smith S, de Lange T. Tankyrase promotes telomere elongation in human cells. *Curr Biol* 2000; **10**: 1299–1302.
11. Artandi SE, DePinho RA. Telomeres and telomerase in cancer. *Carcinogenesis* 2010; **31**: 9–18.
12. Zhu XD, Niedernhofer L, Kuster B, et al. ERCC1/XPF removes the 3' overhang from uncapped telomeres and represses formation of telomeric DNA-containing double minute chromosomes. *Mol Cell* 2003; **12**: 1489–1498.
13. Wu Y, Mitchell TR, Zhu XD. Human XPF controls TRF2 and telomere length maintenance through distinctive mechanisms. *Mech Ageing Dev* 2008; **129**: 602–610.
14. Munoz P, Blanco R, Flores JM, et al. XPF nuclease-dependent telomere loss and increased DNA damage in mice overexpressing TRF2 result in premature aging and cancer. *Nat Genet* 2005; **37**: 1063–1071.
15. Penabaz-Llorca F, Abrial C, Raoelfils I, et al. Comparison of the prognostic significance of Chevallier and Sataloff's pathologic classifications after neoadjuvant chemotherapy of operable breast cancer. *Hum Pathol* 2008; **39**: 1221–1228.
16. Niel F, Martin J, Dastot-Le Moal F, et al. Rapid detection of CFTR gene rearrangements impacts on genetic counselling in cystic fibrosis. *J Med Genet* 2004; **41**: e118.
17. Peñal-Richard C, Kemény S, Gous L, et al. An atypical 0.8 Mb inherited duplication of 22q11.2 associated with psychomotor impairment. *Eur J Med Genet* 2012; **55**: 650–655.
18. Veronese L, Tournilhac O, Callanan M, et al. Telomeres and chromosomal instability in chronic lymphocytic leukemia. *Leukemia* 2013; **27**: 490–493.
19. Cawthon RM. Telomere measurement by quantitative PCR. *Nucleic Acids Res* 2002; **30**: e47.
20. Poncet D, Belleville A, t'kint de Roodenbeke C, et al. Changes in the expression of telomere maintenance genes suggest global telomere dysfunction in B-chronic lymphocytic leukemia. *Blood* 2008; **111**: 2388–2391.
21. Garzinelli M, Mariani P, Cattaneo D, et al. Expression of DNA repair genes in ovarian cancer samples: biological and clinical considerations. *Eur J Cancer* 2011; **47**: 1086–1094.
22. Poonepalli A, Banerjee B, Ramnarayanan K, et al. Telomere-mediated genomic instability and the clinico-pathological parameters in breast cancer. *Genes Chromosomes Cancer* 2008; **47**: 1098–1109.
23. Kwiatkowski F, Girard M, Hacene K, et al. [Sem: a suitable statistical software adapted for research in oncology]. *Bull Cancer* 2000; **87**: 715–721.
24. Bosnyat V, Provenzano E, Symmans WF, et al. Recommendations for standardized pathological characterization of residual disease for neoadjuvant clinical trials of breast cancer by the BIG-NABCG collaboration. *Ann Oncol* 2015; **26**: 1280–1291.
25. Gerhard R, Carvalho A, Carneiro V, et al. Clinicopathological significance of ERCC1 expression in breast cancer. *Pathol Res Pract* 2013; **209**: 331–336.
26. Kim D, Jung W, Koo JS. The expression of ERCC1, RRM1, and BRCA1 in breast cancer according to the immunohistochemical phenotypes. *J Korean Med Sci* 2011; **26**: 352–359.
27. Sidoni A, Cartaginosa F, Colozza M, et al. ERCC1 expression in triple negative breast carcinoma: the paradox revisited. *Breast Cancer Res Treat* 2008; **111**: 569–570.
28. Chen X, Wu J, Lu H, et al. Measuring beta-tubulin III, Bcl-2, and ERCC1 improves pathological complete remission predictive accuracy in breast cancer. *Cancer Sci* 2012; **103**: 262–268.
29. Lee KB, Parker RJ, Bohr V, et al. Cisplatin sensitivity/resistance in UV repair-deficient Chinese hamster ovary cells of complementation groups 1 and 3. *Carcinogenesis* 1993; **14**: 2177–2180.
30. Fu JM, Zhou J, Shi J, et al. Emodin affects ERCC1 expression in breast cancer cells. *J Transl Med* 2012; **10** Suppl 1: S7.
31. Fu JM, Zhou J, Xie JS, et al. [Effect of neoadjuvant chemotherapy on ERCC1 gene expression in breast cancer]. *Nan Fang Yi Ke Da Xue Xue Bao* 2008; **28**: 603–605.
32. Ribeiro E, Garzinelli M, Andreis D, et al. Triple negative breast cancers have a reduced expression of DNA repair genes. *PLoS One* 2013; **8**: e66243.
33. Colleoni M, Viale G, Zahrieh D, et al. Chemotherapy is more effective in patients with breast cancer not expressing steroid hormone receptors: a study of preoperative treatment. *Clin Cancer Res* 2004; **10**: 6622–6628.
34. Ades F, Zardavas D, Bozovic-Spasojevic I, et al. Luminal B breast cancer: molecular characterization, clinical management, and future perspectives. *J Clin Oncol* 2014; **32**: 2794–2803.
35. Deng Q, Yang H, Lin Y, et al. Prognostic value of ERCC1 mRNA expression in non-small cell lung cancer, breast cancer, and gastric cancer in patients from Southern China. *Int J Clin Exp Pathol* 2014; **7**: 8312–8321.
36. Vanhecke E, Valent A, Tang X, et al. 19q13-ERCC1 gene copy number increase in non-small-cell lung cancer. *Clin Lung Cancer* 2013; **14**: 549–557.
37. Fordyce CA, Heaphy CM, Bisoffi M, et al. Telomere content correlates with stage and prognosis in breast cancer. *Breast Cancer Res Treat* 2006; **99**: 193–202.
38. Kammori M, Sugishita Y, Okamoto T, et al. Telomere shortening in breast cancer correlates with the pathological features of tumor progression. *Oncol Rep* 2015; **34**: 627–632.
39. Martínez-Delgado B, Gallardo M, Tanc M, et al. Short telomeres are frequent in hereditary breast tumors and are associated with high tumor grade. *Breast Cancer Res Treat* 2013; **141**: 231–242.
40. Heaphy CM, Asch-Kendrick R, Argani P, et al. Telomere length alterations unique to invasive lobular carcinoma. *Hum Pathol* 2015; **46**: 1197–1203.
41. Heaphy CM, Subhawong AP, Gross AL, et al. Shorter telomeres in luminal B, HER-2 and triple-negative breast cancer subtypes. *Mod Pathol* 2011; **24**: 194–200.
42. Heaphy CM, Baumgartner KB, Bisoffi M, et al. Telomere DNA content predicts breast cancer-free survival interval. *Clin Cancer Res* 2007; **13**: 7037–7043.
43. Simpson K, Jones RE, Grimstead JW, et al. Telomere fusion threshold identifies a poor prognostic subset of breast cancer patients. *Mol Oncol* 2015; **9**: 1186–1193.
44. Lu L, Zhang C, Zhu G, et al. Telomerase expression and telomere length in breast cancer and their associations with adjuvant treatment and disease outcome. *Breast Cancer Res* 2011; **13**: R56.
45. Nagelkerke A, van Kuijk SJ, Martens JW, et al. Poor prognosis of constitutive gamma-H2AX expressing triple-negative breast

- cancers is associated with telomere length. *Biomark Med* 2015; **9**: 383–390.
46. Kirkpatrick KL, Mokbel K. The significance of human telomerase reverse transcriptase (hTERT) in cancer. *Eur J Surg Oncol* 2001; **27**: 754–760.
  47. Taubert H, Wurl P, Greither T, et al. Stem cell-associated genes are extremely poor prognostic factors for soft-tissue sarcoma patients. *Oncogene* 2007; **26**: 7170–7174.
  48. Uen YH, Lin SR, Wu DC, et al. Prognostic significance of multiple molecular markers for patients with stage II colorectal cancer undergoing curative resection. *Ann Surg* 2007; **246**: 1040–1046.
  49. Bieche I, Nogues C, Paradis V, et al. Quantitation of hTERT gene expression in sporadic breast tumors with a real-time reverse transcription-polymerase chain reaction assay. *Clin Cancer Res* 2000; **6**: 452–459.
  50. Salhab M, Jiang WG, Newbold RF, et al. The expression of gene transcripts of telomere-associated genes in human breast cancer: correlation with clinico-pathological parameters and clinical outcome. *Breast Cancer Res Treat* 2008; **109**: 35–46.
  51. Gelmini S, Poggesi M, Distanti V, et al. Tankyrase, a positive regulator of telomere elongation, is over expressed in human breast cancer. *Cancer Lett* 2004; **216**: 81–87.
  52. Riffell JL, Lord CJ, Ashworth A. Tankyrase-targeted therapeutics: expanding opportunities in the PARP family. *Nat Rev Drug Discov* 2012; **11**: 923–936.
  53. Seimiya H. The telomeric PARP, tankyrases, as targets for cancer therapy. *Br J Cancer* 2006; **94**: 341–345.
  54. Diehl MC, Idowu MO, Kimmekhue KN, et al. Elevated TRF2 in advanced breast cancers with short telomeres. *Breast Cancer Res Treat* 2011; **127**: 623–630.
  55. Saito K, Yagihashi A, Nasu S, et al. Gene expression for suppressors of telomerase activity (telomeric-repeat binding factors) in breast cancer. *Jpn J Cancer Res* 2002; **93**: 253–258.
  56. Li Y, Tergaonkar V. Noncanonical functions of telomerase: implications in telomerase-targeted cancer therapies. *Cancer Res* 2014; **74**: 1639–1644.
  57. Palazzo L, Della Monica R, Visconti R, et al. ATM controls proper mitotic spindle structure. *Cell Cycle* 2014; **13**: 1091–1100.
  58. Bao R, Christova T, Song S, et al. Inhibition of tankyrases induces Axin stabilization and blocks Wnt signalling in breast cancer cells. *PLoS One* 2012; **7**: e48670.

## SUPPLEMENTARY MATERIAL ONLINE

### Supplementary methods

Table S1. Clinico-pathological characteristics of breast cancer patients included in this study



## **TERT promoter status and gene copy number gains: effect on TERT expression and association with prognosis in breast cancer**

**Mathilde Gay-Bellile<sup>1,2</sup>, Lauren Véronèse<sup>1,2</sup>, Patricia Combes<sup>1,2</sup>, Eleonore Eymard-Pierre<sup>1,2</sup>, Fabrice Kwiatkowski<sup>1,3</sup>, Marie-Mélanie Dauplat<sup>4</sup>, Anne Cayre<sup>1,4</sup>, Maud Privat<sup>1,5</sup>, Catherine Abrial<sup>1,3</sup>, Yves-Jean Bignon<sup>1,5,6</sup>, Marie-Ange Mouret-Reynier<sup>1,3</sup>, Philippe Vago<sup>1,2</sup>, Frédérique Penault-Llorca<sup>1,4,\*</sup> and Andrei Tchirkov<sup>1,2,\*</sup>**

<sup>1</sup>Université Clermont Auvergne, INSERM, U1240 Imagerie Moléculaire et Stratégies Théranostiques, F-63000 Clermont Ferrand, France

<sup>2</sup>Service de Cytogénétique Médicale, CHU Clermont-Ferrand, F-63003 Clermont-Ferrand, France

<sup>3</sup>Département de Recherche Clinique, Centre Jean Perrin, F-63011 Clermont-Ferrand, France

<sup>4</sup>Département de Pathologie, Centre Jean Perrin, F-63011 Clermont-Ferrand, France

<sup>5</sup>Département d'Oncogénétique, Centre Jean Perrin, F-63011 Clermont-Ferrand, France

<sup>6</sup>Centre de Ressources Biologiques BB-0033-00075, Centre Jean Perrin, F-63011 Clermont-Ferrand, France

\*These authors have contributed equally to this work

Correspondence to: Andrei Tchirkov, email: atchirkov@chu-clermontferrand.fr

**Keywords:** breast cancer, TERT promoter mutation and polymorphism, TERT gene copy number gains, MYC overexpression, prognosis

Received: June 28, 2017

Accepted: July 31, 2017

Published: August 24, 2017

**Copyright:** Gay-Bellile et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License 3.0 (CC BY 3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

### **ABSTRACT**

Upregulation of the telomerase reverse transcriptase (*TERT*) gene in human cancers leads to telomerase activation, which contributes to the growth advantage and survival of tumor cells. Molecular mechanisms of *TERT* upregulation are complex, tumor-specific and can be clinically relevant. To investigate these mechanisms in breast cancer, we sequenced the *TERT* promoter, evaluated *TERT* copy number changes and assessed the expression of the *MYC* oncogene, a known transcriptional *TERT* regulator, in two breast cancer cohorts comprising a total of 122 patients. No activating *TERT* promoter mutations were found, suggesting that this mutational mechanism is not likely to be involved in *TERT* upregulation in breast cancer. The T349C promoter polymorphism found in up to 50% of cases was not correlated with *TERT* expression, but T349C carriers had significantly shorter disease-free survival. *TERT* gains (15-25% of cases) were strongly correlated with increased *TERT* mRNA expression and worse patient prognosis in terms of disease-free and overall survival. Particularly aggressive breast cancers were characterized by an association of *TERT* gains with *MYC* overexpression. These results evidence a significant effect of gene copy number gain on the level of *TERT* expression and provide a new insight into the clinical significance of *TERT* and *MYC* upregulation in breast cancer.

### **INTRODUCTION**

The telomerase reverse transcriptase (*TERT*) gene encodes the catalytic subunit of telomerase that maintains telomere length. Telomerase activity occurs in more

than 90% of cancers [1]. One of the hallmark features of tumor cells, it contributes to their growth advantage and survival, and upregulation of the *TERT* gene is the major mechanism of telomerase activation in human cancer [2]. In breast tumors, *TERT* overexpression was associated

**Table 1: Distributions of *TERT* promoter rs2853669 alleles (T349C SNP) in breast cancer cohorts (with 95%-confidence intervals) and 1000 genome database**

	349 T/T	349 T/C	349 C/C
<b>Breast cancer cohort #1</b>			
pre-NCT biopsies (n= 77)	57.7% (46.7-68.7%)	37.2% (26.5-47.9%)	5.1% (1.1-12.3%)
<b>Breast cancer cohort #2</b>			
post-NCT residual tumors (n= 45)	48.9% (33.4-63.5%)	44.4% (29.9-58.9%)	6.7% (1.7-18.4%)
<b>1000 Genome Database</b>			
All populations	53%	34.5%	12.5%
European population	52.1%	38.2%	9.7%

with tumor aggressiveness [3] and poor survival after adjuvant [4] and neoadjuvant [5] chemotherapy.

Several molecular events can modify *TERT* expression in cancer cells. Somatic mutations and functional single nucleotide polymorphisms (SNP) in the *TERT* promoter can lead to changes in the expression of the gene [6]. Overexpression of the oncogene *MYC* can directly enhance *TERT* expression by increased binding to the *TERT* promoter [7]. Increased copy number or amplification of the *TERT* gene can also result in *TERT* upregulation [8–10].

The *TERT* promoter is the most important element of telomerase expression because it harbors binding sites for numerous cellular transcriptional activators and repressors that directly or indirectly regulate gene expression. Recurrent somatic mutations in the *TERT* promoter were found in cancers of the central nervous system (43%), bladder (59%), thyroid (follicular cell-derived, 10%) and skin (melanoma, 29%) [11, 12]. In particular, two hotspot C228T and C250T mutations create binding motifs for transcription factor ETS2 and increase *TERT* transcriptional activity [13]. In addition, the T349C SNP, chr5:1,295,349 T>C (rs2853669) affects telomerase activity and telomere length [14, 15]. This polymorphism can be studied in both blood and tumor samples since the results are concordant [14, 16].

In breast cancer, only limited data are available concerning the incidence of mutations and SNP in the *TERT* promoter, *TERT* gains and their relationship with *TERT* upregulation and patient prognosis. To address these issues, we investigated the mutational status and genotype of the *TERT* promoter, *TERT* gene copy number and *TERT* and *MYC* mRNA expression in two breast cancer cohorts. The first cohort comprised 77 patients with triple-negative breast cancer (TNBC) from two recent neoadjuvant trials assessing the efficacy and toxicity of anti-EGFR antibodies combined with chemotherapy [17, 18]. The patients had been followed for 5 years. The second cohort consisted of a retrospective series of 45 patients treated with standard neoadjuvant chemotherapy (NCT). Most of

them had common hormone-positive tumors and had been followed for more than 10 years.

## RESULTS

### *TERT* promoter status

We detected no activating *TERT* promoter mutations, including hotspot mutations C228T and C250T, in 77 pre-NCT breast tumor biopsies (cohort #1) and 45 post-NCT residual tumors (cohort #2). The T349C (rs2853669) SNP was identified in 42.3% of tumor biopsies and 48.9% of residual tumors (Table 1). In both #1 and #2 cohorts, most tumors with the variant C allele had a heterozygous 349 T/C genotype (37.2% and 44.4%, respectively) whereas the frequency of 349 C/C homozygotes was low (5.1% and 6.7%). Overall, the frequencies of T349C in the two cohorts were similar to those in the 1000 Genome database for Europe.

### *TERT* copy number aberrations

We found that *TERT* gains were relatively frequent events in both cohorts. Twenty tumors (25.6%) in cohort #1 and seven tumors (15.6%) in cohort #2 had *TERT* gains. *TERT* losses were rare, with the alteration occurring in three tumors (3.9%) in cohort #1 and two tumors (4.4%) in cohort #2.

### Associations of the T349C genotype and *TERT* gene copy number gains with *TERT* and *MYC* expression

We investigated whether T349C genotype and *TERT* gains were associated with changes in the expression of the *TERT* gene (Figure 1A–1B). The results were similar in the two cohorts. Tumors with 349 T/C or C/C polymorphism had increased expression of *TERT*, but the difference from the 349 T/T cases was not significant. In contrast, *TERT* gene copy number gains were associated

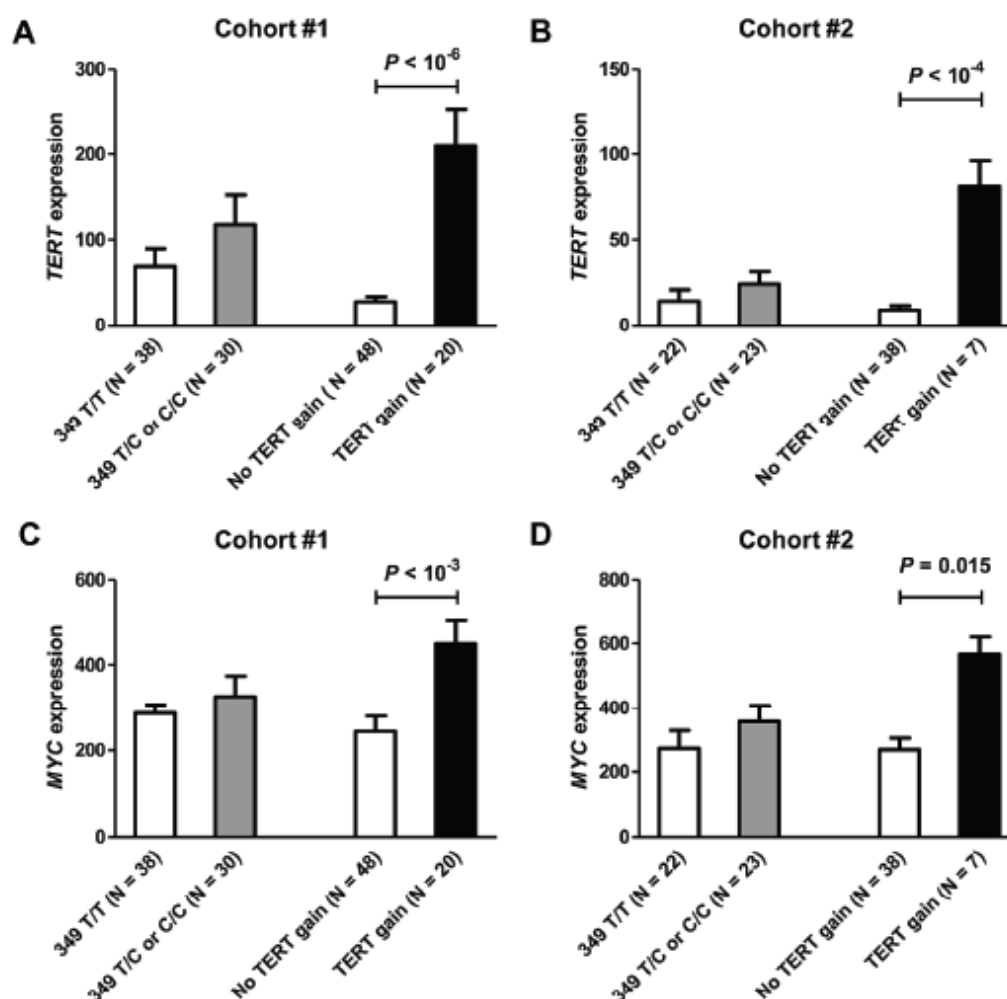
with a highly significant increase in gene expression as compared to cases without *TERT* gain. Of note, *TERT* gains were more frequent in the presence of the T349C SNP. In cases with 349 T/C or C/C genotypes, the relative risk of a *TERT* gain increased 3.2-fold ( $P = 0.0036$ , 95% confidence interval from 1.5 to 6.9) in cohort #1 and 5.7-fold in cohort #2 ( $P = 0.11$ , 95% confidence interval 1.0-32.0), as compared to the risk in 349 T/T cases.

In parallel, we investigated if the presence of *TERT* T349C polymorphism or gain was associated with the expression levels of *MYC* oncogene, a strong positive regulator of *TERT* expression (Figure 1C-1D). We found no association between the presence of the T349C SNP

and *MYC* expression. In contrast, a significantly higher expression of the *MYC* gene were observed in cases with *TERT* gains (cohort #1,  $P < 10^{-3}$ ; cohort #2,  $P = 0.015$ ). Overall, the levels of *TERT* and *MYC* expression were significantly correlated in both cohorts (Pearson  $r = 0.696$ ,  $P < 10^{-3}$  in cohort #1 and  $r = 0.542$ ,  $P < 10^{-3}$  in cohort #2).

#### Clinical significance of the T349C SNP and *TERT* gene copy number gains

The presence of the T349C SNP (349 T/C and C/C genotypes) in cohort #1 was not significantly associated with clinicopathologic characteristics such as tumor size,



**Figure 1:** The levels of *TERT* and *MYC* gene expression according to T349C status and *TERT* gene copy number in breast cancer cohorts #1 (A, C) and #2 (B, D). *TERT* gains were associated with a significant increase in *TERT* and *MYC* expression in both cohorts.

tumor grade, nodal status and pathologic response to NCT (Figure 2A). However, the rate of incomplete pathologic responses tended to be higher in T349C cases than in patients with T349T (69% vs. 51%,  $P = 0.12$ ). Patients with the T349C genotype had significantly shorter DFS and a trend toward a shorter OS (Figure 2B–2C).

In cohort #2, the T349C SNP was not associated with clinicopathologic characteristics (Figure 3A). T3–4 tumors were more frequent in T349C patients than in those with 349 T/T genotype (43% vs 23%), but not to a level of significance ( $P = 0.14$ ). Survival analysis showed, as in cohort #1, a significant effect of T349C on DFS but not on OS (Figure 3B).

