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THESE DE DOCTORAT

NNT : 2024UPASQ019

Rôle du système circadien dans le contrôle de la survie des patients traités par immunothérapie pour cancer. Analyse des mécanismes impliqués.

Role of the circadian timing system in the control of survival of patients receiving immunotherapy for cancer. Analysis of relevant mechanisms.

Thèse de doctorat de l'université Paris-Saclay

École doctorale n°569 : Innovation Thérapeutique : du Fondamental à l'Appliqué (ITFA)
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Thèse préparée dans l'unité de recherche « **Chronothérapie, Cancers et Transplantation (Université Paris-Saclay)** », sous la direction du Professeur **René ADAM**, MD, PhD, la co-direction du Professeur **Francis LÉVI**, MD, PhD, le co-encadrement du Docteur **Sandrine DULONG**, PhD, et du Docteur **Boris DUCHEMANN**, MD, PhD.

Thèse soutenue à Villejuif, le 20 septembre 2024, par

Abdoulaye KARABOUÉ

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université PARIS-SACLAY

ÉCOLE DOCTORALE
Innovation thérapeutique du fondamental à l'appliqué (ITFA)

Titre : Rôle du système circadien dans le contrôle de la survie des patients traités par immunothérapie pour cancer. Analyse des mécanismes impliqués.

Mots clés : Rythme circadien – Cancer – Immunothérapie – Inhibiteurs de point de contrôle immunologique – Administration matinale – Mécanismes de contrôle de la survie

Résumé :

Le système circadien régule la plupart des fonctions cellulaires et organismiques des êtres vivants. Il en résulte que l'heure d'administration des médicaments anticancéreux peut profondément modifier leurs toxicités et leur efficacité. L'objectif principal de ma thèse est d'identifier le rôle de l'heure d'administration des Inhibiteurs de point de Contrôle Immunologique (ICI) sur leur efficacité et leur tolérance chez des patients atteints de cancer, et d'en analyser les mécanismes circadiens principaux. Chez des patients atteints de cancer du poumon non-à petites cellules, je montre un quadruplement de la survie par l'administration matinale majoritaire de nivolumab, un ICI, en comparaison de l'après-midi. Dans la première méta-analyse de 13 études évaluant le rôle de l'heure d'administration sur la survie des patients cancéreux, j'identifie que l'administration matinale de l'immunothérapie permet en moyenne de doubler la survie globale et la survie sans progression des patients atteints de huit types différents de cancer. Dans une revue de synthèse, je mets à jour ces résultats et propose les mécanismes circadiens principaux capables d'influencer l'efficacité de l'immunothérapie au cours des 24 heures. Dans une grande étude franco-chinoise, je mets en évidence que l'administration matinale de l'immunothérapie permet aussi de doubler son efficacité en comparaison d'une administration l'après-midi, malgré son association avec une chimiothérapie immunomodulatrice. Ces résultats me conduisent à proposer un essai clinique randomisé afin d'évaluer l'impact des rythmes circadiens individuels sur l'efficacité et la tolérance de l'immunothérapie. Ma thèse révèle ainsi que l'efficacité de l'immunothérapie des cancers, seule ou associée à une chimiothérapie est améliorée de façon très statistiquement significative par une administration matinale, et pourrait l'être encore davantage par la personnalisation de la chronoimmunothérapie.

université PARIS-SACLAY

ÉCOLE DOCTORALE
Innovation thérapeutique
du fondamental à l'appliqué
(ITFA)

Title : Role of the circadian timing system in the control of survival of patients receiving immunotherapy for cancer. Analysis of relevant mechanisms.

Keywords : Circadian rhythm – Cancer – Immunotherapy – Immune checkpoint inhibitors – Morning administration - Survival control mechanisms

Abstract :

The circadian timing system regulates most cellular and organismic functions in living organisms. As a result, the time of administration of anticancer drugs can profoundly modify their toxicity and efficacy. The main objective of my thesis is to identify the role of the time of administration of Immune Checkpoint Inhibitors (ICIs) on their efficacy and tolerability in cancer patients, and to analyze the main circadian mechanisms involved. In patients with non-small-cell lung cancer (NSCLC), I show a quadrupling of survival with morning compared with afternoon administration of ICI nivolumab. In the first meta-analysis of 13 studies evaluating the role of the time of administration of ICIs on cancer patient survival, I identify that, on the average, morning administration of immunotherapy doubles overall survival and progression-free survival compared to afternoon or evening dosing in patients with eight different types of cancer. In an exhaustive literature review, on these topics, I update these latter results and I analyse the main circadian mechanisms which can influence the efficacy of immunotherapy over the 24 hours. In a large French-Chinese collaborative study involving 713 NSCLC patients, I demonstrate that morning administration of immunotherapy also doubles its efficacy compared with afternoon administration, despite its association with immunomodulating chemotherapy. These results led me to propose a randomized clinical trial to assess the impact of circadian rhythms on the efficacy and tolerability of immunotherapy, and to evaluate the relevance of the personalization of ICI delivery timing. My thesis thus reveals that the efficacy of cancer immunotherapy, alone or in combination with chemotherapy, is improved in a highly statistically significant way by morning administration, and could be further enhanced by the personalization of chrono-immunotherapy according to patient sex and circadian rhythm characteristics.

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- Madame la Professeure **Charna DIBNER**, Université de Genève, Belgique. Merci d'avoir accepté d'évaluer ce travail de thèse et d'en être rapporteure.
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ABBREVIATIONS

AACR: American Association for Cancer Research

ALK: Anaplasic Lymphoma Kinase

ARNTL: Aryl Hydrocarbon Receptor Nuclear Translocator-Like

ASCO: American Society of Clinical Oncology

Atezo : Atezolizumab

Ave: Avelumab

BHLHE40: Basic Helix-Loop-Helix Family