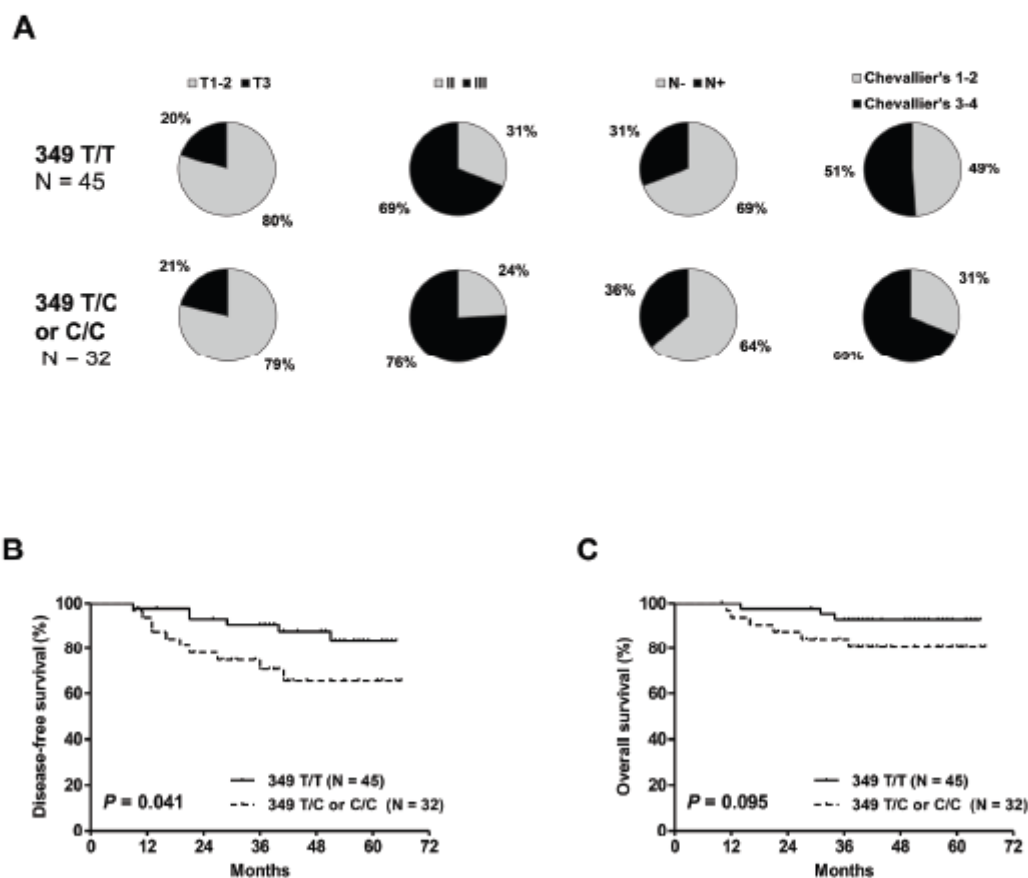
Patients with *TERT* gains in cohort #1 had a trend toward a higher proportion of SBR grade III tumors ( $P = 0.13$ ) and a significantly higher rate of incomplete pathologic responses ( $P < 0.01$ , Figure 4A). No association with other clinicopathologic characteristics was observed.

Both DFS and OS were significantly shorter in patients with *TERT* gains (Figure 4B–4C).

In cohort #2, the number of cases with *TERT* gains was small ( $n=7$ ) and the distribution of clinicopathologic features among cases with and without gains was not significantly different (Figure 5A). However, the occurrence of a *TERT* gain was significantly related to shorter DFS and OS (Figure 5B–5C).

### MYC expression levels impact the prognostic value of *TERT* gains

Univariate Cox analysis for DFS in cohort #1 showed that the presence of the T349C genotype and *TERT* gain increased significantly the risk of tumor recurrence with HR of 2.8 and 3.8, respectively (Table 2). High *MYC* expression ( $>$  median) also had a significant and negative effect on DFS (HR of 3.5). Importantly, very high levels



**Figure 2:** Distribution of clinicopathologic characteristics: tumor size, tumor grade, nodal status and pathologic response (A) disease-free (B) and overall survival (C) in cohort #1 according to T349C status. Patients with 349 T/C or C/C alleles had significantly shorter DFS and a trend toward a shorter OS.

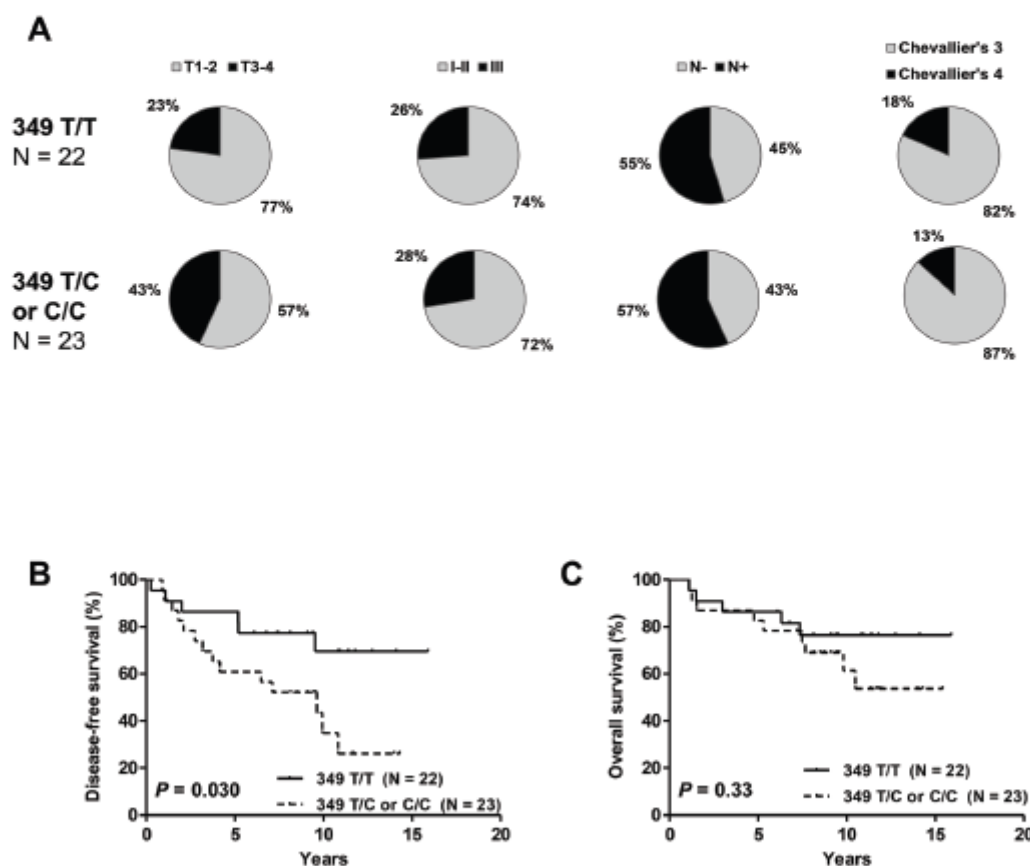


of *MYC* expression (upper quartile, > 421) were associated with a much higher risk of tumor recurrence (HR of 9.4, 95%-CI: 3.2-27.2,  $P < 0.0001$ ). As expected, incomplete pathologic responses to NCT according to Chevallier's classification had a significant negative prognostic effect (HR of 3.6). When adjusted for pathologic responses, *TERT* gain and *MYC* overexpression remained significant prognostic factors (Table 2). As mentioned above, tumors with *TERT* gain frequently had *MYC* overexpression, and it was assumed that the presence of both abnormalities would confer a worse prognosis. Indeed, the combination of *MYC* overexpression with *TERT* gains was associated with the shortest DFS and OS, whereas lower *MYC* levels annulled any negative effect of *TERT* gain on DFS and OS (Figure 6A–6B). In cases without *TERT* gain, *MYC* levels were not prognostic (Figure 6C–6D).

## DISCUSSION

Telomerase activation protects malignant cells from apoptosis and senescence by preventing further telomere loss [2, 3, 19]. Regulation of telomerase activation in cancer is complex and multifactorial and involves transcriptional activation of the *TERT* gene [10].

Activating somatic mutations of the *TERT* promoter have been reported in various cancers [11, 12]. The two most common mutations, C228T and C250T, map -124 and -146 bp upstream of the *TERT* ATG site (chr5: 1,295,228 C>T and 1,295,250 C>T, respectively) and create binding sites for ETS/TCF transcription factors, which leads to a two- to four-fold increase in transcriptional activity [13, 20]. These mutations were not found in limited breast cancer series [11, 12]. Our results from a total of 122



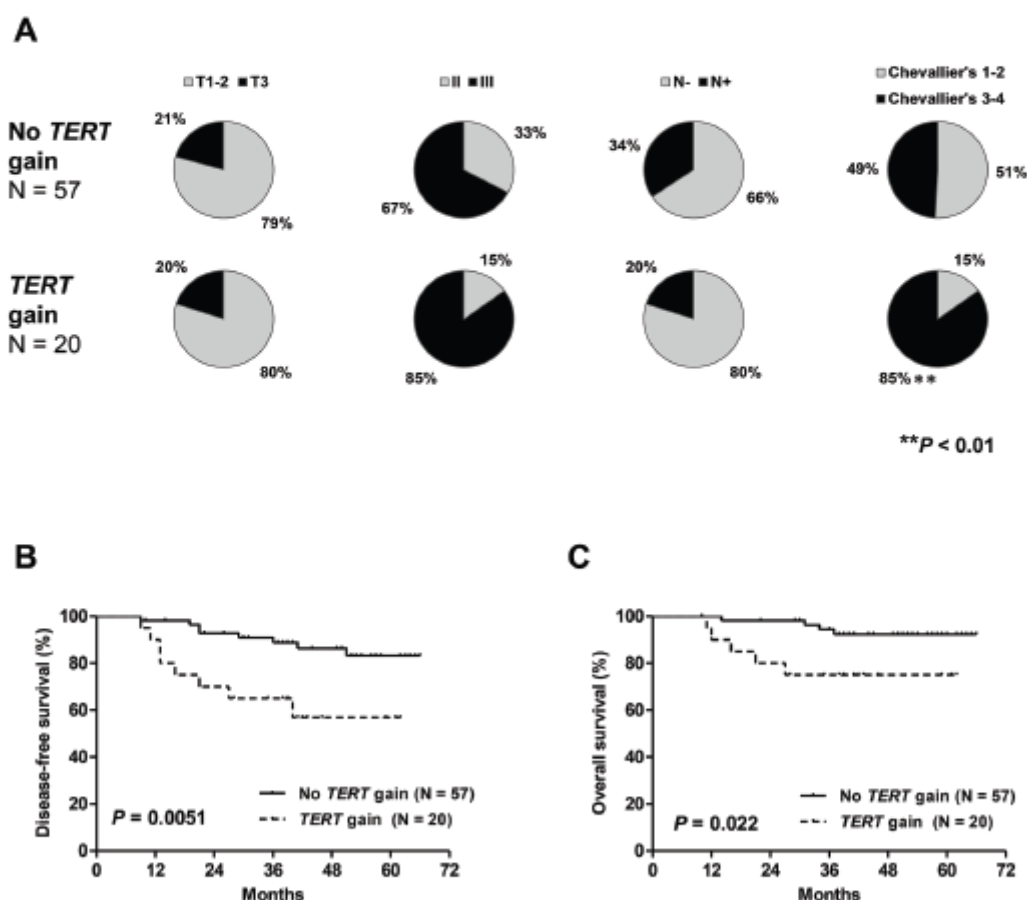
**Figure 3:** Distribution of clinicopathologic characteristics: tumor size, tumor grade, nodal status and pathologic response (A) disease-free (B) and overall survival (C) in cohort #2 according to T349C status. Patients with 349 T/C or C/C alleles had significantly shorter DFS but no difference in OS.

breast tumors evidenced no *TERT* promoter mutations, suggesting that this mutational mechanism is not likely to be involved in *TERT* upregulation in breast cancer.

The variant C-allele of the rs2853669 (T349C) polymorphism disrupts an ETS2-binding site (EtsA) in the proximal region of the *TERT* promoter [14]. Although decreased binding of transcription factor ETS2 can modify the expression of the gene, opinions differ on its effect on the level of *TERT* expression in cancer cells. Our results from cases of breast cancer did not show any significant effect of the T349C SNP on *TERT* expression. Previous studies showed an association of the T349C genotype with reduced *TERT* expression in non-small cell lung cancer, bladder cancer and glioblastoma [16, 21, 22]. However, a recent report on four hepatocellular carcinoma cell lines described increased luciferase *TERT*

promoter activity in the presence of the T349C SNP [23]. The T349C variant site is close to (2 bp downstream) the binding site of transcriptional repressor E2F1, and T/C and C/C variants seem to inhibit E2F1 binding to the *TERT* promoter. In the presence of activating *TERT* mutations, this SNP was suggested to enhance *TERT* transcription levels by blocking E2F1 binding to its promoter. These recent findings suggest that the cooperation of native and changed (mutations, SNP) binding sites for multiple transcriptional factors in the *TERT* promoter could regulate *TERT* expression and result in cancer-specific effects of SNP.

There has been much debate about the clinical effect of the T349C SNP on the survival of patients with malignancies. Several studies reported its positive prognostic value in the context of activating *TERT*



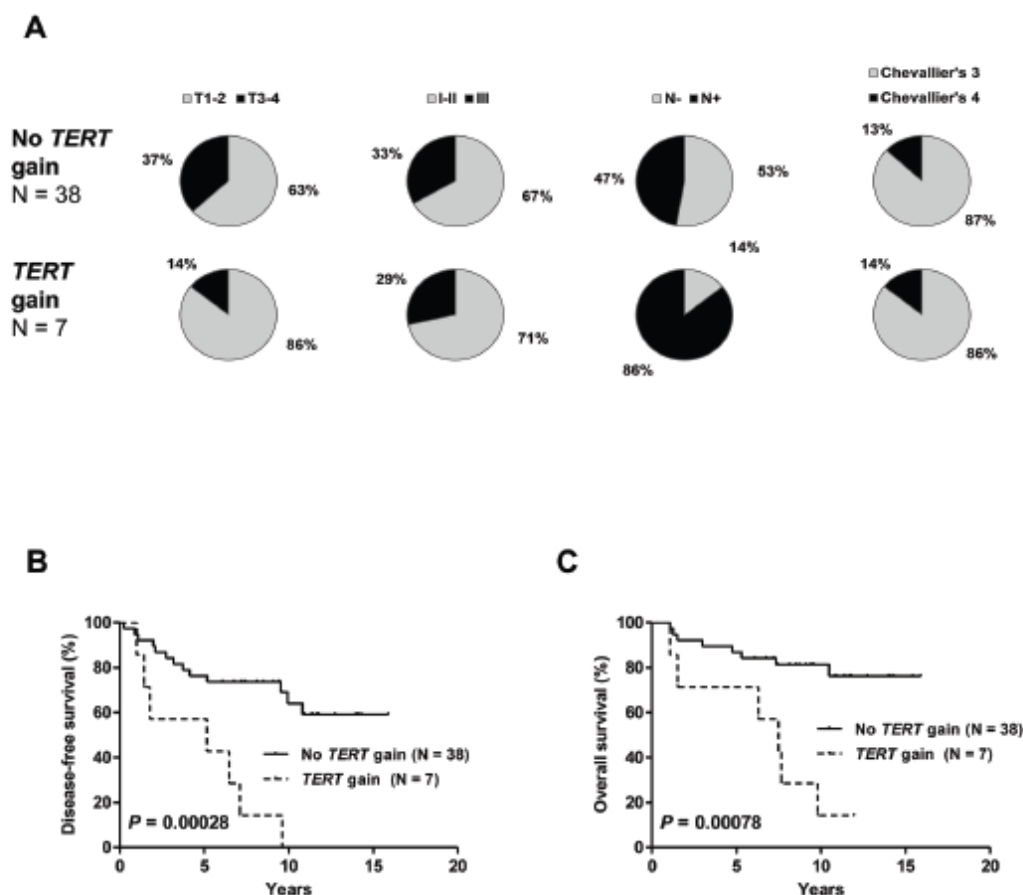
**Figure 4:** Distribution of clinicopathologic characteristics: tumor size, tumor grade, nodal status and pathologic response (A) disease-free (B) and overall survival (C) in patients from cohort #1 with and without *TERT* gene copy number gain. Patients with *TERT* gains had a significantly higher rate of incomplete pathologic responses and significantly shorter DFS and OS.

promoter mutations in bladder cancer and clear cell renal cell carcinoma [22, 24, 25]. However, more recent reports have shown that this SNP has a negative effect in glioblastoma and liver cancer patients bearing activating *TERT* promoter mutations [23, 26]. Among acute myeloid leukemia patients without *TERT* promoter mutations, T349C carriers had significantly shorter survival times [27]. Our study shows for the first time a negative effect of the T349C SNP on survival in breast cancer patients in the absence of activating somatic mutations. However, this SNP was significantly associated with *TERT* copy number gains in our patient series.

Increased *TERT* gene dosage was seen in about 30% of various human tumor-derived cell lines, including breast cancer cells [28]. We found *TERT* copy number gains in more than 20% of primary breast tumors examined.

Previous studies yielded conflicting results regarding the effect of *TERT* gain on *TERT* mRNA expression. *TERT* gene gains were associated with higher *TERT* mRNA expression in non-small-cell lung adenocarcinoma and Merkel cell carcinoma [29, 30] and with high *TERT* protein expression in breast cancer and cervical carcinomas [28, 31]. However, no significant association was found in colorectal carcinomas and squamous-cell lung cancer [30, 32]. Our observation of a highly positive correlation between the *TERT* gene copy number and its mRNA level suggests that *TERT* gain has a functional effect on *TERT* transcription in breast cancer.

We showed that *TERT* gains identified in pre-NCT biopsies were predictive of incomplete pathological responses and shorter survival. In addition, *TERT* gain was a significant predictor of worse survival when assessed in



**Figure 5:** Distribution of clinicopathologic characteristics: tumor size, tumor grade, nodal status and pathologic response (A) disease-free (B) and overall survival (C) in patients from cohort #2 with and without *TERT* gene copy number gain. Patients with *TERT* gains had a significantly higher rate of incomplete pathologic responses and significantly shorter DFS and OS.

Table 2: Cox analysis for disease-free survival in breast cancer cohort #1

Parameters	Unadjusted		Adjusted*	
	HR (95% CI)	P	HR (95% CI)	P
<i>TERT</i> gain (yes vs no)	3.8 (1.4-10.3)	0.0078	3.0 (1.1-8.4)	0.032
<i>TERT</i> T349C (yes vs no)	2.8 (1.0-7.7)	0.047	2.3 (0.9-6.6)	0.096
<i>MYC</i> (high vs low)**	3.5 (1.1-10.8)	0.031	3.2 (1.1-10.1)	0.045
Chevallier's classification (3-4 vs 1-2)	3.6 (1.1-12.6)	0.048	-	-

\*Adjusted for treatment response according to Chevallier's classification: incomplete pathologic response, classes 3-4, vs complete pathologic response, classes 1-2.

\*\*High > median, low ≤ median (181)

Hazard ratio, HR; 95% confidence interval, 95% CI.

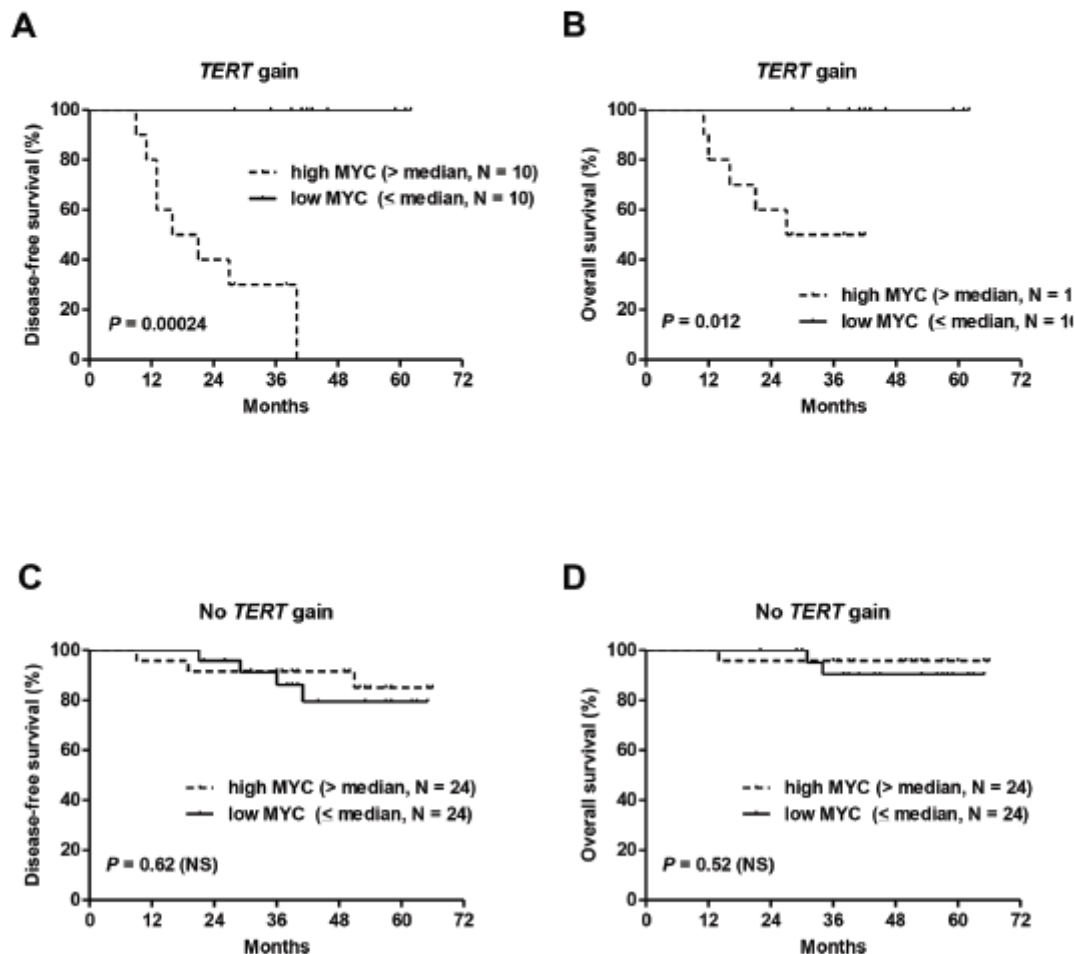


Figure 6: The combination of *TERT* gene copy number gains and *MYC* overexpression was associated with the shortest DFS and OS in cohort #1. Lower *MYC* levels annulled the negative effect of *TERT* gains on DFS and OS (A, B). In cases without *TERT* gains, *MYC* levels had no effect on patient survival (C, D).



residual post-NCT tumors. To our knowledge, only two studies have reported a negative effect of *TERT* gains on survival in non-small cell lung carcinomas and melanomas [30, 33]. For the first time, we show that *TERT* gain is also a negative prognostic marker associated with higher risk of disease recurrence or death from breast cancer and could potentially be used to adapt treatment strategies.

*TERT* upregulation correlates with increased telomerase activity. Inhibition of telomerase in breast tumors might potentiate the effects of standard anti-cancer treatments [34]. This opens up the perspective of using telomerase inhibitors in patients resistant to NCT. Preclinical studies with the telomerase inhibitor GRN163L showed that it can restore sensitivity of HER2+ breast cancer cell lines to trastuzumab and so patients with HER2+ breast cancers were recruited into a phase I clinical trial (NCT 01265927). In combination with chemotherapy, GRN163L was used in a phase II clinical trial in patients with locally recurrent or metastatic breast cancer (NCT01256762). These clinical trials have been completed but the study results are not yet available [34].

Telomerase reactivation and/or *TERT* upregulation correlate with *MYC* overexpression [35, 36]. In line with these findings, we observed that *MYC* and *TERT* expression levels are significantly correlated. *MYC* is a primary transcription factor of the *TERT* gene and stimulates its promoter activity [37, 38]. In breast cancer cells, *MYC* binds to an ETS2 site (EtsA) in the *TERT* gene promoter and increases *TERT* expression levels [15]. As mentioned above, this binding site is disrupted by T349C variants, but we observed no significant changes in *TERT* or *MYC* expression according to the presence or absence of this SNP. In contrast, *MYC* overexpression was strongly associated with the presence of *TERT* gains. Interestingly, a recent study showed that *TERT* regulates *MYC* ubiquitination, stabilization and binding to its target gene promoters [39]. This feed-forward transcriptional loop between *MYC* and *TERT* is functionally critical for oncogenesis. In our study, we found that *MYC* overexpression often coincided with *TERT* gains. Enhanced *MYC* stability and function in cells with high *TERT* and *MYC* levels could potentiate *MYC*-dependent oncogenesis.

By combining the data on *TERT* gain and *MYC* overexpression we were able to isolate a particularly bad prognosis subgroup of breast cancer patients. *MYC* is considered to promote tumor cell survival, proliferation and progression in breast cancer [40, 41] and regulates the expression of 13 different poor-outcome cancer signatures [42]. We found that *MYC* overexpression had a negative prognostic value in the whole patient population and a particularly significant effect within the subgroup of patients with *TERT* gains. A concomitant upregulation of *TERT* and *MYC* identified patients with a high risk of breast cancer recurrence. In contrast, lower *MYC* expression levels were associated with a favorable prognosis, despite the presence of *TERT* gains.

In conclusion, we found no activating *TERT* promoter mutations in 122 breast cancer patients. The T349C *TERT* promoter SNP was not significantly associated with *TERT* expression but T349C carriers had shorter survival. Notably, *TERT* gene copy number gain was significantly related to *TERT* upregulation and, in association with *MYC* overexpression, characterized particularly aggressive disease. These results show a significant effect of gene copy number gain on *TERT* expression level and provide a new insight into the clinical significance of *TERT* and *MYC* upregulation in breast cancer.

## MATERIALS AND METHODS

### Patients and samples

Cohort #1 comprised 77 patients with operable, stage II–III TNBC enrolled in two neoadjuvant trials evaluating the efficacy and toxicity of anti-EGFR antibodies plus chemotherapy: panitumumab combined with a standard neoadjuvant anthracycline/taxane-based [17] and cetuximab combined with docetaxel [18]. After NCT, pathologic complete responses (classes 1 and 2 according to Chevallier's classification) were observed in 32 of 77 cases (41.6%). The remaining 45 (58.4%) patients had incomplete pathologic responses (class 3, N = 39 and class 4, N = 6). The median follow-up in this cohort was 3.8 years (range, 0.9–5.5 years). During follow-up, 16 (20.8%) patients relapsed and 11 (14.3%) died from breast cancer. Cohort #2 consisted of archived samples of residual post-NCT tumors from 45 women diagnosed with invasive breast cancer and treated with NCT protocols [43] between 1996 and 2010 at the Jean Perrin Cancer Center (Clermont-Ferrand, France). Following NCT, patients underwent appropriate surgery and radiotherapy. Those with important residual disease received adjuvant chemotherapy. Menopausal patients with hormone receptor-positive tumors received tamoxifen for 5 years. The mean follow-up was 9.1 years (range, 1.1–15.9 years). During this period, 20 (44.4%) patients relapsed and 14 (31.1%) died from breast cancer. In cohort #1, the study was performed on pre-treatment tumor biopsies and in cohort #2 on post-NCT residual tumor samples cryopreserved in the Biological Resources Center BB-0033-00075. DNA and RNA were simultaneously extracted with the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. The study was approved by the institutional review board.

### Gene expression analysis

Total RNA was converted to cDNA by reverse transcription with Superscript II reverse transcriptase (Invitrogen, Cergy-pontoise, France) according to the

manufacturer's instructions. The expression of *TERT* was quantified by real-time RT-PCR in the Lightcycler 480 System (Roche Diagnostics, Meylan, France) as described previously [44, 45]. *MYC* was quantified with primers described in the literature [46]. The normalized copy numbers were expressed as the ratio between transcript copy numbers of the target and control (*B2M*) genes multiplied by 100.

### *TERT* promotor sequencing

The amplification of genomic DNA for the *TERT* promoter region (267 pb) was performed with the following primers: forward 5'-CCGGGCTCCAGTGGATT-3' and reverse 5'-TTCCACGTGCGCAGCAG-3'. PCR conditions consisted of an initial heating at 95°C for 15 minutes followed by 40 cycles at 95°C for 30 seconds, 60°C for 20 seconds and 72°C for 30 seconds. Amplified PCR products were sequenced with BigDye v1.1 on the ABI 3500 Genetic Analyzer (Applied Biosystems, Courtaboeuf, France).

### *TERT* copy number quantification

*TERT* gene copy number was quantified by Quantitative Multiplex Fluorescent Polymerase Chain Reaction (QMF-PCR) analysis. QMF-PCR was performed on a TProfessional thermocycler (Biometra, Archamps, France) as previously described [47, 48]. Primers were designed for three fragments of the *TERT* gene on chromosome 5p (5'UTR, Exon 9 and 3'UTR) to cover the entire gene. The 5'UTR region is very GC-rich and was difficult to amplify. We therefore had to limit the analysis to exon 9 and 3'UTR. Seven genes on chromosomes 2p, 4p, 7q, 10q, 11p, 11q (*PVRL1*, *BODIL*, *RET*, *ZNF638*, *AGBL2*, *CFTR* and *POR*) were co-amplified as controls. Primers and technical details are described in Supplementary Methods. The fluorescence intensities of PCR products were correlated with the copy number of the relevant exons. Fourteen control DNAs were included in each experiment: 12 normal DNAs and 3 DNAs with known *TERT* copy number (loss, normal and gain, as determined by array-comparative genomic hybridization). A dosage quotient was calculated relative to all the other amplified exons in patients and controls. The range of ratios corresponding to two copies of *TERT* gene was set between 0.8 and 1.2 (mean  $\pm$  3 standard deviations of values obtained in normal DNA samples). Ratios  $>$  1.2 were considered as gains and  $<$  0.8 as losses.

### Statistical analysis

Standard tests (Kruskal-Wallis H test, ANOVA, Student's t-test, chi-squared test, Pearson's correlation) were used to study the relationship between characteristics. Disease-free survival (DFS) was measured from diagnosis until the first relapse (local or distant) and overall survival

(OS) from the start of NCT until the last follow-up report. Survival curves were established by the Kaplan-Meier method and compared with the log-rank test.

### Author contributions

MGB performed the experimental work, analysed the data and drafted the manuscript. LV, PC, EEP, MMD, AC and MP contributed to the experimental work. FK performed statistical analyses. CA, YJB, MAMR and PV contributed to the data analysis. AT and FPL designed and supervised the study. AT wrote the final manuscript. All authors contributed to the final version of the manuscript.

### CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

### GRANT SUPPORT

This work was supported by the Ligue contre le Cancer (Comité du Puy-de-Dôme, France)

### REFERENCES

- Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. *Eur J Cancer Oxf Engl* 1990; 33: 787-91.
- Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; 144: 646-74.
- Poonepalli A, Banerjee B, Ramnarayanan K, Palanisamy N, Putti TC, Hande MP. Telomere-mediated genomic instability and the clinico-pathological parameters in breast cancer. *Genes Chromosomes Cancer* 2008; 47: 1098-109.
- Lu L, Zhang C, Zhu G, Irwin M, Risch H, Menato G, Mitidieri M, Katsaros D, Yu H. Telomerase expression and telomere length in breast cancer and their associations with adjuvant treatment and disease outcome. *Breast Cancer Res BCR* 2011; 13: R56.
- Gay-Bellile M, Romero P, Cayre A, Veronèse L, Privat M, Singh S, Combes P, Kwiatkowski F, Abrial C, Bignon Y-J, Vago P, Penault-Llorca F, Tchirikov A. ERCC1 and telomere status in breast tumours treated with neoadjuvant chemotherapy and their association with patient prognosis. *J Pathol Clin Res* 2016; 2: 234-46.
- Liu T, Yuan X, Xu D. Cancer-Specific Telomerase Reverse Transcriptase (TERT) Promoter Mutations: Biological and Clinical Implications. *Genes* 2016; 7.
- Wu KJ, Grandori C, Amacker M, Simon-Vermot N, Polack A, Lingner J, Dalla-Favera R. Direct activation of TERT transcription by c-MYC. *Nat Genet* 1999; 21: 220-4.
- Cao Y, Bryan TM, Reddel RR. Increased copy number of the TERT and TERC telomerase subunit genes in cancer cells. *Cancer Sci* 2008; 99: 1092-9.
- Hahn WC, Meyerson M. Telomerase activation, cellular immortalization and cancer. *Ann Med* 2001; 33: 123-9.



10. Zhu J, Zhao Y, Wang S. Chromatin and epigenetic regulation of the telomerase reverse transcriptase gene. *Protein Cell*. 2010; 1: 22–32.
11. Killela PJ, Reitman ZJ, Jiao Y, Bettgeowda C, Agrawal N, Diaz LA, Friedman AH, Friedman H, Gallia GL, Giovannella BC, Grollman AP, He T-C, He Y, et al. TERT promoter mutations occur frequently in gliomas and a subset of tumors derived from cells with low rates of self-renewal. *Proc Natl Acad Sci U S A*. 2013; 110: 6021–6.
12. Vinagre J, Almeida A, Pópulo H, Batista R, Lyra J, Pinto V, Coelho R, Celestino R, Prazeres H, Lima L, Melo M, da Rocha AG, Preto A, et al. Frequency of TERT promoter mutations in human cancers. *Nat Commun*. 2013; 4: 2185.
13. Huang FW, Hodis E, Xu MJ, Kryukov GV, Chin L, Garraway LA. Highly recurrent TERT promoter mutations in human melanoma. *Science*. 2013; 339: 957–9.
14. Hsu C-P, Hsu N-Y, Lee L-W, Ko J-L. Ets2 binding site single nucleotide polymorphism at the hTERT gene promoter—effect on telomerase expression and telomere length maintenance in non-small cell lung cancer. *Eur J Cancer Oxf Engl* 1990. 2006; 42: 1466–74.
15. Xu D, Dwyer J, Li H, Duan W, Liu J-P. Ets2 maintains hTERT gene expression and breast cancer cell proliferation by interacting with c-Myc. *J Biol Chem*. 2008; 283: 23567–80.
16. Park C-K, Lee S-H, Kim JY, Kim JE, Kim TM, Lee S-T, Choi SH, Park S-H, Kim IH. Expression level of hTERT is regulated by somatic mutation and common single nucleotide polymorphism at promoter region in glioblastoma. *Oncotarget*. 2014; 5: 3399–407.
17. Nabholz JM, Abrial C, Mouret-Reynier MA, Dauplat MM, Weber B, Gligorov J, Forest AM, Tredan O, Vanlemmens L, Petit T, Guin S, Van Praagh I, Jouannaud C, et al. Multicentric neoadjuvant phase II study of panitumumab combined with an anthracycline/taxane-based chemotherapy in operable triple-negative breast cancer: identification of biologically defined signatures predicting treatment impact. *Ann Oncol Off J Eur Soc Med Oncol ESMO*. 2014; 25: 1570–7.
18. Nabholz JM, Chalabi N, Radosevic-Robin N, Dauplat MM, Mouret-Reynier MA, Van Praagh I, Servent V, Jacquin JP, Benmammar KE, Kullab S, Bahadoor MRK, Kwiatkowski F, Cayre A, et al. Multicentric neoadjuvant pilot Phase II study of cetuximab combined with docetaxel in operable triple negative breast cancer. *Int J Cancer*. 2016; 138: 2274–80.
19. Bièche I, Noguès C, Paradis V, Olivi M, Bedossa P, Lidereau R, Vidaud M. Quantitation of hTERT gene expression in sporadic breast tumors with a real-time reverse transcription-polymerase chain reaction assay. *Clin Cancer Res Off J Am Assoc Cancer Res*. 2000; 6: 452–9.
20. Brennan CW, Verhaak RGW, McKenna A, Campos B, Nounshahr H, Salama SR, Zheng S, Chakravarty D, Sanborn JZ, Berman SH, Beroukhim R, Bernard B, Wu C-J, et al. The somatic genomic landscape of glioblastoma. *Cell*. 2013; 155: 462–77.
21. Labussière M, Di Stefano AL, Gleize V, Boisselier B, Giry M, Mangesius S, Bruno A, Paterra R, Marie Y, Rahimian A, Finocchiaro G, Houlston RS, Hoang-Xuan K, et al. TERT promoter mutations in gliomas, genetic associations and clinico-pathological correlations. *Br J Cancer*. 2014; 111: 2024–32.
22. Rachakonda PS, Hosen I, de Verdier PJ, Fallah M, Heidenreich B, Ryk C, Wiklund NP, Steineck G, Schadendorf D, Hemminki K, Kumar R. TERT promoter mutations in bladder cancer affect patient survival and disease recurrence through modification by a common polymorphism. *Proc Natl Acad Sci U S A*. 2013; 110: 17426–31.
23. Ko E, Seo H-W, Jung ES, Kim B-H, Jung G. The TERT promoter SNP rs2853669 decreases E2F1 transcription factor binding and increases mortality and recurrence risks in liver cancer. *Oncotarget*. 2016; 7: 684–99.
24. Hosen I, Rachakonda PS, Heidenreich B, de Verdier PJ, Ryk C, Steineck G, Hemminki K, Kumar R. Mutations in TERT promoter and FGFR3 and telomere length in bladder cancer. *Int J Cancer J Int Cancer*. 2015; 137: 1621–9. <https://doi.org/10.1002/ijc.29526>.
25. Hosen I, Rachakonda PS, Heidenreich B, Sitaram RT, Ljungberg B, Roos G, Hemminki K, Kumar R. TERT promoter mutations in clear cell renal cell carcinoma. *Int J Cancer J Int Cancer*. 2015; 136: 2448–52.
26. Mosrati MA, Malmström A, Lysiak M, Krysztofiak A, Hallbeck M, Milos P, Hallbeck A-L, Brathall C, Strandéus M, Stenmark-Askmal M, Söderkvist P. TERT promoter mutations and polymorphisms as prognostic factors in primary glioblastoma. *Oncotarget*. 2015; 6: 16663–73.
27. Mosrati MA, Willander K, Falk U, Hermanson M, Höglund M, Stockelberg D, Wei Y, Lotfi K, Söderkvist P. Association between TERT promoter polymorphisms and acute myeloid leukemia risk and prognosis. *Oncotarget*. 2015; 6: 25109–20.
28. Zhang A, Zheng C, Lindvall C, Hou M, Ekedahl J, Lewensohn R, Yan Z, Yang X, Henriksson M, Blennow E, Nordenskjöld M, Zetterberg A, Björkholm M, et al. Frequent amplification of the telomerase reverse transcriptase gene in human tumors. *Cancer Res*. 2000; 60: 6230–5.
29. Xie H, Liu T, Wang N, Björnhagen V, Höög A, Larsson C, Lui W-O, Xu D. TERT promoter mutations and gene amplification: promoting TERT expression in Merkel cell carcinoma. *Oncotarget*. 2014; 5: 10048–57.
30. Zhu C-Q, Cutz J-C, Liu N, Lau D, Shepherd FA, Squire JA, Tsao M-S. Amplification of telomerase (hTERT) gene is a poor prognostic marker in non-small-cell lung cancer. *Br J Cancer*. 2006; 94: 1452–9.
31. Zhang A, Zheng C, Hou M, Lindvall C, Wallin K-L, Angström T, Yang X, Hellström A-C, Blennow E,

- Björkholm M, Zetterberg A, Gruber A, Xu D. Amplification of the telomerase reverse transcriptase (hTERT) gene in cervical carcinomas. *Genes Chromosomes Cancer*. 2002; 34: 269–75.
32. Palmqvist R, Zhang A, Xu D, Golovleva I, Norrback K-F, Gruber A, Oberg A, Stenling R, Roos G. hTERT gene copy number is not associated with hTERT RNA expression or telomerase activity in colorectal cancer. *Int J Cancer*. 2005; 116: 395–400.
33. Diaz A, Puig-Butillé JA, Muñoz C, Costa D, Díez A, Garcia-Herrera A, Carrera C, Badenas C, Solé F, Malvehy J, Puig S, Alos L. TERT gene amplification is associated with poor outcome in acral lentiginous melanoma. *J Am Acad Dermatol*. 2014; 71: 839–41.
34. Ruden M, Puri N. Novel anticancer therapeutics targeting telomerase. *Cancer Treat Rev*. 2013; 39: 444–56.
35. Latil A, Vidaud D, Valéri A, Fournier G, Vidaud M, Lidereau R, Cussenot O, Bièche I. htert expression correlates with MYC over-expression in human prostate cancer. *Int J Cancer J Int Cancer*. 2000; 89: 172–6.
36. Wang J, Xie LY, Allan S, Beach D, Hannon GJ. Myc activates telomerase. *Genes Dev*. 1998; 12: 1769–74.
37. Dwyer J, Li H, Xu D, Liu J-P. Transcriptional regulation of telomerase activity: roles of the Ets transcription factor family. *Ann N Y Acad Sci*. 2007; 1114: 36–47.
38. Li H, Xu D, Li J, Berndt MC, Liu J-P. Transforming Growth Factor  $\beta$  Suppresses Human Telomerase Reverse Transcriptase (hTERT) by Smad3 Interactions with c-Myc and the hTERT Gene. *J Biol Chem*. 2006; 281: 25588–600.
39. Koh CM, Khattar E, Leow SC, Liu CY, Muller J, Ang WX, Li Y, Franzoso G, Li S, Guccione E, Tergaonkar V. Telomerase regulates MYC-driven oncogenesis independent of its reverse transcriptase activity. *J Clin Invest*. 2015; 125: 2109–22.
40. Chen Y, Yang Y, van Overbeek M, Donigian JR, Baciú P, de Lange T, Lei M. A shared docking motif in TRF1 and TRF2 used for differential recruitment of telomeric proteins. *Science*. 2008; 319: 1092–6.
41. Wolfer A, Ramaswamy S. MYC and metastasis. *Cancer Res*. 2011; 71: 2034–7.
42. Wolfer A, Wittner BS, Irimia D, Flavin RJ, Lupien M, Gunawardane RN, Meyer CA, Lightcap ES, Tamayo P, Mesirov JP, Liu XS, Shioda T, Toner M, et al. MYC regulation of a “poor-prognosis” metastatic cancer cell state. *Proc Natl Acad Sci U S A*. 2010; 107: 3698–703.
43. Penault-Llorca F, Abrial C, Raoelfils I, Cayre A, Mouret-Reynier M-A, Leheutier M, Durando X, Achard J-L, Gimbergues P, Chollet P. Comparison of the prognostic significance of Chevallier and Sataloff's pathologic classifications after neoadjuvant chemotherapy of operable breast cancer. *Hum Pathol*. 2008; 39: 1221–8.
44. Poncet D, Belleville A, t'kint de Roodenbeke C, Roborel de Climens A, Ben Simon E, Merle-Beral H, Callet-Bauchu E, Salles G, Sabatier L, Delic J, Gilson E. Changes in the expression of telomere maintenance genes suggest global telomere dysfunction in B-chronic lymphocytic leukemia. *Blood*. 2008; 111: 2388–91.
45. Véronèse L, Tournilhac O, Callanan M, Prie N, Kwiatkowski F, Combes P, Chauvet M, Davi F, Gouas L, Verrelle P, Guéze R, Vago P, Bay JO, et al. Telomeres and chromosomal instability in chronic lymphocytic leukemia. *Leukemia*. 2013; 27: 490–3.
46. Mitas M, Mikhitarian K, Walters C, Baron PL, Elliott BM, Brothers TE, Robison JG, Metcalf JS, Palesch YY, Zhang Z, Gillanders WE, Cole DJ. Quantitative real-time RT-PCR detection of breast cancer micrometastasis using a multigene marker panel. *Int J Cancer*. 2001; 93: 162–71.
47. Niel F, Martin J, Dastot-Le Moal F, Costes B, Boissier B, Delattre V, Goossens M, Girodon E. Rapid detection of CFTR gene rearrangements impacts on genetic counselling in cystic fibrosis. *J Med Genet*. 2004; 41: e118.
48. Pebrel-Richard C, Kemeny S, Gouas L, Eymard-Pierre E, Blanc N, Francannet C, Tchirkov A, Goumy C, Vago P. An atypical 0.8 Mb inherited duplication of 22q11.2 associated with psychomotor impairment. *Eur J Med Genet*. 2012; 55: 650–5.

Article

# Telomere Status of Advanced Non-Small-Cell Lung Cancer Offers a Novel Promising Prognostic and Predictive Biomarker

Eve Faugeras <sup>1,2</sup>, Lauren Véronèse <sup>3,4</sup>, Gaëlle Jeannin <sup>1</sup>, Henri Janicot <sup>1</sup>, Sébastien Bailly <sup>5</sup>, Jacques-Olivier Bay <sup>4,5</sup>, Bruno Pereira <sup>6</sup>, Anne Cayre <sup>7</sup>, Frédérique Penault-Llorca <sup>2,7</sup>, Florent Cachin <sup>2,8</sup>, Patrick Merle <sup>1,2</sup> and Andrei Tchirkov <sup>3,4,\*</sup>

- <sup>1</sup> Service de Pneumologie, CHU Clermont-Ferrand, 63000 Clermont-Ferrand, France
- <sup>2</sup> UMR1240 "Imagerie Moléculaire et Stratégies Théranostiques", Université Clermont Auvergne, INSERM, 63000 Clermont-Ferrand, France
- <sup>3</sup> Service de Cytogénétique Médicale, CHU Clermont-Ferrand, 63000 Clermont-Ferrand, France
- <sup>4</sup> EA7453 "Clonal Heterogeneity, Leukemic Environment, Therapy Resistance of Chronic Leukemias", Université Clermont Auvergne, 63000 Clermont-Ferrand, France
- <sup>5</sup> Service d'Hématologie Clinique, CHU Clermont-Ferrand, 63000 Clermont-Ferrand, France
- <sup>6</sup> Délégation à la Recherche Clinique et à l'Innovation, CHU de Clermont-Ferrand, 63000 Clermont-Ferrand, France
- <sup>7</sup> Centre Jean Perrin, Département de Pathologie, 63000 Clermont-Ferrand, France
- <sup>8</sup> Service de Médecine Nucléaire, Centre Jean Perrin, 63000 Clermont-Ferrand, France

\* Correspondence: atchirkov@chu-clermontferrand.fr; Tel.: +33-4-73-750-707



Citation: Faugeras, E.; Véronèse, L.; Jeannin, G.; Janicot, H.; Bailly, S.; Bay, J.-O.; Pereira, B.; Cayre, A.; Penault-Llorca, F.; Cachin, F.; et al. Telomere Status of Advanced Non-Small-Cell Lung Cancer Offers a Novel Promising Prognostic and Predictive Biomarker. *Cancers* **2023**, *15*, 290. <https://doi.org/10.3390/cancers15010290>

Academic Editor: Yatsuya Nagano

Received: 30 November 2022

Revised: 18 December 2022

Accepted: 19 December 2022

Published: 31 December 2022



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

**Simple Summary:** Short, dysfunctional telomeres represent the genetic biomarkers of cancer. Studies in early-stage non-small-cell lung cancer (NSCLC) have shown that telomere length and telomerase levels are correlated with survival. In patients with advanced NSCLC, telomere status has not yet been investigated, and its clinical significance remains unknown. We studied telomere length and the expression of telomerase and shelterin genes in a cohort of 79 patients with advanced NSCLC, and evaluated these parameters as potential prognostic and predictive factors. Telomere shortening, high levels of telomerase and aberrant expression of shelterin genes *TRF2*, *RAP1* and *TIN2* were significantly correlated with shorter survival. Furthermore, a worse response to immunotherapy was observed in patients with shorter telomeres. The determination of telomere parameters in advanced NSCLC could be useful for individualized treatment decisions.

**Abstract:** Telomere length appears to correlate with survival in early non-small-cell lung cancer (NSCLC), but the prognostic impact of telomere status in advanced NSCLC remains undetermined. Our purpose was to evaluate telomere parameters as prognostic and predictive biomarkers in advanced NSCLC. In 79 biopsies obtained before treatment, we analyzed the telomere length and expression of *TERT* and shelterin complex genes (*TRF1*, *TRF2*, *POT1*, *TPP1*, *RAP1*, and *TIN2*), using quantitative PCR. Non-responders to first-line chemotherapy were characterized by shorter telomeres and low *RAP1* expression ( $p = 0.0035$  and  $p = 0.0069$ ), and tended to show higher *TERT* levels ( $p = 0.058$ ). In multivariate analysis, short telomeres were associated with reduced event-free (EFS,  $p = 0.0023$ ) and overall survival (OS,  $p = 0.00041$ ). *TERT* and *TRF2* overexpression correlated with poor EFS ( $p = 0.0069$  and  $p = 0.00041$ ) and OS ( $p = 0.0051$  and  $p = 0.007$ ). Low *RAP1* and *TIN2* expression-levels were linked to reduced EFS ( $p = 0.00032$  and  $p = 0.0069$ ) and OS ( $p = 0.000051$  and  $p = 0.02$ ). Short telomeres were also associated with decreased survival after nivolumab therapy ( $p = 0.097$ ). Evaluation of telomere status in advanced NSCLC emerges as a useful biomarker that allows for the selection of patient groups with different clinical evolutions, to establish personalized treatment.

**Keywords:** advanced NSCLC; telomere length; *TERT*; shelterin complex; prognosis



## 1. Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide [1]. Non-small-cell lung cancer (NSCLC) is the most frequent lung cancer, accounting for 85% of all cases. NSCLC is frequently diagnosed at an advanced stage, and the overall prognosis is poor. Nevertheless, treatment responses and survival times are different in individual patients, which could be explained by biological heterogeneity of NSCLC [2]. This underlines the importance of biomarkers to identify subjects at higher risk of drug resistance and disease progression, who would benefit from more specific therapies. Furthermore, therapies targeting immune checkpoints have changed the treatment of NSCLC, enabling longer survival for patients with advanced disease. However, progression rates reported with nivolumab are above 30% [3,4], and approximately 14% of cases have hyperprogressive disease [5]. An early switch to salvage treatment in these patients should be considered, but no predictive factor has yet been identified.

Dysfunctional telomeres represent the genetic biomarkers of cancer [6,7]. Telomeres are nucleoprotein complexes essential for the protection of chromosomal ends. Due to incomplete DNA replication, telomeres shorten with every cell division [8]. Critical telomere shortening contributes to genomic instability and promotes cancerogenesis [9]. To maintain shortened telomeres, cancer cells activate telomerase, a specialized ribonucleoprotein that regenerates telomeric DNA, which enables survival and the limitless proliferation of tumors [10]. Upregulation of *TERT* (telomerase reverse transcriptase) gene leads to telomerase activation in cancers [11]. Telomeric DNA is protected by the shelterin protein complex, which regulates telomere length (TL) and prevents the inappropriate DNA damage response at chromosomal ends [12]. Shelterin contains six proteins: POT1 (protection of telomeres 1), TRF1 and TRF2 (telomeric repeat-binding factor 1 and 2), TIN2 (TRF1-interacting nuclear protein 2), RAP1 (repressor/activator protein 1) and TPP1 (TIN2 and POT1-interacting protein). Severe telomere shortening and abnormal expression of *TERT* and shelterin-complex genes were previously correlated with cancer prognosis [6,13,14].

Although relatively rare, telomere studies in NSCLC have shown that TL and telomerase levels were correlated with survival [15,16]. However, these clinical correlations have been evaluated only for early-stage surgically resected NSCLC. Moreover, only one study explored the prognostic value of shelterin gene expression in early-stage tumors [17]. In patients with advanced NSCLC, telomere status has not yet been investigated and its clinical significance remains unknown. In the present study, we measured TL and the expression of *TERT* and shelterin genes in a cohort of 79 patients with advanced NSCLC, and evaluated these parameters as potential prognostic and predictive factors.

## 2. Materials and Methods

### 2.1. Patients and Samples

This retrospective study was performed on 79 patients with histologically proven NSCLC from the Pulmonology Department of the University Hospital of Clermont-Ferrand. For this cohort, we collected the data on demographic characteristics, performance and smoking status, histological diagnosis, mutation status, TNM stage, treatment modalities, tumor-response evaluation, and follow-up. Tumor tissue samples were obtained using fibroscopy or transparietal biopsy and snap frozen until molecular analysis. Each sample was analyzed using histology, for diagnostic purposes, which also enabled us to verify the adequate tumor-cell content (>30%).

### 2.2. DNA and RNA Extraction

DNA and RNA were simultaneously extracted from snap-frozen tumor biopsies with the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Courtaboeuf, France), in accordance with the manufacturer's instructions.

### 2.3. Assessment of Telomere Length Using Quantitative PCR

Average telomere length in tumor DNA was evaluated with quantitative real-time PCR in a LightCycler 480 System (Roche Diagnostics, Meylan, France) using SYBR Green I technology (SYBR Green Kit, Roche Diagnostics), as described elsewhere [18]. This method measures the template amounts of telomere repeat (T) and a reference single-copy gene (S), which are then used to determine the relative telomere length as the T/S ratio [19]. Glyceraldehyde-3-phosphate dehydrogenase gene was used as the reference gene. Each PCR series also included normal DNA control. The T/S ratio of tumor samples was normalized relative to the ratio obtained in the control DNA. The normalized T/S ratio of 1 corresponds to the average length of telomeres which is identical to that of the normal control DNA, whereas ratios  $T/S < 1$  indicate different degrees of telomeric shortening.

### 2.4. Quantitative RT-PCR for TERT and Shelterin Complex GENE expression

Total RNA was converted to complementary DNA by reverse transcription, using Superscript II reverse transcriptase (Invitrogen, Cergy-Pontoise, France), in accordance with the manufacturer's instructions. The expression of TERT and the shelterin complex genes TRF1, TRF2, POT1, TPP1, RAP1, and TIN2 were quantified using real-time RT-PCR in a LightCycler 480 System (Roche Diagnostics, Meylan, France), as described previously [18,20]. The normalized copy numbers (NCN) were expressed as the ratio of the numbers of transcript copies of the target and control (beta2-microglobulin) genes, multiplied by 100.

### 2.5. Statistical Analysis

Statistical analysis was performed using SEM software V1 [21]. The Student's, Kruskal-Wallis, and Mann-Whitney tests were used for comparisons between the telomere data and clinical parameters. Event-free survival (EFS) was defined as the time between the date of diagnosis and the date of occurrence of the first event. The events were death from any cause, or clinical and/or radiological progression evaluated according to RECIST criteria [22]. Overall survival (OS) was defined as the time between the date of diagnosis and the date of death from any cause. Survival analysis was performed using the univariate Cox regression models, and survival curves were established in accordance with the Kaplan-Meier method and compared using the log-rank test. Multivariate Cox regression models were used to test the independent prognostic value of the telomere parameters. A sensitivity analysis was used to determine the best cutoff values associated with EFS and OS for the telomere parameters. To be precise, for each parameter value, EFS and OS were compared between cases that were below and above this value, using the Cox proportional hazards regression. The proportional hazard hypothesis was studied using Schoenfeld's test. A sensitivity analysis was realized to find the best cutoff value for each parameter. An example of the best cutoff determination for TL is presented in Figure S1. An unsupervised hierarchical clustering was performed with SEM software [21]. Distances between clusters were calculated using 1-Pearson's correlation-coefficient values, and the dendrogram was constructed in accordance with Ward's algorithm.

## 3. Results

### 3.1. Patient Characteristics

The characteristics of the study cohort are presented in Table 1. Male smokers (active smokers or ex-smokers) represented the majority of the population. Adenocarcinoma was the most frequent type (58.2%) followed by squamous-cell carcinoma (31.7%). Eight patients had other histological types (six large-cell neuroendocrine carcinomas and two large-cell lung undifferentiated carcinomas). Two-thirds of NSCLC (70.8%) were metastatic at diagnosis. Thirteen patients had a KRAS-activating mutation, and four patients an EGFR mutation. Twenty-one patients with early NSCLC received primary local treatment with surgery (eleven patients), concomitant or sequential radiochemotherapy (eight patients) or radiotherapy alone (two patients). In advanced NSCLC, the first-line regimen was



platinum-based chemotherapy (cisplatin or carboplatin) for fifty-two patients, and anti-EGFR tyrosine-kinase-inhibitor therapy for four patients with mutated EGFR.

**Table 1.** Demographic and disease characteristics of the NSCLC cohort ( $n = 79$ ).

Characteristics	Median (Range) or No. (%)
Median age, years	65 (43–82)
Men	38 (48.1)
Smoking	74 (93.7)
Adenocarcinoma	46 (58.2)
Squamous cell carcinoma	25 (31.7)
Other types	8 (10.1)
Stages	
I and II	11 (13.9)
III with local treatment	10 (12.7)
III with chemotherapy	1 (1.3)
IV	56 (70.8)
Not known <sup>1</sup>	1 (1.3)
Genetics	
KRAS mutation	13 (16.5)
EGFR mutation	4 (5.1)
ALK translocation	1 (1.3)
Treatments	
None <sup>1</sup>	2 (2.5)
1st line treatment	
Local	
Surgery	11 (13.9)
Radiotherapy alone	2 (2.5)
Radiochemotherapy	8 (10.1)
Chemotherapy	52 (65.8)
Tyrosine kinase inhibitors	4 (5.1)
2nd line nivolumab	36 (45.6)
3rd line nivolumab	2 (2.5)
4th line nivolumab	1 (1.3)

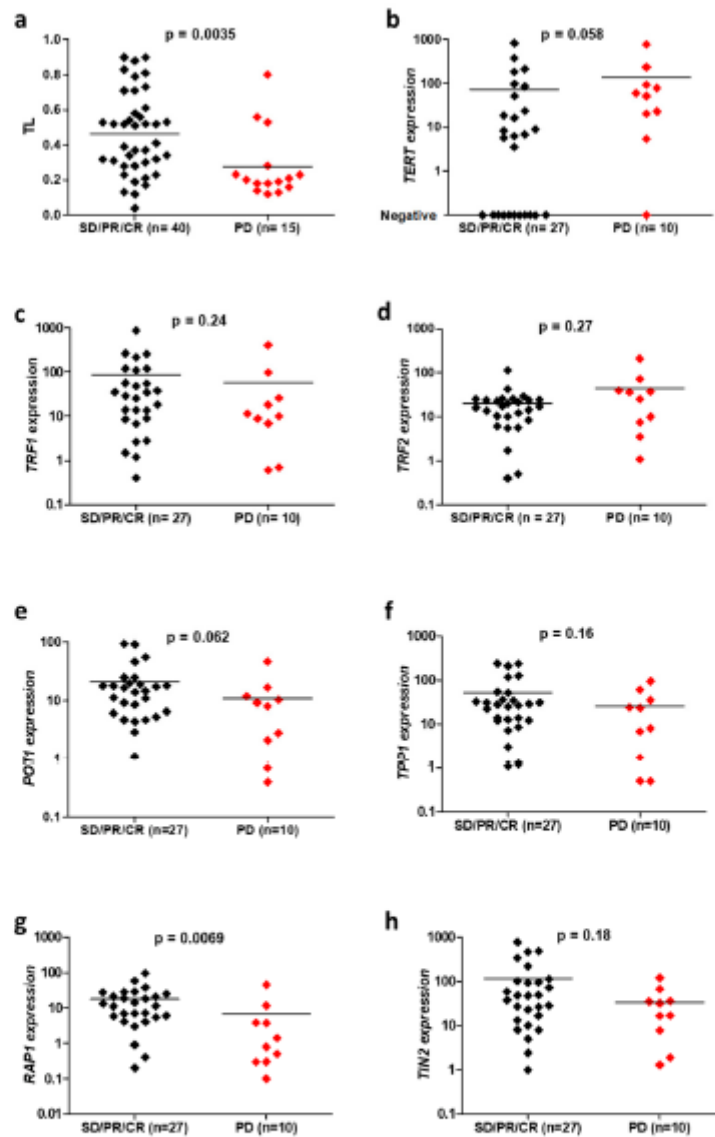
<sup>1</sup> Early death.

### 3.2. Telomere Parameters in Early and Advanced NSCLC

We compared telomere characteristics at diagnosis between early ( $n = 21$ ) and advanced NSCLC ( $n = 56$ ) patients. TL was significantly shorter in advanced than in early NSCLC tumors (median T/S ratios: 0.4 vs. 0.59;  $p = 0.025$ ). Expression levels of *RAP1* and *TPP1* were significantly lower in advanced NSCLC cases (median NCN: 11.65 vs. 30.74;  $p = 0.0020$  and 33.7 vs. 106.3;  $p = 0.012$ , respectively). *TIN2* and *TRF1* tended to be more weakly expressed in advanced NSCLC cases (68.2 vs. 218.7;  $p = 0.054$  and 49.5 vs. 177.6;  $p = 0.070$ , respectively). There was no significant difference in expression levels of *TERT*, *TRF2*, and *POT1* between early and advanced NSCLC cases.

### 3.3. Association of Telomere Parameters with the Response to First-Line Therapy in Advanced NSCLC

Fifty-six patients with advanced NSCLC were treated with first-line chemotherapy. A scanner assessment of response was performed after three months of treatment. TL was significantly shorter ( $p = 0.0035$ , Figure 1a) and *TERT* expression tended to be higher ( $p = 0.058$ , Figure 1b) in cases with progressive disease, in comparison to patients who responded to therapy or had stable disease. The expression of all shelterin complex genes except *TRF2* tended to be lower in the group of patients who progressed after first-line chemotherapy (Figure 1c–h). In particular, a very significant decrease was observed for *RAP1* expression ( $p = 0.0069$ , Figure 1g).



**Figure 1.** Comparison of telomeric parameters, including telomere length (TL) (a), *TERT* (b), *TRF1* (c), *TRF2* (d), *POT1* (e), *TPP1* (f), *RAP1* (g) and *TIN2* (h) expressions, depending on the response to the first-line chemotherapy for advanced NSCLC, in accordance with RECIST 1.1 criteria: progressive disease (PD) versus stable disease (SD), partial response (PR) or complete response (CR).

#### 3.4. Association of Telomere Parameters with Survival in Advanced NSCLC

For 56 patients who received first-line chemotherapy for advanced NSCLC, the associations of telomere parameters with EPS and OS were tested in univariate analyses, and the results are summarized in Table 2.

**Table 2.** Univariate analyses for event-free and overall survival in the advanced NSCLC cohort

Variable	Univariate Cox for EFS		Univariate Cox for OS	
	HR (95% CI)	p-Value	HR (95% CI)	p-Value
Telomere length				
Decreased (<0.34; median)	1.85 (1.00–3.33)	0.049	1.92 (1.03–3.57)	0.04
Short (<0.23; best cutoff)	6.67 (2.86–14.29)	0.0000062	6.25 (3.03–14.29)	0.0000017
TERT expression				
Increased (>12.8; median)	1.95 (0.88–4.34)	0.1	3.01 (1.24–7.03)	0.011
High (>18.4; best cutoff)	4.84 (1.81–12.93)	0.0016	4.75 (2.02–11.21)	0.00037
Shelterin gene expression				
Decreased TRF1 (<18.2; median)	1.39 (0.68–2.86)	0.37	1.72 (0.77–3.85)	0.18
Increased TRF2 (>20.4; median)	1.11 (0.53–2.32)	0.78	1.28 (0.57–2.21)	0.55
High TRF2 (>29; best cutoff)	9.40 (3.43–25.76)	0.000013	8.68 (2.77–27.25)	0.00021
Decreased POT1 (<11; median)	1.25 (0.60–2.56)	0.56	1.54 (0.68–3.45)	0.3
Decreased TPP1 (<24.5; median)	2.13 (0.051)	0.051	1.43 (0.61–3.23)	0.4
Decreased RAP1 (<7; median)	2.70 (1.22–6.25)	0.015	1.61 (0.73–3.57)	0.035
Low RAP1 (<1.4; best cutoff)	8.33 (2.70–25.00)	0.00022	14.29 (4.00–50.00)	0.016
Decreased TIN2 (<31.9; median)	2.63 (1.22–5.56)	0.013	2.04 (0.87–4.76)	0.016
Low TIN2 (<36; best cutoff)	2.94 (1.35–6.25)	0.0065	3.85 (1.61–9.09)	0.0026

EFS: event-free survival; OS: overall survival; HR: hazard ratio, 95% CI: 95% confidence interval.

Advanced NSCLC with short telomeres (<0.23) had significantly reduced EFS and OS, with a median EFS and OS of 4 months vs. 9 and 25 months, respectively, in the subgroup with longer telomeres (Figure 2a;  $p < 10^{-3}$ ). High TERT expression (>18.4) negatively affected EFS, with a median EFS and OS of 5 months compared with 10 and 32 months, respectively, in patients with low TERT expression (Figure 2b;  $p = 0.0018$  and  $p = 0.00035$ ). High tumor TRF2 expression (>29) was significantly associated with reduced survival, with a median EFS and OS of 2 and 4 months vs. 8 and 29 months, respectively, in cases showing low TRF2 expression (Figure 2c;  $p < 10^{-6}$  and  $p = 0.000071$ ). Patients with low RAP1 expression in their tumors (<1.4) showed a median EFS and OS of 4 months, whereas the median EFS and OS were 8 and 32 months, respectively, when RAP1 expression was high (Figure 2d;  $p = 0.00023$  and  $p = 0.0000018$ ). Patients with low tumor TIN2 expression (<36) also had a significantly worse clinical evolution (an EFS of 5 and OS of 6 months) compared with patients with high TIN2 (Figure 2e; EFS of 11 and OS of 32 months;  $p = 0.015$  and  $p = 0.0018$ ).

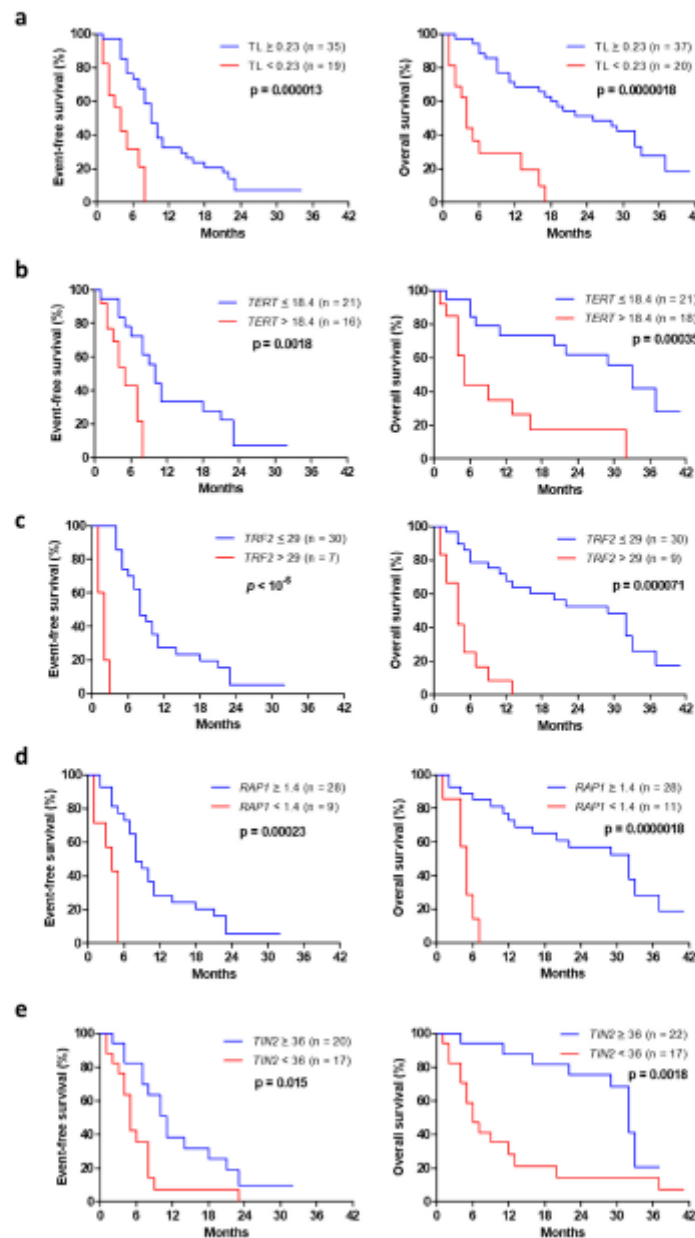
A multivariate analysis, including age and performance status, indicated that short telomeres (<0.23), high TERT (>18.4), high TRF2 (>29) and low levels of RAP1 (<1.4) and TIN2 (<36) expression remained significant prognostic factors (Table 3).

In addition, an unsupervised hierarchical-clustering analysis was performed, to identify patient subgroups in accordance with the distribution of the telomeric markers (Figures S2 and 3a). Survival analysis showed that the profiles of the telomeric biomarkers were highly correlated with EFS and OS (Figure 3b,c;  $p < 10^{-6}$ ). In particular, cluster #1 patients with negative telomeric markers had a longer EFS and OS (a median of 14 and 34 months). In cluster #2, the main features were low TIN2 and high TERT, and patients had an intermediate prognosis (an EFS of 7.4 months and OS of 16.7 months). In clusters #3 and #4, tumors had multiple telomeric markers, and patient survival was considerably shortened (an EFS of 4.3 and 2.2 months and an OS of 5.5 and 2.5 months, respectively).

### 3.5. Correlation of Telomere Length with Survival in Advanced NSCLC Treated with Immunotherapy

In advanced NSCLC, nivolumab was given after the failure of at least one prior platinum-based chemotherapy regimen. Most patients received nivolumab in the second-line setting. To assess the impact of TL on the survival of patients treated with nivolumab, OS was defined as death from any cause, and determined from the date of the first infusion of nivolumab. In advanced NSCLC treated with nivolumab, short telomeres (<0.23) nega-

tively affected overall survival, with a median OS of 4 months vs. 12 months when TL was  $\geq 0.23$  (Figure 4: HR = 2.33 [0.89–5.88],  $p = 0.097$ ).



**Figure 2.** Event-free (left) and overall (right) survival from the date of diagnosis in advanced NSCLC as a function of telomere length (a), TERT expression (b), TRF2 expression (c), RAPI expression (d) and TIN2 expression (e).

Table 3. Multivariate analyses for EFS and OS in the advanced NSCLC cohort.

Variable	Multivariate Cox for EFS *		Multivariate Cox for OS *	
	HR (95% CI)	p-Value	HR (95% CI)	p-Value
Short telomeres	4.35 (1.69–11.11)	0.0023	5.26 (2.08–12.5)	0.00041
High <i>TERT</i>	4.12 (1.47–11.53)	0.0069	3.99 (1.51–10.50)	0.0051
High <i>TRF2</i>	7.31 (2.43–22.01)	0.00041	8.24 (2.43–27.93)	0.007
Low <i>RAP1</i>	9.09 (2.70–33.33)	0.00032	14.29 (3.85–50.00)	0.000051
Low <i>TIN2</i>	3.33 (1.39–8.33)	0.0069	3.03 (1.19–7.69)	0.02

\* Adjusted for age and performance status.

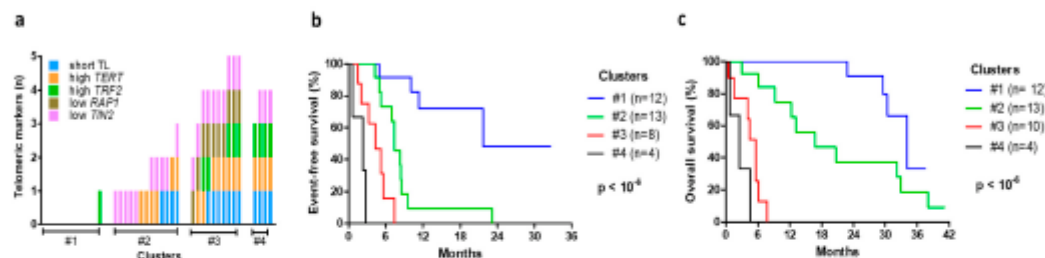


Figure 3. Telomeric markers combined together (a), statistically correlated with event-free (b) and overall survival (c).

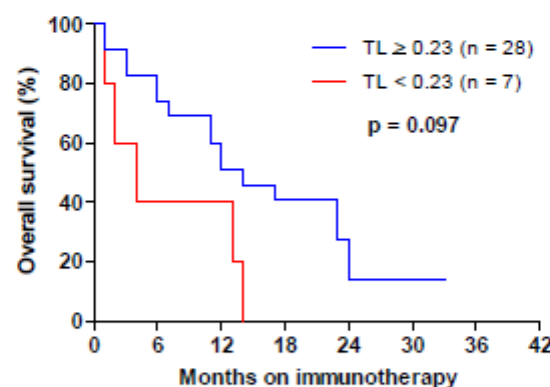


Figure 4. Overall survival from the date of the initiation of immunotherapy in advanced NSCLC as a function of telomere length.

#### 4. Discussion

Telomere attrition is one of the initiating events in lung cancerogenesis [23]. Subsequent activation of telomerase activity favors cancer-cell immortality and tumor progression. Short telomeres and the presence of telomerase activity in tumor tissue were significantly associated with reduced disease-free survival after curative surgery in early-stage NSCLC [15,16,24]. However, it is unknown whether telomere parameters may have prognostic value in advanced NSCLC. To address this issue, we assessed the impact of tumor telomere characteristics on treatment response and survival in advanced NSCLC patients, who represented the large majority of our cohort.

We found that telomeres were significantly shorter in advanced than in localized stages, which is in line with a previous report showing that telomeres were significantly



shorter in the IIIB-IV stage than in the I-IIIa tumors [24]. We correlated telomere status with treatment response and survival for patients at advanced stages. At diagnosis, we identified approximately 35% of patients whose tumors displayed very short telomeres. In this sub-group, the response to first-line chemotherapy was considerably worse, and EFS, as well as OS, was significantly shorter than in patients with longer telomeres in their tumors. Thus, enhanced telomere shortening was strongly associated with tumor resistance and a worse prognosis in advanced NSCLC.

Among lung-cancer cases, 85% of tumors express telomerase independently of the disease stage [16]. We did not observe any significant difference in *TERT* expression levels between early and advanced cases. In early, resectable NSCLC, the presence of telomerase activity was shown to negatively affect survival after surgery [24,25]. In advanced NSCLC, we found that the EFS and OS of patients with higher *TERT* levels were significantly worse. Also, higher *TERT* expression was detected in patients who were resistant to first-line chemotherapy. Thus, telomerase overexpression is strongly associated with tumor aggressiveness in advanced NSCLC.

Expression levels of shelterin complex genes were previously studied in lung cancer, but only *TRF1*, *TRF2*, *POT1*, and *RAP1* mRNA were measured in early NSCLC, and no consistent pattern of expression was identified [17,24,26]. We examined the expression of all six core members of shelterin in early and advanced disease, and found significantly lower expression of *RAP1* and *TPP1* and reduced expression of *TIN2* and *TRF1* in advanced NSCLC. No change was found for *TRF2* and *POT1* expression. Interestingly, *RAP1* expression was significantly lower in patients who did not respond to chemotherapy. Moreover, we found that expression levels of *TRF2*, *RAP1*, and *TIN2* were significantly correlated with the survival of patients with advanced NSCLC.

High *TRF2* expression was significantly associated with shorter PFS and OS. *TRF2* has been increasingly recognized as involved in telomere maintenance and DNA damage response (DDR) [27]. Short telomeres are perceived as double-strand DNA breaks and induce DDR mediated by p53/ATM (ataxia-telangiectasia mutated) proteins. *TRF2* has been shown to inhibit the action of the ATM protein [28,29]. Increased expression of *TRF2* in response to short telomeres is a factor of poor prognosis, because ATM-dependent apoptosis may be more strongly inhibited, and this allows the cell to survive and cancer to progress [30]. High expression of *TRF2* is associated with poor prognosis in other cancers such as hepatocellular carcinomas or advanced-stage cervical cancers [31,32].

Low expression of *RAP1* at diagnosis was a poor-prognostic factor, with reduced EFS and OS. *RAP1* protects the telomere ends from the attacks by the DNA repair mechanisms similarly to other proteins of the shelterin complex, but by different mechanisms [33]. In particular, *RAP1* plays an important role in the repression of aberrant homology-directed DNA repair at the telomeres, which prevents tumor cells from further telomere loss and genomic rearrangements [34]. Altered *RAP1* expression has been found in multiple types of human cancer, including NSCLC [35–38]. In early NSCLC, low *RAP1* expression has been previously correlated with short survival [17]. Our observation shows that the decreased *RAP1* expression is correlated with the resistance to first-line chemotherapy and poor survival, in advanced NSCLC.

We found that low *TIN2* expression was associated with reduced EFS and OS. To our knowledge, this is the first study showing the prognostic impact of low *TIN2* in NSCLC. *TIN2* binds to *TRF1*, *TRF2*, and *TPP1*, thus forming a bridge between the double-stranded telomeric DNA-related proteins and those related to single-stranded sequences [12]. The *TRF1*-*TIN2* interaction is mediated by the *TRFH* domain and a pattern specific for the C-terminal region of *TIN2*, while the N-terminal region of *TIN2* associates with the hinge domain of *TRF2* [39]. When these interactions are simultaneous, *TIN2* connects *TRF1* to *TRF2*, and this link contributes to the stabilization of *TRF2* on telomeres. *TIN2* also recruits *TPP1* (and thus *POT1*) via a third interaction site located in its N-terminal region [40]. *TIN2* is a key component of the shelterin complex, and depletion of *TIN2* has a powerful destabilizing effect [41]. *TIN2* deficiency in aging mice was shown to lead to

telomere fragility, accumulation of DNA damage at chromosomal ends and an enhanced lymphoma formation [42]. This mechanism may be relevant to the development of human malignancies. As compared to normal tissue, *TIN2* transcription levels are lower in tumor tissue in breast cancer [43], gastric cancer [44] and chronic lymphocytic leukemia [20]. Low *TIN2* expression was also shown to be a factor of poor prognosis in chronic lymphocytic leukemia [45–47].

Immune checkpoint inhibitors have changed the management of treatment of advanced NSCLC, with improved survival and better tolerance compared with standard chemotherapy. Nivolumab is a humanized monoclonal antibody against programmed death 1 (PD-1), approved as second-line treatment in patients with advanced NSCLC [48]. Current tests to predict the response to immunotherapy such as tumor PD-L1 overexpression or mutation burden have so far yielded inconsistent results [49]. We found that in patients treated with nivolumab, short telomeres negatively affected survival. To our knowledge, this is the first evidence that telomere length could be a prognostic factor and, possibly, a response predictor in advanced NSCLC treated with immunotherapy. Recently, genomic instability involving arm and whole-chromosome copy-number aberration has been proposed as a predictive biomarker for cancer immunotherapy [50]. Telomere dysfunctions can lead to the formation of cancer with a heavily rearranged genome [9]. Indeed, the unprotected chromosome ends generate end-to-end fusions and dicentric chromosomes, leading to many forms of genome instability, including global chromosomal copy-number alterations [51]. Therefore, the presence of short telomeres is consistent with enhanced genome instability of this particular type, which could lead to a poor response to nivolumab in advanced NSCLC.

Patients with newly diagnosed advanced NSCLC are currently tested for the presence of actionable mutations in tumor DNA [52]. In patients with no identified driver, RNA-based testing is considered, to search for druggable fusion genes. Including the telomeric biomarkers into the clinical-routine setting would be relatively easy, since the tests can be performed on very small amounts of already available DNA and RNA samples. The analytical performance of the tests has to be certified in accordance with medical laboratory quality-requirements. The tests can be recommended for all patients, to provide prognostic and predictive information. In addition, telomere targeting has been proposed for prolonging disease control of therapy-resistant NSCLC patients, and clinical benefits from telomerase inhibition seem to be greater in patients with tumors showing a more pronounced telomere dysfunction [53,54].

## 5. Conclusions

We have shown that short telomere-length, high levels of *TERT* and *TRF2*, and low expression of *RAP1* and *TIN2* are significantly associated with poor EFS and OS in advanced NSCLC. Moreover, enhanced telomere shortening in tumors could be a biomarker for worse survival in patients treated with nivolumab. Thus, evaluation of telomere status emerges as a useful molecular tool that allows for the selection of groups of NSCLC patients with different clinical evolutions, to establish personalized therapy protocols.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cancers15010290/s1>, Figure S1: The best cutoff determination for telomere length values. Forest-plot for hazard ratios (HR) and 95% confidence intervals (95% CI) associated with the overall survival (OS) for each value of telomere length (between 0.17 and 0.39). This sensitivity analysis shows that the best cutoff of TL associated with the OS is 0.23. Figure S2: An unsupervised hierarchical clustering was performed by combining telomere length (TL) and *TERT*, *TRF2*, *RAP1* and *TIN2* gene expression as dichotomic parameters relative to best cutoff values used for survival analysis. Negative prognostic factors (short TL, high *TERT* and *TRF2*, low *RAP1* and *TIN2*) are shown in red and their absence is indicated in green. The different color intensities of dichotomic parameters reflect normalized values and not original binary (0/1) values.



**Author Contributions:** Conceptualization, E.F., P.M. and A.T.; methodology, E.F., L.V. and A.T.; software, B.P.; validation, E.F., L.V., P.M. and A.T.; formal analysis, E.F., L.V., P.M. and A.T.; investigation, E.F., L.V., G.J., H.J., S.B., J.-O.B., B.P., A.C., F.P.-L., E.C., P.M. and A.T.; resources, E.F., B.P., P.M. and A.T.; data curation, E.F., B.P., P.M. and A.T.; writing—original draft preparation, E.F., B.P., P.M. and A.T.; writing—review and editing, E.F., L.V., G.J., H.J., S.B., J.-O.B., B.P., A.C., F.P.-L., E.C., P.M. and A.T. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** The study was approved by the institutional review board (IRB00008526, Comité de Protection des Personnes SUD-EST VI, CHU Clermont-Ferrand).

**Informed Consent Statement:** This retrospective monocentric study was performed on samples remaining after routine diagnostic tests. Written informed consent was obtained from patients to use the residual samples for research purposes. All samples and data were anonymized.

**Data Availability Statement:** The data supporting reported results are available from the corresponding author on request.

**Acknowledgments:** The authors are grateful to Farida Godeau and Delphine Voisin for technical support.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Ferlay, J.; Colombet, M.; Soerjomataram, I.; Mathers, C.; Parkin, D.M.; Piñeros, M.; Znaor, A.; Bray, F. Estimating the Global Cancer Incidence and Mortality in 2018: GLOBOCAN Sources and Methods. *Int. J. Cancer* **2019**, *144*, 1941–1953. [\[CrossRef\]](#) [\[PubMed\]](#)
2. Zito Marino, F.; Bianco, R.; Accardo, M.; Ronchi, A.; Cozzolino, L.; Morgillo, F.; Rossi, G.; Franco, R. Molecular Heterogeneity in Lung Cancer: From Mechanisms of Origin to Clinical Implications. *Int. J. Med. Sci.* **2019**, *16*, 981–989. [\[CrossRef\]](#) [\[PubMed\]](#)
3. Borghaei, H.; Paz-Ares, L.; Horn, L.; Spigel, D.R.; Steins, M.; Ready, N.E.; Chow, L.Q.; Vokes, E.E.; Felip, E.; Holgado, E.; et al. Nivolumab versus Docetaxel in Advanced Nonsquamous Non-Small-Cell Lung Cancer. *N. Engl. J. Med.* **2015**, *373*, 1627–1639. [\[CrossRef\]](#)
4. Brahmer, J.; Reckamp, K.L.; Baas, P.; Crinò, L.; Eberhardt, W.E.E.; Poddubskaya, E.; Antonia, S.; Pluzanski, A.; Vokes, E.E.; Holgado, E.; et al. Nivolumab versus Docetaxel in Advanced Squamous-Cell Non-Small-Cell Lung Cancer. *N. Engl. J. Med.* **2015**, *373*, 123–135. [\[CrossRef\]](#)
5. Ferrara, R.; Mezquita, L.; Texier, M.; Lahmar, J.; Audigier-Valette, C.; Tessonnier, L.; Mazieres, J.; Zalcman, G.; Brosseau, S.; Le Moulec, S.; et al. Hyperprogressive Disease in Patients with Advanced Non-Small Cell Lung Cancer Treated With PD-1/PD-L1 Inhibitors or With Single-Agent Chemotherapy. *JAMA Oncol.* **2018**, *4*, 1543–1552. [\[CrossRef\]](#) [\[PubMed\]](#)
6. Bisoffi, M.; Heaphy, C.M.; Griffith, J.K. Telomeres: Prognostic Markers for Solid Tumors. *Int. J. Cancer* **2006**, *119*, 2255–2260. [\[CrossRef\]](#)
7. Gaspar, T.B.; Sá, A.; Lopes, J.M.; Sobrinho-Simões, M.; Soares, P.; Vinagre, J. Telomere Maintenance Mechanisms in Cancer. *Genes* **2018**, *9*, 241. [\[CrossRef\]](#)
8. Bernadotte, A.; Mikhelson, V.M.; Spivak, I.M. Markers of Cellular Senescence: Telomere Shortening as a Marker of Cellular Senescence. *Aging* **2016**, *8*, 3–11. [\[CrossRef\]](#)
9. Maciejowski, J.; de Lange, T. Telomeres in Cancer: Tumour Suppression and Genome Instability. *Nat. Rev. Mol. Cell Biol.* **2017**, *18*, 175–186. [\[CrossRef\]](#)
10. Kim, N.W.; Piatyszek, M.A.; Prowse, K.R.; Harley, C.B.; West, M.D.; Ho, P.L.; Coviello, G.M.; Wright, W.E.; Weinrich, S.L.; Shay, J.W. Specific Association of Human Telomerase Activity with Immortal Cells and Cancer. *Science* **1994**, *266*, 2011–2015. [\[CrossRef\]](#)
11. Leão, R.; Apolônio, J.D.; Lee, D.; Figueiredo, A.; Tabori, U.; Castelo-Branco, P. Mechanisms of Human Telomerase Reverse Transcriptase (HTRT) Regulation: Clinical Impacts in Cancer. *J. Biomed. Sci.* **2018**, *25*, 22. [\[CrossRef\]](#) [\[PubMed\]](#)
12. Palm, W.; de Lange, T. How Shelterin Protects Mammalian Telomeres. *Annu. Rev. Genet.* **2008**, *42*, 301–334. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Wang, K.; Wang, R.-L.; Liu, J.-J.; Zhou, J.; Li, X.; Hu, W.-W.; Jiang, W.-J.; Hao, N.-B. The Prognostic Significance of HTRT Overexpression in Cancers: A Systematic Review and Meta-Analysis. *Medicine* **2018**, *97*, e11794. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Cookson, J.C.; Laughton, C.A. The Levels of Telomere-Binding Proteins in Human Tumours and Therapeutic Implications. *Eur. J. Cancer* **2009**, *45*, 536–550. [\[CrossRef\]](#)
15. Jeon, H.-S.; Choi, Y.Y.; Choi, J.E.; Lee, W.K.; Lee, E.; Yoo, S.S.; Lee, S.Y.; Lee, J.; Cha, S.I.; Kim, C.H.; et al. Telomere Length of Tumor Tissues and Survival in Patients with Early Stage Non-Small Cell Lung Cancer. *Mol. Carcinog.* **2014**, *53*, 272–279. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Fernández-Marcelo, T.; Gómez, A.; Pascua, I.; de Juan, C.; Head, J.; Hernando, F.; Jarabo, J.-R.; Calatayud, J.; Torres-García, A.-J.; Iniesta, P. Telomere Length and Telomerase Activity in Non-Small Cell Lung Cancer Prognosis: Clinical Usefulness of a Specific Telomere Status. *J. Exp. Clin. Cancer Res.* **2015**, *34*, 78. [\[CrossRef\]](#)

17. Lin, X.; Gu, J.; Lu, C.; Spitz, M.R.; Wu, X. Expression of Telomere-Associated Genes as Prognostic Markers for Overall Survival in Patients with Non-Small Cell Lung Cancer. *Clin. Cancer Res.* **2006**, *12*, 5720–5725. [\[CrossRef\]](#)
18. Véronèse, L.; Tournilhac, O.; Callanan, M.; Prie, N.; Kwiatkowski, F.; Combes, P.; Chauvet, M.; Davi, F.; Gouas, L.; Verrelle, P.; et al. Telomeres and Chromosomal Instability in Chronic Lymphocytic Leukemia. *Leukemia* **2013**, *27*, 490–493. [\[CrossRef\]](#)
19. Cawthon, R.M. Telomere Measurement by Quantitative PCR. *Nucleic Acids Res.* **2002**, *30*, e47. [\[CrossRef\]](#)
20. Poncet, D.; Belleville, A.; t'kint de Roodenbeke, C.; Roborel de Climens, A.; Ben Simon, E.; Merle-Beral, H.; Callet-Bauchu, E.; Salles, G.; Sabatier, L.; Delic, J.; et al. Changes in the Expression of Telomere Maintenance Genes Suggest Global Telomere Dysfunction in B-Chronic Lymphocytic Leukemia. *Blood* **2008**, *111*, 2388–2391. [\[CrossRef\]](#)
21. Kwiatkowski, F.; Girard, M.; Hacene, K.; Berlie, J. Sem: A suitable statistical software adapted for research in oncology. *Bull. Cancer* **2000**, *87*, 715–721.
22. Eisenhauer, E.A.; Therasse, P.; Bogaerts, J.; Schwartz, L.H.; Sargent, D.; Ford, R.; Dancey, J.; Arbuck, S.; Gwyther, S.; Mooney, M.; et al. New Response Evaluation Criteria in Solid Tumours: Revised RECIST Guideline (Version 1.1). *Eur. J. Cancer* **2009**, *45*, 228–247. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Lantuejoul, S.; Raynaud, C.; Salameire, D.; Gazzeri, S.; Moro-Sibilot, D.; Soria, J.-C.; Brambilla, C.; Brambilla, E. Telomere Maintenance and DNA Damage Responses during Lung Carcinogenesis. *Clin. Cancer Res.* **2010**, *16*, 2979–2988. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Frias, C.; García-Aranda, C.; De Juan, C.; Morán, A.; Ortega, P.; Gómez, A.; Hernando, F.; López-Asenjo, J.-A.; Torres, A.-J.; Benito, M.; et al. Telomere Shortening Is Associated with Poor Prognosis and Telomerase Activity Correlates with DNA Repair Impairment in Non-Small Cell Lung Cancer. *Lung Cancer Amst. Neth.* **2008**, *60*, 416–425. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Zhu, C.-Q.; Cutz, J.-C.; Liu, N.; Lau, D.; Shepherd, F.A.; Squire, J.A.; Tsao, M.-S. Amplification of Telomerase (HTRT) Gene Is a Poor Prognostic Marker in Non-Small-Cell Lung Cancer. *Br. J. Cancer* **2006**, *94*, 1452–1459. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Hsu, C.-P.; Ko, J.-L.; Shai, S.-E.; Lee, L.-W. Modulation of Telomere Shelterin by TRF1 and TRF2 Interacts with Telomerase to Maintain the Telomere Length in Non-Small Cell Lung Cancer. *Lung Cancer Amst. Neth.* **2007**, *58*, 310–316. [\[CrossRef\]](#)
27. Rizzo, A.; Iachettini, S.; Salvati, E.; Zizza, P.; Maresca, C.; D'Angelo, C.; Benarroch-Popivker, D.; Capolupo, A.; Del Gaudio, F.; Cosconati, S.; et al. SIRT6 Interacts with TRF2 and Promotes Its Degradation in Response to DNA Damage. *Nucleic Acids Res.* **2017**, *45*, 1820–1834. [\[CrossRef\]](#)
28. van Gent, D.C.; Hoeijmakers, J.H.; Kanaar, R. Chromosomal Stability and the DNA Double-Stranded Break Connection. *Nat. Rev. Genet.* **2001**, *2*, 196–206. [\[CrossRef\]](#)
29. Khanna, K.K.; Jackson, S.P. DNA Double-Strand Breaks: Signaling, Repair and the Cancer Connection. *Nat. Genet.* **2001**, *27*, 247–254. [\[CrossRef\]](#)
30. Lavin, M.F.; Kozlov, S. ATM Activation and DNA Damage Response. *Cell Cycle* **2007**, *6*, 931–942. [\[CrossRef\]](#)
31. Kim, H.; Yoo, J.E.; Cho, J.Y.; Oh, B.-K.; Yoon, Y.-S.; Han, H.-S.; Lee, H.S.; Jang, J.J.; Jeong, S.H.; Kim, J.W.; et al. Telomere Length, TERT and Shelterin Complex Proteins in Hepatocellular Carcinomas Expressing “Stemness”-Related Markers. *J. Hepatol.* **2013**, *59*, 746–752. [\[CrossRef\]](#)
32. Ozden, S.; Tiber, P.M.; Ozgen, Z.; Ozyurt, H.; Serakinci, N.; Orun, O. Expression of TRF2 and Its Prognostic Relevance in Advanced Stage Cervical Cancer Patients. *Biol. Res.* **2014**, *47*, 61. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Patel, T.N.; Vasan, R.; Gupta, D.; Patel, J.; Trivedi, M. Shelterin Proteins and Cancer. *Asian Pac. J. Cancer Prev.* **2015**, *16*, 3085–3090. [\[CrossRef\]](#)
34. Rai, R.; Chen, Y.; Lei, M.; Chang, S. TRF2-RAP1 Is Required to Protect Telomeres from Engaging in Homologous Recombination-Mediated Deletions and Fusions. *Nat. Commun.* **2016**, *7*, 10881. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Braig, M.; Pfallmann, N.; Preukschas, M.; Steinemann, D.; Hofmann, W.; Gompf, A.; Streichert, T.; Braunschweig, T.; Copland, M.; Rudolph, K.L.; et al. A ‘telomere-Associated Secretory Phenotype’ Cooperates with BCR-ABL to Drive Malignant Proliferation of Leukemic Cells. *Leukemia* **2014**, *28*, 2028–2039. [\[CrossRef\]](#)
36. Aoude, L.G.; Pritchard, A.L.; Robles-Espinoza, C.D.; Wadt, K.; Harland, M.; Choi, J.; Gartside, M.; Quesada, V.; Johansson, P.; Palmer, J.M.; et al. Nonsense Mutations in the Shelterin Complex Genes ACD and TERF2IP in Familial Melanoma. *J. Natl. Cancer Inst.* **2015**, *107*, dju408. [\[CrossRef\]](#)
37. Massion, P.P.; Zou, Y.; Chen, H.; Jiang, A.; Coulson, P.; Amos, C.I.; Wu, X.; Wistuba, I.; Wei, Q.; Shyr, Y.; et al. Smoking-Related Genomic Signatures in Non-Small Cell Lung Cancer. *Am. J. Respir. Crit. Care Med.* **2008**, *178*, 1164–1172. [\[CrossRef\]](#)
38. Sweet-Cordero, A.; Tseng, G.C.; You, H.; Douglass, M.; Huey, B.; Albertson, D.; Jacks, T. Comparison of Gene Expression and DNA Copy Number Changes in a Murine Model of Lung Cancer. *Genes. Chromosomes Cancer* **2006**, *45*, 338–348. [\[CrossRef\]](#) [\[PubMed\]](#)
39. Chen, Y.; Yang, Y.; van Overbeek, M.; Donigian, J.R.; Baciu, P.; de Lange, T.; Lei, M. A Shared Docking Motif in TRF1 and TRF2 Used for Differential Recruitment of Telomeric Proteins. *Science* **2008**, *319*, 1092–1096. [\[CrossRef\]](#)
40. Frescas, D.; de Lange, T. TRF2-Tethered TIN2 Can Mediate Telomere Protection by TPP1/POT1. *Mol. Cell. Biol.* **2014**, *34*, 1349–1362. [\[CrossRef\]](#)
41. Ye, J.Z.-S.; Donigian, J.R.; van Overbeek, M.; Loayza, D.; Luo, Y.; Krutchinsky, A.N.; Chait, B.T.; de Lange, T. TIN2 Binds TRF1 and TRF2 Simultaneously and Stabilizes the TRF2 Complex on Telomeres. *J. Biol. Chem.* **2004**, *279*, 47264–47271. [\[CrossRef\]](#) [\[PubMed\]](#)



42. Hartmann, K.; Illing, A.; Leithäuser, F.; Baisantray, A.; Quintanilla-Martinez, L.; Rudolph, K.L. Gene Dosage Reductions of Trf1 and/or Trf2 Induce Telomere DNA Damage and Lymphoma Formation in Aging Mice. *Leukemia* **2016**, *30*, 749–753. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Salhab, M.; Jiang, W.G.; Newbold, R.F.; Mokbel, K. The Expression of Gene Transcripts of Telomere-Associated Genes in Human Breast Cancer: Correlation with Clinico-Pathological Parameters and Clinical Outcome. *Breast Cancer Res. Treat.* **2008**, *109*, 35–46. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Yamada, M.; Tsuji, N.; Nakamura, M.; Moriai, R.; Kobayashi, D.; Yagihashi, A.; Watanabe, N. Down-Regulation of TRF1, TRF2 and TIN2 Genes Is Important to Maintain Telomeric DNA for Gastric Cancers. *Anticancer Res.* **2002**, *22*, 3303–3307.
45. Guéze, R.; Pages, M.; Veronèse, L.; Combes, P.; Lemal, R.; Gay-Bellile, M.; Chauvet, M.; Callanan, M.; Kwiatkowski, F.; Pereira, B.; et al. Telomere Status in Chronic Lymphocytic Leukemia with TP53 Disruption. *Oncotarget* **2016**, *7*, 56976–56985. [\[CrossRef\]](#)
46. Augereau, A.; T'kint de Roodenbeke, C.; Simonet, T.; Bauwens, S.; Horard, B.; Callanan, M.; Leroux, D.; Jallades, L.; Salles, G.; Gilson, E.; et al. Telomeric Damage in Early Stage of Chronic Lymphocytic Leukemia Correlates with Shelterin Dysregulation. *Blood* **2011**, *118*, 1316–1322. [\[CrossRef\]](#)
47. Ishdorj, G.; Kost, S.E.F.; Beiggi, S.; Zang, Y.; Gibson, S.B.; Johnston, J.B. A Novel Spliced Variant of the TIN2 Shelterin Is Present in Chronic Lymphocytic Leukemia. *Leuk. Res.* **2017**, *59*, 66–74. [\[CrossRef\]](#)
48. Somasundaram, A.; Socinski, M.A.; Villaruz, L.C. Immune Checkpoint Blockade in Lung Cancer. *Discov. Med.* **2016**, *22*, 55–65.
49. Bodor, J.N.; Bumber, Y.; Borghaei, H. Biomarkers for Immune Checkpoint Inhibition in Non-Small Cell Lung Cancer (NSCLC). *Cancer* **2020**, *126*, 260–270. [\[CrossRef\]](#)
50. Davoli, T.; Uno, H.; Wooten, E.C.; Elledge, S.J. Tumor Aneuploidy Correlates with Markers of Immune Evasion and with Reduced Response to Immunotherapy. *Science* **2017**, *355*, eaaf8399. [\[CrossRef\]](#)
51. Roger, L.; Jones, R.E.; Heppel, N.H.; Williams, G.T.; Sampson, J.R.; Baird, D.M. Extensive Telomere Erosion in the Initiation of Colorectal Adenomas and Its Association with Chromosomal Instability. *J. Natl. Cancer Inst.* **2013**, *105*, 1202–1211. [\[CrossRef\]](#) [\[PubMed\]](#)
52. Aisner, D.L.; Riely, G.J. Non-Small Cell Lung Cancer: Recommendations for Biomarker Testing and Treatment. *J. Natl. Compr. Cancer Netw.* **2021**, *19*, 610–613. Available online: [https://jncn.org/configurable/content/journals\\$002fjncn\\$002f19\\$002f5.5\\$002farticlep610.xml?ac=journals%24002fjncn%24002f19%24002f5.5%24002farticle-p610.xml](https://jncn.org/configurable/content/journals$002fjncn$002f19$002f5.5$002farticlep610.xml?ac=journals%24002fjncn%24002f19%24002f5.5%24002farticle-p610.xml) (accessed on 14 December 2022). [\[CrossRef\]](#)
53. Chiappori, A.A.; Kolevska, T.; Spigel, D.R.; Hager, S.; Rarick, M.; Gadgil, S.; Blais, N.; Von Pawel, J.; Hart, L.; Reck, M.; et al. A Randomized Phase II Study of the Telomerase Inhibitor Imetelstat as Maintenance Therapy for Advanced Non-Small-Cell Lung Cancer. *Ann. Oncol.* **2015**, *26*, 354–362. [\[CrossRef\]](#) [\[PubMed\]](#)
54. Mender, I.; LaRanger, R.; Luitel, K.; Peyton, M.; Girard, L.; Lai, T.-P.; Batten, K.; Cornelius, C.; Dalvi, M.P.; Ramirez, M.; et al. Telomerase-Mediated Strategy for Overcoming Non-Small Cell Lung Cancer Targeted Therapy and Chemotherapy Resistance. *Neoplasia* **2018**, *20*, 826–837. [\[CrossRef\]](#)

**Disclaimer/Publisher's Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.



## LYMPHOID NEOPLASIA

# Genetic events associated with venetoclax resistance in CLL identified by whole-exome sequencing of patient samples

Jasneet Kaur Khalsa,<sup>1,2,\*</sup> Justin Cha,<sup>2,\*</sup> Filippo Utrio,<sup>3,\*</sup> Aishath Naeem,<sup>1,2,\*</sup> Ishwarya Murali,<sup>1,2,\*</sup> Yanan Kuang,<sup>4</sup> Kevin Vasquez,<sup>4</sup> Liang Li,<sup>2</sup> Svitlana Tyekucheva,<sup>5</sup> Stacey M. Fernandes,<sup>1</sup> Lauren Veronese,<sup>6,7</sup> Romain Guieze,<sup>7,8</sup> Binu Kandathilparambil Sasi,<sup>1,2</sup> Zou Wang,<sup>5</sup> John-Hanson Machado,<sup>1,2</sup> Harrison Bai,<sup>1</sup> Maryam Alasfour,<sup>1</sup> Kahn Rhrissorakrai,<sup>3</sup> Chaya Levovitz,<sup>3</sup> Brian P. Danysh,<sup>2</sup> Kara Slowik,<sup>2</sup> Raquel A. Jacobs,<sup>2</sup> Matthew S. Davids,<sup>1,9</sup> Cloud P. Pawelczak,<sup>4</sup> Ignaty Leshchiner,<sup>2</sup> Laxmi Parida,<sup>3,†</sup> Gad Getz,<sup>2,10,11,†</sup> and Jennifer R. Brown<sup>1,2,9,†</sup>

<sup>1</sup>Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA; <sup>2</sup>Cancer Program, Broad Institute of MIT and Harvard, Cambridge, MA; <sup>3</sup>IBM Research, Yorktown Heights, New York, NY; <sup>4</sup>Belfer Center for Applied Cancer Science, Dana-Farber Cancer Institute, Boston, MA; <sup>5</sup>Department of Data Sciences, Dana-Farber Cancer Institute, Harvard TH Chan School of Public Health, Boston, MA; <sup>6</sup>Service de Cytogénétique Médicale, CHU Clermont-Ferrand, Clermont-Ferrand, France; <sup>7</sup>EA7453 CHLTER, Université Clermont Auvergne, Clermont-Ferrand, France; <sup>8</sup>Service d'Hématologie clinique et thérapie cellulaire, CHU Clermont-Ferrand, Clermont-Ferrand, France; <sup>9</sup>Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA; and <sup>10</sup>Cancer Center and <sup>11</sup>Department of Pathology, Massachusetts General Hospital, Boston, MA

### KEY POINTS

- Clonal evolution on venetoclax selects for 8p deletion, which leads to increased venetoclax resistance in vitro.
- Elevated Erk signaling and constitutively high BCR signaling are associated with venetoclax progression.

Although *BCL2* mutations are reported as later occurring events leading to venetoclax resistance, many other mechanisms of progression have been reported though remain poorly understood. Here, we analyze longitudinal tumor samples from 11 patients with disease progression while receiving venetoclax to characterize the clonal evolution of resistance. All patients tested showed increased in vitro resistance to venetoclax at the posttreatment time point. We found the previously described acquired *BCL2*-G101V mutation in only 4 of 11 patients, with 2 patients showing a very low variant allele fraction (0.03%-4.68%). Whole-exome sequencing revealed acquired loss(8p) in 4 of 11 patients, of which 2 patients also had gain (1q21.2-21.3) in the same cells affecting the *MCL1* gene. In vitro experiments showed that CLL cells from the 4 patients with loss(8p) were more resistant to venetoclax than cells from those without it, with the cells from 2 patients also carrying gain (1q21.2-21.3) showing increased sensitivity to *MCL1* inhibition. Progression samples with gain (1q21.2-21.3) were more susceptible to the combination of *MCL1* inhibitor and venetoclax. Differential gene expression analysis comparing bulk RNA sequencing data from pretreatment and progression time points of all patients showed upregulation of proliferation, B-cell receptor (BCR), and NF- $\kappa$ B gene sets including MAPK genes. Cells from progression time points demonstrated upregulation of surface immunoglobulin M and higher pERK levels compared with those from the preprogression time point, suggesting an upregulation of BCR signaling that activates the MAPK pathway. Overall, our data suggest several mechanisms of acquired resistance to venetoclax in CLL that could pave the way for rationally designed combination treatments for patients with venetoclax-resistant CLL.

## Introduction

Chronic lymphocytic leukemia (CLL) remains largely incurable<sup>1</sup> despite the development of targeted agents including *BCL2* inhibitors. The first-in-class *BCL2* inhibitor venetoclax<sup>2</sup> is a highly selective US Food and Drug Administration–approved small molecule that acts as a BH3 mimetic inhibiting *BCL2*.<sup>3</sup> In patients who are heavily pretreated, venetoclax given continuously showed a median progression-free survival of 28.2

months.<sup>4</sup> However, some patients fail to respond, and others initially respond but relapse soon after.

Mechanisms of acquired resistance to venetoclax remain poorly understood. The primary identified mechanism of venetoclax resistance is mutation of the venetoclax-binding site of *BCL2*.<sup>5</sup> However, these mutations were observed in patients with late disease progression, at a median of 32 months, and were only found in 7 of 15 (50%) patients, often at very low variant allele



fractions (VAFs) (VAF <2% at progression in 6 of 7 patients tested positive for *BCL2* p.G101V mutation). Most patients with this *BCL2* mutation also have other mutations in *BCL2*.<sup>6,7</sup> Other resistance mechanisms have also been proposed. A whole-exome sequencing (WES) study of 8 patients with disease progression on venetoclax with loss(17p), including several who developed Richter transformation, identified the loss of *CDKN2A/B* and mutations in the antiproliferative *BTG1* gene as resistance-associated genetic aberrations, along with acquired nonrecurrent mutations in known CLL driver genes.<sup>8,9</sup> Other studies have identified the upregulation of *MCL1* and *NF- $\kappa$ B*,<sup>10</sup> increased *BCL-XL* in venetoclax-resistant lymphoma cell lines and samples from patients with CLL,<sup>11</sup> and increased AMP-activated protein kinase signaling as mechanisms of resistance.<sup>12,13</sup>

However, detailed genomic clonal evolution analysis, which could help identify growing resistant clones, has not been performed on clinical samples collected through the course of venetoclax treatment. Hence, in this study, we characterized the clonal evolution of resistance via WES and RNA sequencing (RNA-seq) data analysis using longitudinal samples from patients with CLL who had developing progressive disease while receiving venetoclax. Furthermore, we performed *in vitro* validation of the key findings using CLL cells from patients with CLL collected at pre and postprogression time points on venetoclax.

## Methods

### Patient sample preparation

Matched peripheral blood (tumor) and saliva (healthy) samples were collected from patients who consented to our Dana-Farber Harvard Cancer Center institutional review board-approved CLL tissue banking protocol. All patients provided written informed consent before any samples were drawn.

### Identification of somatic mutations

WES was performed at the Broad Institute as detailed in the supplemental Methods, available on the *Blood* website. High confidence somatic mutations were identified using previously published pipelines<sup>14-16</sup> developed in the Cancer Genome Analysis group at the Broad Institute (<https://software.broadinstitute.org/cancer/cga/>). The somatic single nucleotide variants (sSNVs) were detected using MuTect1,<sup>15</sup> and somatic short insertions and deletions were detected using Strelka and annotated MuTect2. The somatic mutation calls were filtered using a realignment filter, removing variants supported by ambiguously mapped reads.<sup>14</sup> All the filtered mutation events were manually visualized and reviewed using the Integrative Genomics Viewer.<sup>17</sup> Total copy number analysis was performed using Genome Analysis Toolkit and segmented into allelic copy number using AllelicCapSeg.<sup>18</sup>

### Estimation of clonality using ABSOLUTE

The local copy number and the cancer cell fraction of each mutation (the fraction of cancer cells harboring the mutation) were estimated using ABSOLUTE,<sup>19</sup> which calculates the sample's purity (fraction of tumor cells) and ploidy (average DNA per cancer cell in units of haploid human genome) and local absolute copy number.

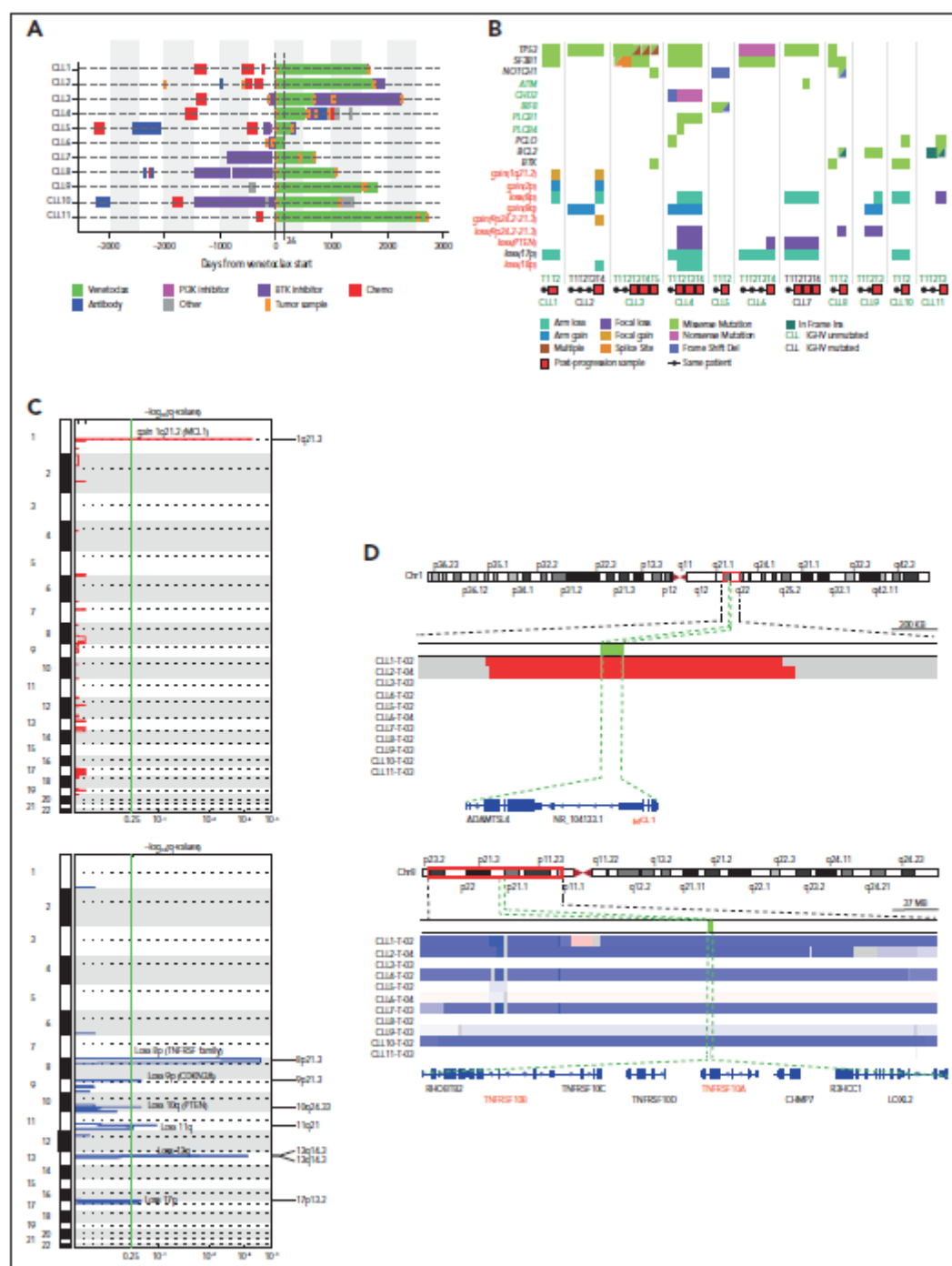
Additional methods can be found in supplemental Methods.

## Results

### WES and evaluation of *BCL2* mutations in patient samples before and after progression with venetoclax

To determine whether recurrent genetic alterations result in venetoclax resistance, we evaluated matched serial CLL and germ line samples from 11 patients whose disease progressed while receiving venetoclax. Samples were taken before venetoclax and at progression from 10 patients with acquired resistance and 1 patient with primary refractory disease. A median of 3 longitudinal tumor samples (range, 2-5 samples) were sequenced per patient ( $N_{\text{samples}} = 35$ ). The patients had received a median of 2 prior therapies, including 8 patients with prior chemoimmunotherapy and 5 patients treated with prior Bruton tyrosine kinase (BTK) inhibitors (Figure 1A; supplemental Tables 1 and 2). Before initiation of venetoclax, del(17p) as well as complex karyotype (>3 aberrations) was observed in 4 of 11 (36%) patients (supplemental Table 3). Our cohort was also enriched in patients with CLL with TP53 (63%; 7 of 11) and SF3B1 mutations (45%; 5 of 11) (supplemental Table 3). The best response to venetoclax in all patients was partial remission per the criteria of the International Workshop on CLL, with a median time to progression of 35.76 months (range, 1.2-89.6 months) while receiving venetoclax (supplemental Table 2).

We assessed the presence of G101V mutation in the *BCL2* gene.<sup>5</sup> In our WES data, using MuTect,<sup>15</sup> we did not identify any sSNVs in *BCL2* or *BCL2*-family genes at baseline, as in previous reports.<sup>12,14</sup> On further visualization of the sequencing data using the Integrative Genomics Viewer,<sup>17</sup> 2 patients (CLL8 and CLL9) had *BCL2* G101V mutation at progression while receiving venetoclax. CLL3 and CLL4 also had 1 read each (1 of 63 and 1 of 71, respectively) for the mutation, in the progression samples (supplemental Figure 1A). Because of the extremely low allelic frequency of the mutation in CLL3 and CLL4, we performed a droplet digital polymerase chain reaction assay (ddPCR) using previously described probes,<sup>5</sup> in peripheral blood mononuclear cell (PBMC) and/or bone marrow DNA from pre-venetoclax and postprogression samples from our cohort. G101V mutation was detected via ddPCR in CLL8 and CLL9 at progression. In addition, G101V mutation was detected, at progression, in CLL1 and CLL4 at low VAFs of 0.03% and 4.68%, respectively (CLL1, 4 of 16064 droplets; CLL4, 640 of 13680 droplets; supplemental Table 4). We detected 1 droplet positive for G101V mutation in 2 other patients in bone marrow samples at venetoclax progression (CLL2 and CLL3). To confirm these very low frequency events, we examined postprogression PBMC RNA. We could not detect the mutations in RNA-seq data but were able to detect the G101V mutations via ddPCR using the complementary DNA from 2 patients (CLL1 and CLL4), for whom we detected mutations in DNA (supplemental Table 4). However, we could not confirm the mutations in patients CLL2 and CLL3 via ddPCR of the complementary DNA derived from the RNA. Similar to the findings from a recent report,<sup>6</sup> we did not observe any G101V mutations in the *BCL2* gene in patients who only had prior BTK inhibitor (ibrutinib) therapy (CLL6 and CLL7). Because of the absence or very low VAF of the *BCL2*



**Figure 1. Genetic profile of patients on venetoclax therapy.** (A) Swimmer plot for 11 evaluated patients with CLL who received venetoclax, showing treatment received before and after initiation of venetoclax. Venetoclax treatment shown in green for CLL6 at 36 days from venetoclax start. For all other patients, days from venetoclax start is per scale as defined on the x-axis. (B) Co-Mut plot representation of individual mutations and copy number aberrations present in 11 patients with CLL as derived from WES data. Half-filled squares in different colors represent distinct mutations. Mutations written in black are recurrent nonsynonymous mutations, whereas those in green are nonsynonymous mutations acquired in a single patient. (C) GISTIC plot of genomic regions with CN gain (red) or loss (blue) for 22 autosomes (y-axis) with  $-\log_{10}(p\text{-value})$  on the x-axis. (D) Visual validation of chromosomal segment loss and gains in integrative genomic viewer at the progression time point on venetoclax for all 11 patients. Blue indicates segment loss and red indicates segment gain.

G101V mutations in 9 of 11 patients, other mechanisms of acquired resistance appear important.

### Genetic alterations acquired while receiving venetoclax therapy

To evaluate for recurrent somatic alterations leading to venetoclax resistance, we performed WES and copy number analysis on samples from 11 patients ( $N_{\text{samples}} = 35$ ). MutSig analysis of WES data ( $q < .1$ ) showed recurrent nonsynonymous clonal mutations in *TP53* and *BTK* genes (Figure 1B). Three patients had PCLO (piccolo presynaptic cytomatrix protein) mutation, as previously reported,<sup>20</sup> with CLL10 and CLL11 acquiring this mutation at progression while receiving venetoclax (Figure 1B). Gain(8q) and loss(17p), known CLL driver events, were observed in 2 and 5 patients, respectively, before venetoclax treatment, and gain(8q) was acquired in 1 patient receiving venetoclax. Focal gain(9p24.2-21.3) affecting the *CDKN2A/B* gene was acquired in CLL2, whereas CLL4, CLL8, and CLL9 showed acquired loss(9p24.2-21.3).

GISTIC2.0<sup>21</sup> analysis of the copy number data from all 11 patients in this cohort detected some known CLL drivers, such as loss(17p), loss(11q), and loss(13q)<sup>14</sup> along with loss(9p21.3)(*CDKN2A/B*) and loss(10q24.33)(*PTEN*) (Figure 1C). GISTIC also revealed 1q21.2 as significantly amplified and arm-level 8p deletion as well as focal 8p21.3 deletion as significantly deleted regions in venetoclax progression samples (Figure 1C-D; supplemental Figure 1B). Six patients showed loss(8p), of which 2 patients (CLL7 and CLL10) had this genetic event before venetoclax initiation, whereas 4 patients (CLL1, CLL2, CLL4, and CLL9), acquired it during venetoclax treatment. In addition, CLL11 appeared to acquire a focal 68-kb loss(8p) affecting the *TNFRSF10A* gene. Because of its subclonal nature and small size with limited probes in the region (Figure 1B-D), we could not independently confirm this event. Arm loss(8p), containing the *TRAF6* receptor genes, has been linked to ibrutinib resistance in CLL.<sup>20</sup> Furthermore, acquired loss(8p) cooccurred with acquired gain(1q21.2-21.3) affecting the *MCL1* gene in 2 (CLL1 and CLL2) of 4 patients. *MCL1* overexpression as a mechanism of resistance to venetoclax<sup>12</sup> has been reported recently.<sup>10,11</sup>

### Patients with CLL who are resistant to venetoclax exhibit clonal evolution

Next, we used PhyloclonDT<sup>22</sup> and Concerti<sup>23</sup> to study the subclonal architecture and expansion in 11 of our patients, using between 2 and 5 serial CLL samples per patient. We observed that CLL progression while receiving venetoclax was associated with marked clonal evolution in all patients (Figures 2 and 3; supplemental Figure 2). Clonal analysis of the pre-venetoclax and progression time points in patients with long-term venetoclax treatment of nearly 4.5 years (CLL1 and CLL2) showed that treatment with venetoclax resulted in large clonal expansions of a clone containing arm-level loss(8p) and gain(1q21.2)-containing *MCL1* (Figure 2A-B). The pre-venetoclax CLL population in CLL1 was predominantly composed of a clone harboring mutation in known CLL drivers *SF3B1*, *TP53*, *KRAS*, *XPO1*, and loss(17p), whereas in CLL2 the clonal population contained the known driver *TP53* (Figure 2A-B). To validate whether loss(8p) and gain(1q21.2) were cooccurring genetic events in the same cells, we performed

fluorescence in situ hybridization using telomeric 8p/8q probes and a locus-specific *MCL1* hybridization probe in pre- and postprogression samples for CLL1 and CLL2 (Figure 2C; supplemental Table 5). Costaining with these probes confirms 8p loss with concomitant *MCL1* gain in the same cells in accordance with the PhyloclonDT prediction.

CLL4 and CLL9 are the remaining 2 of 4 patients with acquired arm-level deletion of 8p, however, in the absence of *MCL1* amplification at progression (Figure 3A-B). CLL4, like CLL1, was treated with chemoimmunotherapy before venetoclax and had clonal driver mutations in *SF3B1* and *TP53*. However, in CLL4, with relatively early relapse while receiving venetoclax (~1.4 years), loss(8p) cooccurred with gain(8q), loss(17p), loss(18q22.1-23), loss(10q23.31), and loss(9p23-21.2) (containing the *MYC*, *TP53*, *BCL2*, *PTEN*, and *CDKN2A/B* genes, respectively) (Figure 3A). Venetoclax treatment in CLL9 resulted in the expansion of CLL cells with loss(9p) and loss(3p), whereas loss(8p) was acquired in a subclone also harboring *BCL2* G101V mutation (Figure 3B).

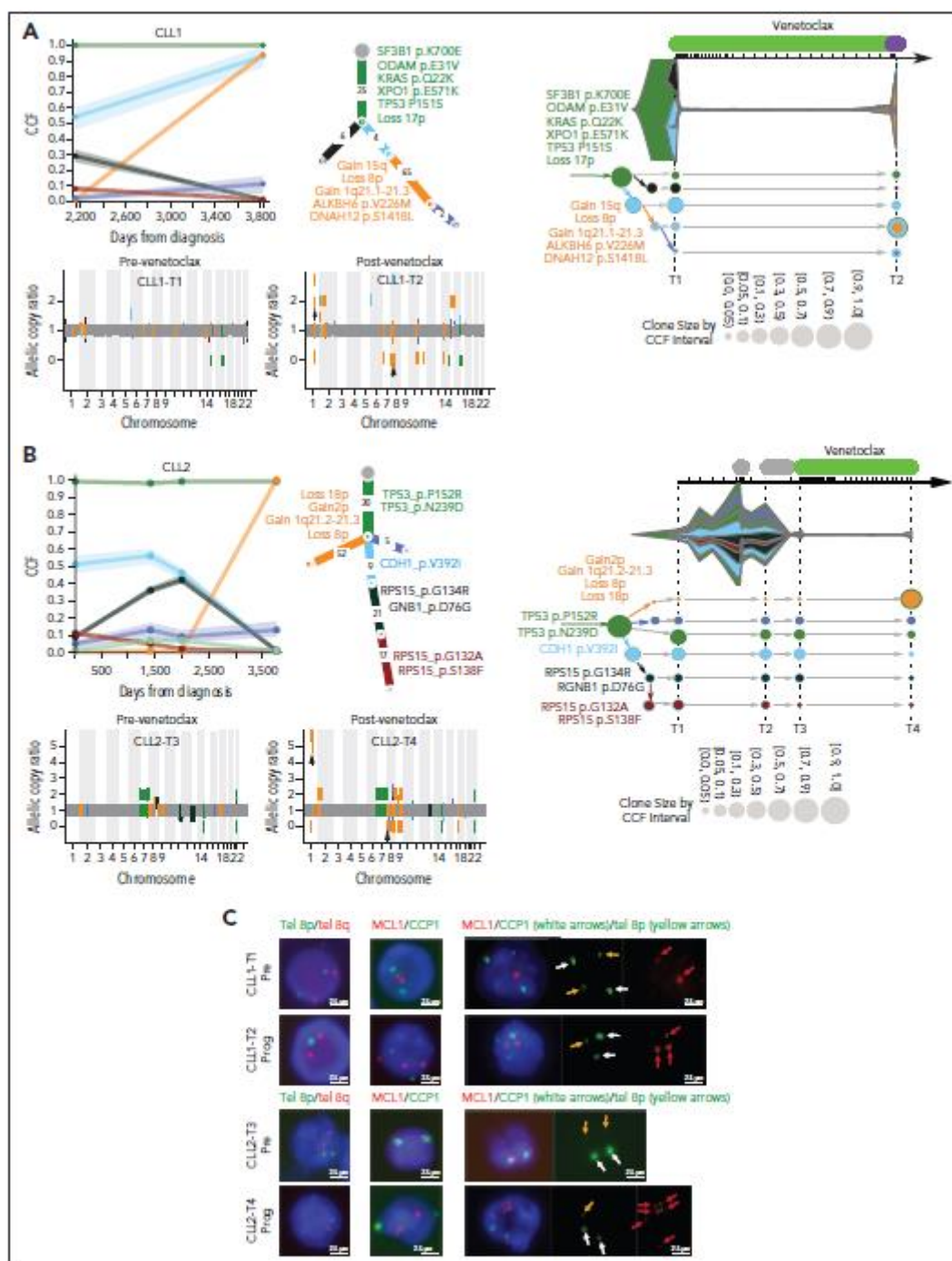
Copy number data confirmed acquired loss(8p) in 4 patients (CLL1, CLL2, CLL4, and CLL9) in accordance with GISTIC2.0 analysis (Figure 2A-B; supplemental Figure 2A). Intriguingly, the clonal dynamics in all 4 patients with loss(8p) showed a branching evolution in which the clone harboring the loss(8p) and gain(1q21.2) in CLL1 and CLL2, and loss(8p) in CLL4 and CLL9, was selected for and became the dominant clone at progression. These clones all arose from a background clone with *TP53* loss, in accordance with our previous report on loss(8p) after administration of venetoclax.<sup>12</sup>

CLL3 exhibited complex branching evolution in which several *TP53* mutations were acquired in clones that expanded during venetoclax treatment as well as other subclones containing *TP53* and *SF3B1* mutations that initially expanded but then relatively decreased during the evolution of venetoclax resistance (supplemental Figure 2A). CLL5 exhibited linear evolution with acquired *IRF8* mutations (S283C and A16fs) emerging as the dominant subclones at the time of relapse (supplemental Figure 2B). In 2 other patients (CLL6 and CLL7), who were chemo-naïve with prior progression while receiving ibrutinib, the marked clonal expansion of a clone containing loss(10q23.31-24.1) that includes *PTEN* was observed in CLL6, whereas the *PTEN* clone showed a decline in CLL7 at the progression time point (supplemental Figure 2C-D).

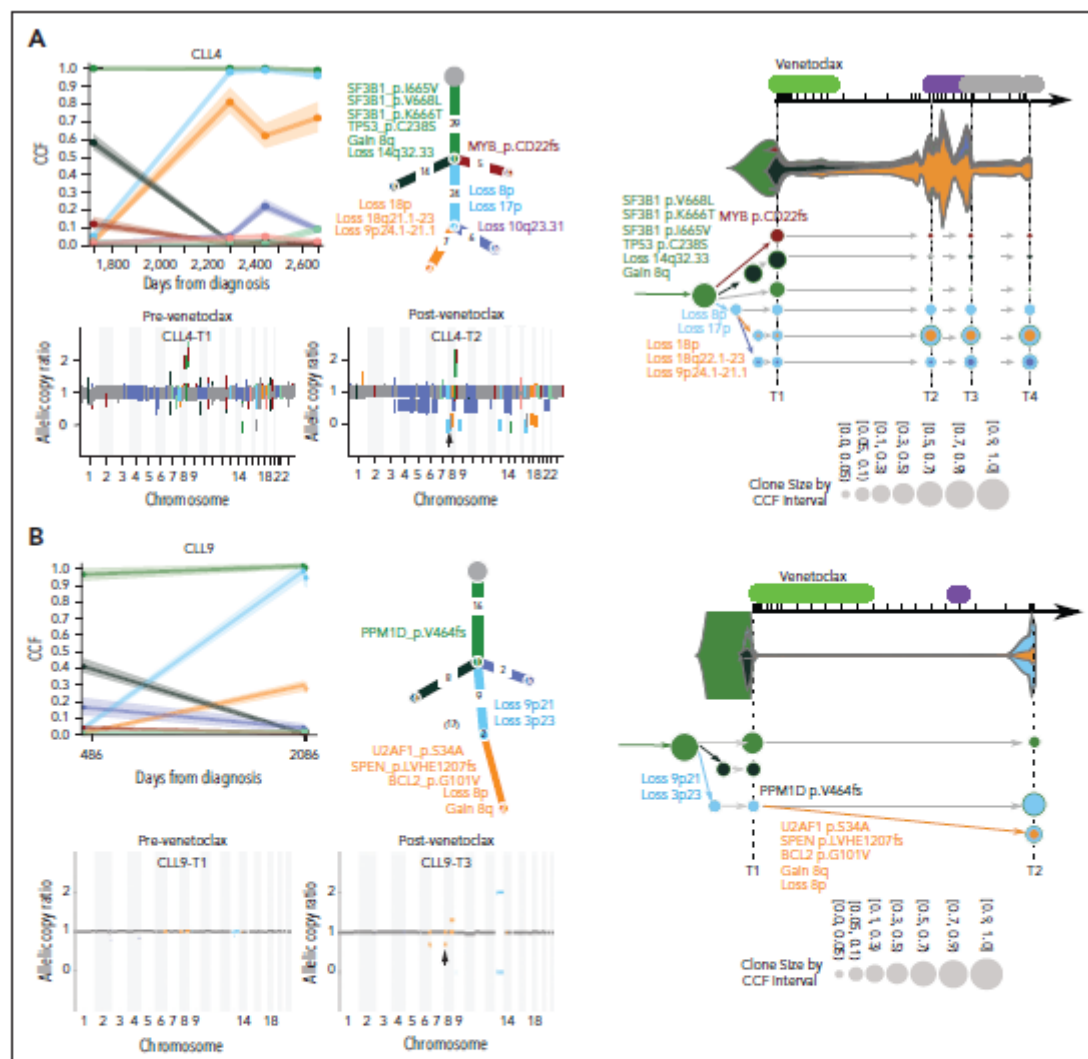
CLL8, previously treated with ibrutinib and acalabrutinib, had *TP53* and *SF3B1* as truncal mutations with *BTK* p.C481S mutation in a major subclone that regressed upon venetoclax therapy (supplemental Figure 2E). On progression, a new subclone emerged with a new *SF3B1* mutation (R775G) and *NOTCH1* mutation (P2514fs). This subclone had a daughter subclone with 2 different *BCL2* mutations: RRYR insertion mutation at *BCL2* p109 (previously reported<sup>7,24</sup>) and *BCL2* p.G101V, another *NOTCH1* and *BRAF* mutation.

CLL7 and CLL10 showed a clonal loss(8p), possibly acquired earlier with ibrutinib resistance. CLL10 also had the ibrutinib resistance mutation at *BTK* p.C481S (supplemental Figure 2D,F). The emerging dominant subclone at the time of progression on venetoclax in CLL10 harbors PCLO mutation as





**Figure 2. Clonal evolution of somatic mutations in CLL patients with concomitant loss(8p) and gain (1q21.2).** Subclonal structure and clonal evolution of somatic mutations derived using PhyloGNTD (left, middle) and Concert (right) for CLL1 (A) and CLL2 (B). (Left) Each line represents the cancer cell fraction (CCF) of mutation clusters with shading representing a 95% confidence interval. Each dot represents a different time point. Phylogenetic trees (middle) represent the best estimated clonal and subclonal architecture corresponding to the same clones (represented in the same colors) as in the left panels. (Right) Time-scaled Concert plots. Clones are sized proportionally to their prevalence and represented by the same color as in the PhyloGNTD plots. Copy number data are shown under the PhyloGNTD plots, as derived from ABSOLUTE in



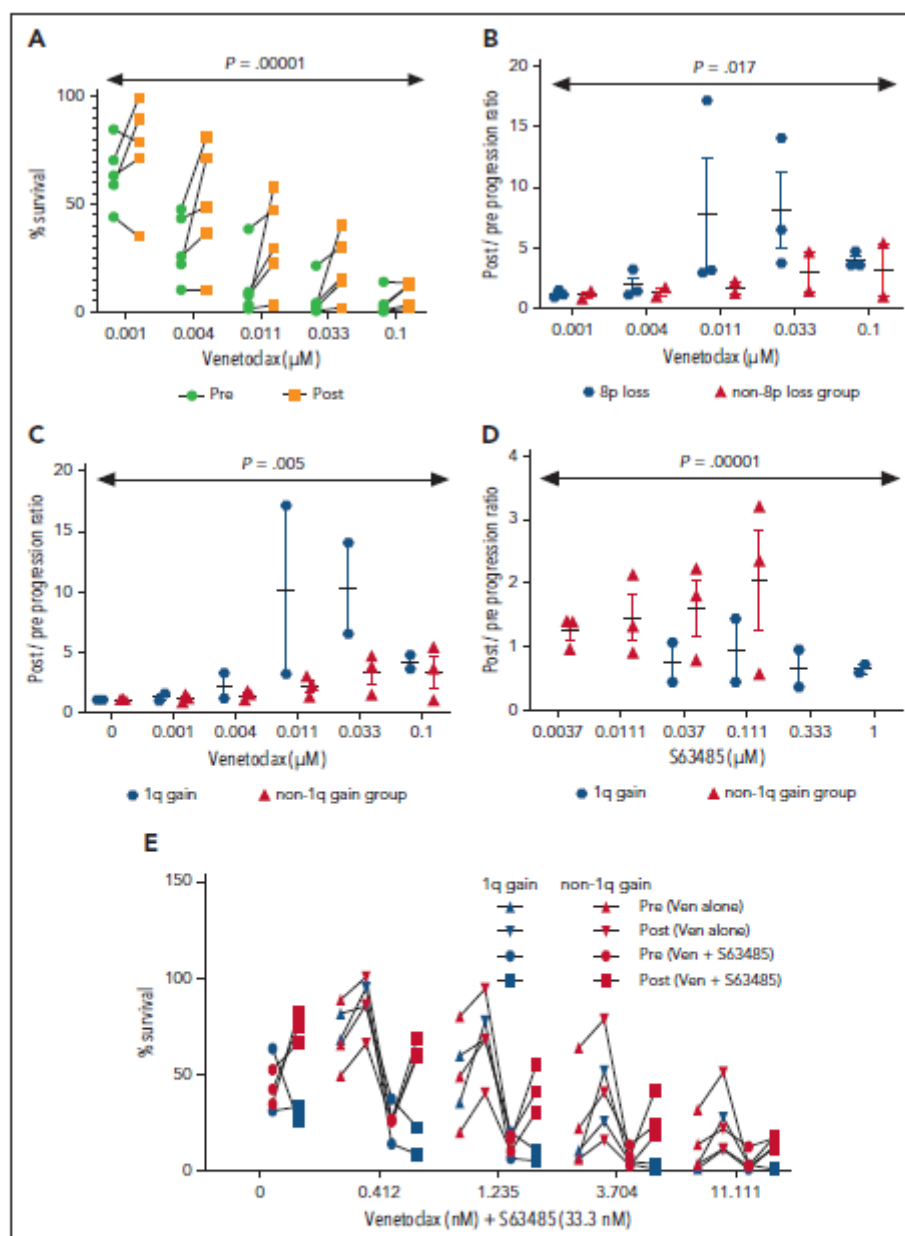
previously reported in patients with ibrutinib-relapsed CLL.<sup>25</sup> CLL11 had the longest time to progression on treatment (2724 days) with only 1 prior therapy (bendamustine-rituximab). CLL11, along with CLL8, had a BCL2\_p109 mutation in their growing clusters at the progression time point (supplemental Figure 2E,G). Altogether, these results suggest that acquired resistance to venetoclax may result from several mechanisms including loss(8p) (36%, 4 of 11), gain(1q) (18%, 2 of 11), loss(9p21) (28%, 3 of 11), and loss(PTEN) (28%, 3 of 11). Patients

in our cohort also acquired the known *BCL2* mutations *BCL2* p.G101V (18%, 2 of 11) and *BCL2* p109 (18%, 2 of 11).

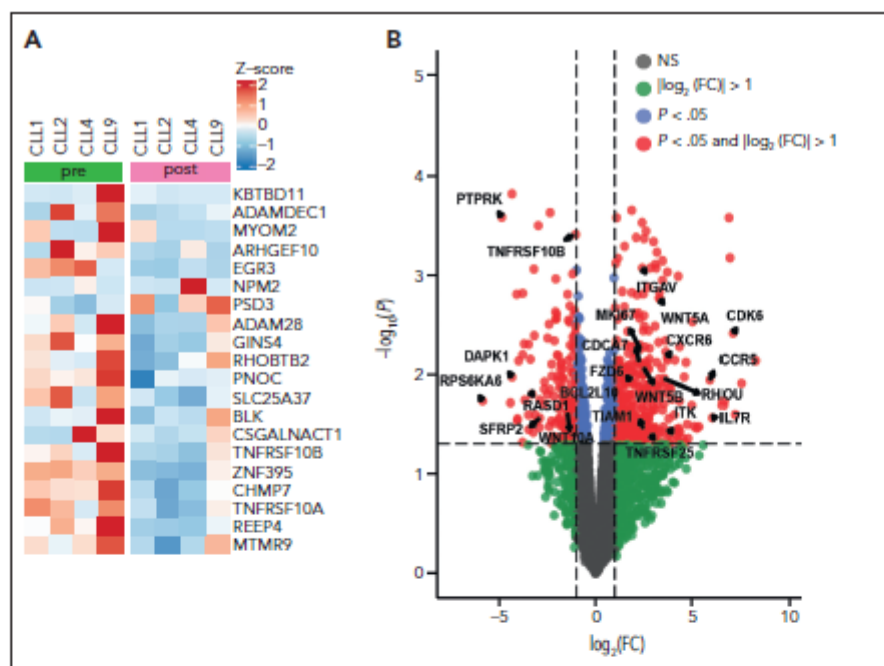
**In vitro response to venetoclax is worse in a cohort of patients with loss(8p)/gain(1q)**

We evaluated the cell viability in pre-venetoclax and progression PBMC samples treated *ex vivo* for 24 hours with venetoclax. We found that in the 5 patients tested (CLL1, CLL2, CLL4, CLL5, and CLL7), progression samples were significantly more

**Figure 2 (continued)** acquired patients with loss(8p) and gain(1q21.2) CLL1 and CLL2. See Figure 3 and supplemental Figure 2 for the remaining patients. (C) Multiplexed fluorescence in situ hybridization performed on pre- and postprogression venotocax samples for CLL1 and CLL2. The probe combination was tel(8p) (green) and tel(8q) (red) for the first column, MCL1 (red) and CCP1 (green) for the second column, and a combination of MCL1 (red) denoted by red arrows, CCP-1 (green) denoted by white arrows and tel(8q) denoted by yellow arrows for the third column.



**Figure 4. In vitro response to venetoclax in patient samples collected pre- and postprogression on venetoclax.** Pre- and postprogression time point PBMC samples (refer to the classification column of supplemental Table 1) were treated with escalating concentrations of venetoclax (A-C) for 24 hours. (A) Representation of survival as analyzed using CellTiter-Glo and the line connects matched pre- and postprogression samples for each patient. Data from panel A was split as patients with and without loss(8p) (B) and with and without gain(1q) (C). The x-axis represents the ratio of surviving cells in postprogression/pre-treatment samples. (D) Pre- and postprogression samples were treated with the MCL1 inhibitor S63485 for 24 hours and surviving cells were estimated with CellTiter-Glo. Patients with and without gain(1q) were compared. The x-axis represents the ratio of surviving pre/postprogression samples. (E) Pre- and postprogression samples were treated with 33.3 nM S63485 for 24 hours in combination with escalating concentrations of venetoclax, and surviving cells were determined by CellTiter-Glo. The line connects matched pre- and postprogression samples for each patient. Patients with and without gain(1q) were compared. The x-axis represents cell survival upon treatment with various drugs.



**Figure 5. Transcriptomic changes associated with venetoclax progression in patients with loss(8p).** (A) Normalized counts from RNA-seq data for the top 25 genes transcribed from chromosome 8p, based on mean difference in pre- and postvenetoclax progression in samples of the loss(8p) cohort (CLL1, CLL2, CLL4, and CLL9). (B) Volcano plot showing differentially expressed genes from RNA-seq data comparing samples from patients with or without loss(8p). The  $\log_2(FC)$  is plotted on the x-axis, and the negative  $\log_{10}$  adjusted P value (using FDR) is plotted on the y-axis. Genes marked in red show differentially expressed genes with an absolute value of  $\log_2(FC)$  no less than 1 ( $FC = 2$ ) and adjusted P value  $< .05$ .

resistant to venetoclax compared with corresponding pre-venetoclax time points across multiple concentrations of venetoclax ( $n = 5$ ;  $P < .001$ ) (Figure 4A). Moreover, we found that the progression samples of patients with loss(8p) (CLL1, CLL2, and CLL4) were more viable after venetoclax treatment compared with those from patients without loss(8p) (CLL5 and CLL7) (Figure 4B), which suggests that patients with loss(8p) have more resistant disease ( $P < .05$ ).

The region loss(8p) includes TRAIL receptors (TNFRSF10A/TNFRSF10B) (Figure 1D) that mediate the TRAIL apoptosis pathway and have previously been implicated in ibrutinib resistance.<sup>20</sup> To examine whether loss(8p) results in higher resistance to TRAIL-mediated apoptosis, we compared TRAIL susceptibility in 8p-intact preprogression samples and 8p-loss postprogression samples (supplemental Figure 3). Cells were activated with CD40L for 24 hours followed by exposure to titrating concentrations of TRAIL for 24 hours. CLL8, a patient without 8p-loss, did not show any difference in cell viability between preprogression and postprogression samples. In contrast, cells from patient CLL9, who acquired 8p-loss during progression, became more resistant to TRAIL-mediated death. Interestingly, cells from patient CLL11, which may harbor a focal 8p-loss affecting TNFRSF10A, also showed a similar increased resistance to TRAIL-mediated killing at the progression time point. These data are suggestive of the direct impact of loss(8p) and increased resistance to apoptosis that might explain progression of the disease in these patients.

Gain(1q) resulting in MCL1 upregulation was previously linked with venetoclax resistance in CLL cell lines and patient cells<sup>10-12</sup> as well as in acute myeloid leukemia (AML) cells.<sup>25</sup> We performed RNA-seq using pre and postprogression samples for all patients and found that the MCL1 gene was upregulated at progression in 2 patients (CLL2 and CLL7), including CLL2 with gain(1q), and marginally in CLL1 (the other patient with gain(1q)), CLL8, and CLL9 (supplemental Figure 4A). We looked at the gene expression of other antiapoptotic genes BCL2, BCL-XL/BCL2L1, BCL-W/BCL2L2, and BFL1A and found that all patients had at least 1 antiapoptotic gene that was upregulated upon progression, suggesting a compensatory mechanism in response to BCL2 inhibition by venetoclax (supplemental Figure 4A). We confirmed this finding via a western blot for BCL-XL and BFL1A in which postprogression samples had higher levels of these antiapoptotic proteins (supplemental Figure 4B).

Next, we examined the association of gain(1q) with viability. We demonstrated that, after treatment for 24 hours, the gain(1q) group (CLL1 and CLL2) was more resistant to venetoclax compared with those without gain(1q) (CLL4, CLL5, and CLL7) ( $P < .05$ ; Figure 4C). Because the gain(1q) region includes the MCL1 gene, we tested the viability of PBMC samples from pre- and postprogression time points after treatment with the MCL1 inhibitor, S63845, for 24 hours. Progression samples of the gain(1q) cohort showed enhanced susceptibility to the MCL1 inhibitor compared with their prevenetoclax patient-matched time points ( $P < .05$ ; Figure 4D). Furthermore, we tested the



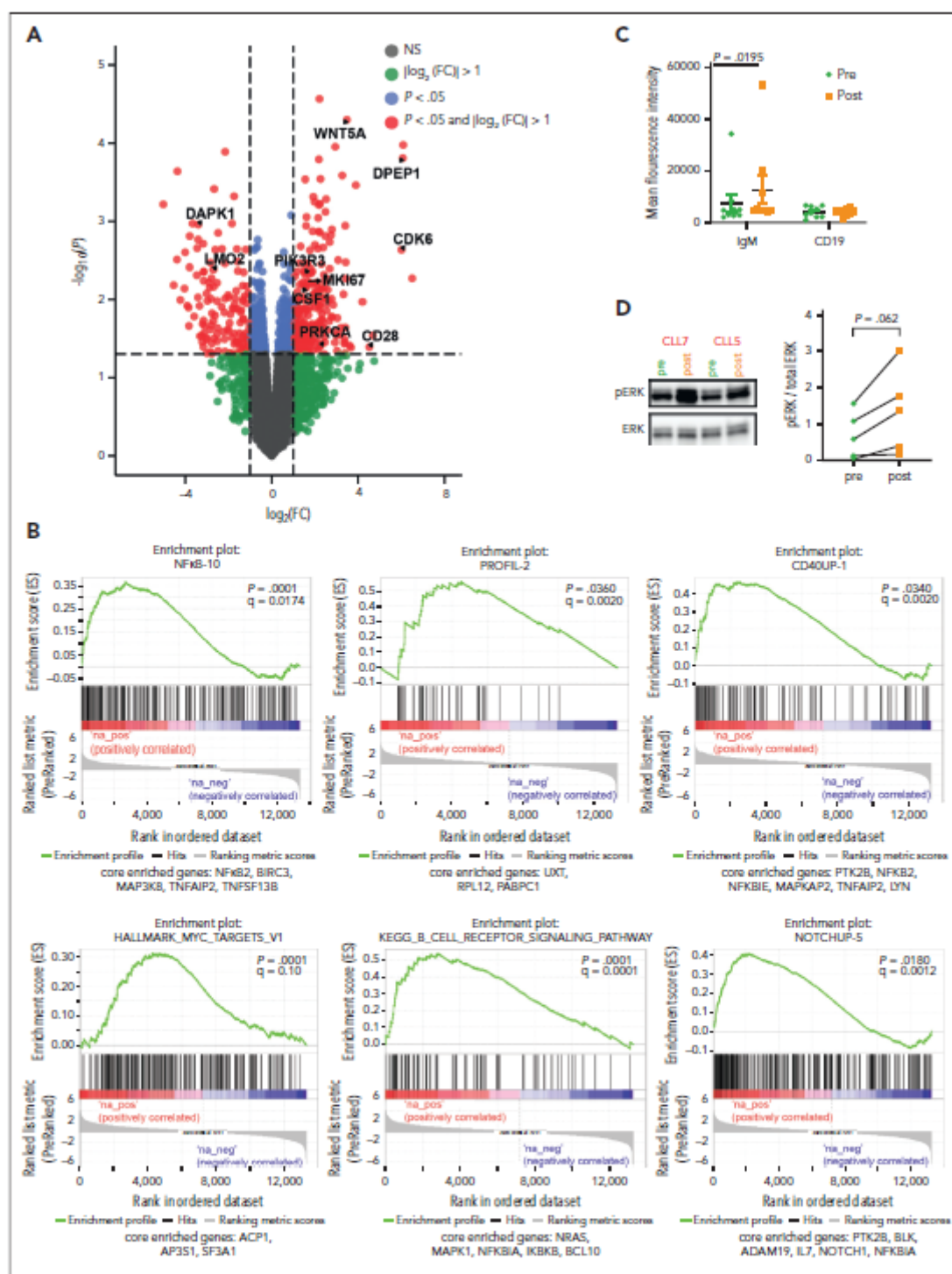


Figure 6.

combination of venetoclax and MCL1 inhibitor for 24 hours and found that progression samples of the gain(1q) cohort were more susceptible to the combination (Figure 4E). These results suggest that the combination therapy may be an effective treatment for some patients with gain(1q) venetoclax resistance, and, if used earlier, may have the potential to prevent the emergence of gain(1q), thus delaying resistance in some patients.

### Transcriptomic changes associated with venetoclax progression in patients with loss(8p)

To understand the expression changes associated with venetoclax resistance in patients with loss(8p) with or without gain(1q), we looked at RNA-seq data for the genes included in the 8p deletion in 4 patients (CLL1, CLL2, CLL4, and CLL9). Postvenetoclax resistant samples from patients with loss(8p) showed an overall downregulation of genes encoded by the 8p region as compared with prevenetoclax samples (supplemental Figure 5A). The top 20 genes on 8p that showed differential gene expression (DGE) are represented in Figure 5A. RNA-seq analysis comparing prevenetoclax with postvenetoclax resistant samples from patients with loss(8p) showed a significant downregulation of the *TNFRSF10A/10B* genes (TRAIL-Rs; Limma-Voom,  $P < .1$ ; Figure 5A-B). Gene network analysis using differentially expressed genes ( $P < .01$ ;  $|\log_2FC| > 1$  [FC, fold change]) in STRING, yielded multiple significantly altered gene ontology terms related to death pathways in which *TNFRSF10A/10B* genes are central (supplemental Figure 5). These include the regulation of the extrinsic apoptotic signaling pathway via death domain receptors (strength = 1.07; false discovery rate [FDR] = 0.021), the apoptotic pathway (strength = 0.55; FDR = 0.024), and the apoptotic process pathway (strength = 0.41; FDR = 0.047; marked in green) (supplemental Table 6).

DGE analysis showed the upregulation of *CDK6* that has been identified as a potential prognostic biomarker in AML<sup>27</sup> (Figure 5B). *WNT5A* and *FZD6*, central to the noncanonical Wnt pathway, were upregulated in the loss(8p) group (Figure 5B). High levels of *WNT5A* have been reported in the plasma of patients with CLL, and a recent report showed that *WNT5A* enhances proliferation of CLL<sup>28</sup> by binding to ROR1, which is overexpressed in CLL cells. Moreover, increased ROR1 and ROR1 signaling was observed in patients with CLL on venetoclax therapy.<sup>29</sup> These results suggest that the poor outcome of patients with loss(8p) might be mediated via the downregulation of TRAIL-R signaling and upregulation of *WNT5A* signaling pathways.

### Patients with disease progression on venetoclax show elevated ERK1/2 signaling

Next, we performed RNA-seq analysis of the entire cohort to evaluate transcriptomic adaptation of the cells during development of acquired resistance to venetoclax. To determine the consequence of venetoclax inhibition *in vivo*, we profiled 11 patients treated with venetoclax (both pre- and postvenetoclax time points) and performed DGE analysis (Figure 6A). Gene set

enrichment analysis using the Staudt Lab gene set database (<https://lymphochip.nih.gov/signaturedb/>) (with FDR < 0.1) and MSigDB<sup>30,31</sup> curated hallmark gene sets showed positive enrichment of several proliferation-related gene sets, suggesting a higher proliferative capacity of cells on acquiring resistance to venetoclax (supplemental Tables 7 and 8; Figure 6B). *WNT5A* was transcriptionally upregulated in progression samples and *WNT5A* is central to proliferation and upstream of extracellular signal-regulated kinase (ERK) phosphorylation (Figure 6A). Gene set enrichment analysis showed the positive enrichment of MYC targets and tumor necrosis factor  $\alpha$  signaling via NF- $\kappa$ B response gene set that are both downstream of ERK signaling (supplemental Table 8). In melanoma, the ERK pathway induces c-MYC stabilization via the attenuation of its ubiquitin-mediated protein degradation mechanism.<sup>32</sup> MYC upregulation also leads to higher proliferation. Genes related to B-cell receptor (BCR) activation and STAT-3 upregulation (supplemental Table 7), both of which lead to B-cell signaling upregulation, were also overrepresented. To test this, we compared surface immunoglobulin M (sIgM) levels in pre- and postprogression PBMCs by flow cytometry. We found consistently higher sIgM levels at progression compared with that in preprogression samples ( $n = 9$ ;  $P = .02$ ; Figure 6C), suggestive of constitutively higher BCR signaling. CD19 expression did not differ, suggesting that the increase in IgM surface receptor is specific (Figure 6C). The PBMC samples from pre- and postprogression time points were then profiled for downstream BCR signaling proteins. We observed higher pPLCG2 and pBTK expression in progression samples as observed through western blot, although not statistically significant because of the small sample size (supplemental Figure 6). We also observed that ERK phosphorylation (Thr 202/Tyr 204) showed a trend toward elevation in the venetoclax progression time points when compared with corresponding prevenetoclax samples for all patients tested ( $n = 5$ ;  $P = .06$ ; Figure 6D). ERK may be downstream of *WNT5A* or the BCR. In summary, these data suggest that upregulation of the BCR signaling pathway and extracellular signal-regulated kinase (ERK) phosphorylation contribute to venetoclax resistance.

## Discussion

In this study, we identified multiple potential mechanisms of resistance in patients with CLL who progressed while receiving venetoclax. The WES of 11 such patient samples revealed no recurrent sSNV involved in the resistance to venetoclax, other than *BCL2*, as previously reported. However, we identified several somatic copy-number alterations showing robust clonal shifts and evolution in patients whose disease progressed while receiving venetoclax. These include the acquired loss(8p) (36%; 4 of 11) and gain(1q) (18%; 2 of 11) in particular but also acquired loss(9p21.3) affecting *CDKN2A* (27%, 3 of 11) and acquired loss(*PTEN*) (18%; 2 of 11). In patients with relapsed/refractory disease who were previously exposed to chemo-immunotherapy, loss(9p21.3) was particularly enriched, as

**Figure 6. Patients with disease progression on venetoclax show elevated ERK1/2 signaling.** (A) Volcano plot showing differentially expressed genes between pre and postvenetoclax progression time points from RNA-seq data with adjusted  $P$  value < .05 and  $|\log_2FC| > 1.0$ . (B) Enrichment plots obtained from gene set enrichment analysis, which was performed on a list of all genes from DGE analysis and ranked based on  $t$  statistic. (C) sIgM and CD19 staining was performed on PBMCs collected from patients ( $n = 5$ ) at pre- and postprogression on venetoclax time points. Mean fluorescent intensity was measured on gated CD19<sup>+</sup> cells. (D) Representative western blot image showing pERK and ERK for PBMC lysates from pre- and postvenetoclax progression time points for 2 patients, together with quantification of pERK:ERK ratio ( $n = 5$ ).



described previously.<sup>9,33</sup> Furthermore, we identified constitutive pERK activation across many of our samples from patients with venetoclax resistance.

Previously identified venetoclax resistance mutations in BCL2 G101V were found in 3 of 11 patients (CLL8, CLL9, and CLL11) in our cohort. We did not see these mutations in 2 patients who had received prior treatment with ibrutinib (CLL6 and CLL7), raising the possibility that the clonal diversity at start of venetoclax might be reduced in patients who have not received prior chemoimmunotherapy, although confounded by short times on venetoclax for both of these patients. The extraordinarily low VAFs of 2 of the detected mutations suggest that other mechanisms are contributing to resistance.

By evaluating PBMC viability before and after ex vivo venetoclax treatment, we found that in all the patients tested, progression samples showed significantly higher survival after venetoclax treatment compared with cells from corresponding pre-venetoclax time points, consistent with acquired intrinsic resistance. Moreover, in comparing the viability of venetoclax progression samples from the loss(8p) and without loss(8p) groups, we found that progression samples from patients with loss(8p) better survived treatment with venetoclax compared with samples from patients without loss(8p) (Figure 3B), suggesting that patients with loss(8p) are more resistant and therefore likely to have a worse prognosis, as previously observed in breast cancer cells.<sup>34-36</sup> Loss of TRAIL-Rs has also previously been linked with ibrutinib resistance in CLL.<sup>20</sup> A recent study suggested that induction of cell death through TRAIL is a BAX-independent mechanism for overcoming venetoclax resistance; this observation is potentially consistent with our observation that deletion of TRAIL-Rs in CLL cells is an alternative mechanism of venetoclax resistance.<sup>11</sup>

Similarly, at progression, cells from patients with gain(1q) showed higher viability when compared with those from patients without gain(1q), and treatment with a MCL1 inhibitor alone or in combination with venetoclax significantly decreased viability in progression samples only in patients with gain(1q), suggesting this combination therapy as an option for treating this group of patients. These findings support and expand prior work that demonstrated 1q gain in patients who are resistant to venetoclax.<sup>12</sup> Furthermore, our results provide rationale for initial combination therapy with venetoclax and a MCL1 inhibitor to prevent the development of resistance via this mechanism. The aforementioned combination resensitized the cells to venetoclax despite upregulation of other antiapoptotic proteins (supplemental Figure 3; Figure 3E). It is interesting that 1q gain in our cohort occurred exclusively in the same cells with 8p deletion; this mechanism of potential synergy remains to be elucidated.

Western blot analysis of pre- and postprogression samples showed elevated pERK levels at the time of progression in all the patient samples tested (CLL1, CLL2, CLL4, CLL5, and CLL7), demonstrating a potentially significant role for MAPK signaling in acquired resistance to venetoclax in CLL. Using venetoclax-resistant clones from multiple myeloma cell lines, Chakraborty et al showed that acquired resistance to venetoclax is mainly due to the activation of pERK.<sup>37</sup> They also showed elevated expression of MCL1 and BCL-XL in the resistant clones.<sup>37</sup> Furthermore, in venetoclax-resistant AML cell lines, Zhang et al

demonstrated that resistance was primarily mediated by upregulation of MAPK signaling, resulting in increased MCL1 and BCL-XL expression.<sup>38</sup> In follicular lymphoma cells, pERK and pBIM (S67) levels were higher in cells treated with venetoclax.<sup>39</sup>

We also observed elevated sIgM levels in patients with progressive disease, indicating that BCR signaling could be partially contributing to elevated pERK levels. Anti-IgM stimulation reportedly protects CLL cells from venetoclax-induced apoptosis through MCL1 upregulation,<sup>40</sup> in accordance with our observation.

In summary, our findings identify potential targets to assess therapeutic opportunities for the treatment of patients with CLL who have acquired resistance to venetoclax. Our study shows that a subset of BCL2 mutations occur at very low VAFs, suggesting the existence of other mechanisms of resistance. We identified upregulation of sIgM and MAPK signaling as a mechanism of acquired resistance to venetoclax, suggesting that treatment with MEK or ERK inhibitors of patients with venetoclax-resistant disease could be beneficial. Moreover, we also observed that cells from patients with certain genetic abnormalities (ie, loss(8p) and gain(1q)) are more resistant to venetoclax than those from patients without these genetic abnormalities, possibly because of the loss of TRAIL receptors, upregulation of WNT5A, and MYC signaling. Treatment with MCL1 inhibitor either alone or with venetoclax decreased viability in progression samples in patients with gain(1q), suggesting that combining a MCL1 inhibitor with venetoclax might prevent the outgrowth of clones with this genetic abnormality. In addition, combination therapy may prevent the emergence of gain(1q), potentially delaying onset of resistance in some patients. Our results point to several future directions for combination therapy in clinical trials, with the goal of overcoming or avoiding venetoclax resistance.

## Acknowledgments

The authors thank all the patients and their families who participated in the venetoclax clinical trials and were willing to donate samples for this study. The authors also thank Delphine Voisin for technical assistance and Francesca Morelli and Roberta Santos Azevedo for providing clinical data analysis support.

This research was funded by the National Institutes of Health, National Cancer Institute grant R01 CA 213442 (principal investigator, J.R.B.) and by Broad/IBM Cancer Resistance Research Project (principal investigators, G.G. and L.P.).

## Authorship

Contribution: J.R.B., G.G., L.P., C.P.P., I.M., and J.K.K. designed the research; J.K.K., I.M., J.C., F.U., A.N., L.V., R.G., B.K.S., I.L., Y.K., K.V., K.R., C.L., and M.S.D. performed research and collected data; J.R.B., G.G., and L.P. contributed vital new reagents or analytical tools; I.M., J.K.K., J.C., F.U., L.L., Z.W., I.L., Y.K., K.V., K.R., C.L., and S.T. analyzed, interpreted, and performed statistical analysis; J.K.K., I.M., J.C., F.U., I.L., A.N., Y.K., K.V., S.T., S.M.F., J.-H.M., H.B., M.A., K.R., C.L., B.P.D., K.S., R.A.J., C.P.P., L.P., G.G., and J.R.B. wrote the manuscript; S.M.F., J.-H.M., H.B., M.A., B.P.D., K.S., and R.A.J. provided administrative support (ie, biobanking, managing and organizing samples, and sequencing support); and J.R.B. supervised the study.

Conflict-of-interest disclosure: J.R.B. has served as a consultant for AbbVie, Acerta/AstraZeneca, BeiGene, Genentech/Roche, Gilead, Worldwide Operations, Hutchmed, iOnctura, Janssen, Kite, Loxo/Lilly,

MEI Pharma, Numab Therapeutics, Pfizer, and Pharmacyclics; and received research funding from BeiGene, Gilead, iOncare, Loxo/Lilly, MEI Pharma, and TG Therapeutics. G.G. receives research funds from IBM and Pharmacyclics; is an inventor on patent applications related to MSMTect, MSMTect, POLYSOLVER, TensorQL, and MinimuMM-seq; and is a founder, consultant, and holds privately held equity in Scorpion Therapeutics. I.L. serves as a consultant for PACT Pharma Inc; has stock, is on the board, and serves as a consultant, for emov1 LLC; and is on the board of and holds equity in Nord Bio Inc. M.S.D. declares consultancy for AbbVie, Adaptive Biotechnologies, Ascentage Pharma, AstraZeneca, BeiGene, Bristol Myers Squibb, Eli Lilly, Genentech, Genmab, Janssen, Merck, MingSight Pharmaceuticals, Ono Pharmaceuticals, Secura Bio, Takeda, and TG Therapeutics; declares having received research funding from AbbVie, Ascentage Pharma, AstraZeneca, Genentech, Novartis, Secura Bio, and TG Therapeutics; has received honoraria from Aptitude Health, AXIS Medical Education, BioAscend, Curio Science, Medscape Education, PeerView Institute for Medical Education, Physician's Education Resource, PlatformQ Health Education, and Plexus Communications; and declares royalties for Research to Practice Other Potential Financial Relationship. The remaining authors declare no competing financial interests.

ORCID profiles: J.K.K., 0000-0003-4656-8454; F.U., 0000-0003-3226-7642; K.V., 0000-0003-0062-9389; S.T., 0000-0002-3119-6507; L.V., 0000-0002-4414-7974; B.K.S., 0000-0002-3325-9874; J.H.M., 0000-0003-0477-3476; K.R., 0000-0002-1567-9090; J.R.B., 0000-0003-2040-4961.

Correspondence: Jennifer R. Brown, Dana-Farber Cancer Institute, 450 Brookline Ave, Boston, MA 02215; email: [jennifer\\_brown@dfci.harvard.edu](mailto:jennifer_brown@dfci.harvard.edu).

## Footnotes

Submitted 28 April 2022; accepted 4 April 2023; prepublished online on Blood First Edition 5 May 2023. <https://doi.org/10.1182/blood.2022016600>.

\*J.K.K., J.C., F.U., A.N., and I.M. contributed equally to this study.

†L.P., G.G., and J.R.B. contributed equally to this study.

Presented in abstract form at the AACR Annual Meeting (virtual, 2021), and the International Workshop on CLL meeting (virtual, 2021).

Data are available on request from the corresponding author, Jennifer R. Brown ([jennifer\\_brown@dfci.harvard.edu](mailto:jennifer_brown@dfci.harvard.edu)).

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

## REFERENCES

- Skarland SS, Mato AR. Overcoming resistance to targeted therapies in chronic lymphocytic leukemia. *Blood Adv*. 2021;5(1):334-343.
- Souers AJ, Levenson JD, Boghaert ER, et al. ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. *Nat Med*. 2013;19(2):202-208.
- Kipps TJ, Stevenson FK, Wu CJ, et al. Chronic lymphocytic leukaemia. *Nat Rev Dis Primers*. 2017;3:16096.
- Roberts AW, Ma S, Kipps TJ, et al. Efficacy of venetoclax in relapsed chronic lymphocytic leukemia is influenced by disease and response variables. *Blood*. 2019;134(2):111-122.
- Blomberg P, Anderson MA, Gong JN, et al. Acquisition of the recurrent Gly101Val mutation in BCL2 confers resistance to venetoclax in patients with progressive chronic lymphocytic leukemia. *Cancer Discov*. 2019;9(3):342-353.
- Lucas F, Larkin K, Gregory CT, et al. Novel BCL2 mutations in venetoclax-resistant, ibrutinib-resistant CLL patients with BTK/PLCG2 mutations. *Blood*. 2020;135(24):2192-2195.
- Blomberg P, Thompson ER, Nguyen T, et al. Multiple BCL2 mutations cooccurring with Gly101Val emerge in chronic lymphocytic leukemia progression on venetoclax. *Blood*. 2020;135(10):773-777.
- Sedlarikova L, Petrickova A, Papajik T, Turcsanyi P, Kriegova E. Resistance-associated mutations in chronic lymphocytic leukemia patients treated with novel agents. *Front Oncol*. 2020;10:894.
- Herling CD, Abedpour N, Weiss J, et al. Clonal dynamics towards the development of venetoclax resistance in chronic lymphocytic leukemia. *Nat Commun*. 2018;9(1):727.
- Thijssen R, Tian L, Anderson MA, et al. Single-cell multiomics reveal the scale of multilayered adaptations enabling CLL relapse during venetoclax therapy. *Blood*. 2022;140(20):2127-2141.
- Thomalla D, Beckmann L, Grimm C, et al. Deregulation and epigenetic modification of BCL2-family genes cause resistance to venetoclax in hematologic malignancies. *Blood*. 2022;140(20):2113-2126.
- Guieze R, Liu VM, Rosebrock D, et al. Mitochondrial reprogramming underlies resistance to BCL-2 inhibition in lymphoid malignancies. *Cancer Cell*. 2019;36(4):369-384.e13.
- Konopleva M, Pollyea DA, Poturi J, et al. Efficacy and biological correlates of response in a phase II study of venetoclax monotherapy in patients with acute myelogenous leukemia. *Cancer Discov*. 2016;6(10):1106-1117.
- Landau DA, Tausch E, Taylor-Weiner AN, et al. Mutations driving CLL and their evolution in progression and relapse. *Nature*. 2015;526(7574):525-530.
- Cibulskis K, Lawrence MS, Carter SL, et al. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. *Nat Biotechnol*. 2013;31(3):213-219.
- Lawrence MS, Stojanov P, Mermel CH, et al. Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature*. 2014;505(7484):495-501.
- Robinson JT, Thorvaldsdottir H, Winckler W, et al. Integrative genomics viewer. *Nat Biotechnol*. 2011;29(1):24-26.
- Stachler MD, Taylor-Weiner A, Peng S, et al. Paired exome analysis of Barrett's esophagus and adenocarcinoma. *Nat Genet*. 2015;47(9):1047-1055.
- Carter SL, Cibulskis K, Helman E, et al. Absolute quantification of somatic DNA alterations in human cancer. *Nat Biotechnol*. 2012;30(5):413-421.
- Burger JA, Landau DA, Taylor-Weiner A, et al. Clonal evolution in patients with chronic lymphocytic leukaemia developing resistance to BTK inhibition. *Nat Commun*. 2016;7:11589.
- Mermel CH, Schumacher SE, Hill B, Meyerson ML, Berukhim R, Getz G. GISTIC2.0 facilitates sensitive and confident localization of the targets of focal somatic copy-number alteration in human cancers. *Genome Biol*. 2011;12(4):R41.
- Leshchiner I, Luvit D, Gainer JF, et al. Comprehensive analysis of tumour initiation, spatial and temporal progression under multiple lines of treatment. *bioRxiv*. Preprint posted online 16 February 2019. <https://doi.org/10.1101/508127>.
- Uro F, Levovitz C, Rhrissomkrai K, Parida L. A common methodological phylogenomics framework for intra-patient heteroplasms to infer SARS-CoV-2 sublineages and tumor clones. *BMC Genomics*. 2021;22(suppl 5):518.
- Popovic R, Dunbar F, Lu C, et al. Identification of recurrent genomic alterations in the apoptotic machinery in chronic lymphocytic leukemia patients treated with venetoclax monotherapy. *Am J Hematol*. 2022;97(2):E47-e51.

25. Kadri S, Lee J, Fitzpatrick C, et al. Clonal evolution underlying leukemia progression and Richter transformation in patients with ibrutinib-relapsed CLL. *Blood Adv.* 2017; 1(12):715-727.
26. Li H, Zhang M, Linghu E, et al. Epigenetic silencing of TMEM176A activates ERK signaling in human hepatocellular carcinoma. *Clin Epigenetics.* 2018;10(1):137.
27. Liu W, Yi JM, Liu Y, et al. CDK6 is a potential prognostic biomarker in acute myeloid leukemia. *Front Genet.* 2020; 11:600227.
28. Hasan MK, Rassenti LZ, Wicherop II GF, Shen Z, Briggs SP, Kipps TJ. Wnt5a induces ROR1 to interact Grb2 to enhance Ras activation in chronic lymphocytic leukemia [abstract]. *Blood.* 2021;138(suppl 1). Abstract 247.
29. Ghia EM, Rassenti LZ, Choi MY, et al. High expression level of ROR1 and ROR1-signaling associates with venetoclax resistance in chronic lymphocytic leukemia. *Leukemia.* 2022;36(6):1609-1618.
30. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A.* 2005;102(43):15545-15550.
31. Mootha VK, Lindgren CM, Eriksson KF, et al. PGC-1 $\alpha$ -responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet.* 2003;34(3):267-273.
32. Tsai W-B, Aliba I, Long Y, et al. Activation of Ras/P3K/ERK pathway induces c-Myc stabilization to upregulate argininosuccinate synthetase, leading to arginine deiminase resistance in melanoma cells. *Cancer Res.* 2012;72(10):2622-2633.
33. Edelmann J, Holzmann K, Tausch E, et al. Genomic alterations in high-risk chronic lymphocytic leukemia frequently affect cell cycle key regulators and NOTCH1-regulated transcription. *Haematologica.* 2020;105(5):1379-1390.
34. Chang B, Liu G, Yang G, Mercado-Uribe I, Huang M, Liu J. REDD1 is required for RAS-mediated transformation of human ovarian epithelial cells. *Cell Cycle.* 2009;8(5):780-786.
35. Brown JR, Hanna M, Tesar B, et al. Integrative genomic analysis implicates gain of PIK3CA at 3q26 and MYC at 8q24 in chronic lymphocytic leukemia. *Clin Cancer Res.* 2012; 18(14):3791-3802.
36. Lebeck P, Mittenzwei A, Kuth M, et al. 8p deletion is strongly linked to poor prognosis in breast cancer. *Cancer Biol Ther.* 2015; 16(7):1080-1087.
37. Chakraborty C, Xu Y, Yao Y, et al. Activation of the ERK pathway drives acquired resistance to venetoclax in MM cell models [abstract]. *Blood.* 2020;136(suppl 1):21-22.
38. Zhang Q, Riley-Gillis B, Han L, et al. Activation of RAS/MAPK pathway confers MCL-1 mediated acquired resistance to BCL-2 inhibitor venetoclax in acute myeloid leukemia. *Signal Transduct Target Ther.* 2022;7(1):51.
39. Bodo J, Zhao X, Durkin L, et al. Acquired resistance to venetoclax (ABT-199) in t(14;18) positive lymphoma cells. *Oncotarget.* 2016; 7(43):70000-70010.
40. Bojarczuk K, Sasi BK, Gobessi S, et al. BCR signaling inhibitors differ in their ability to overcome Mcl-1-mediated resistance of CLL B cells to ABT-199. *Blood.* 2016;127(25):3192-3201.

© 2023 by The American Society of Hematology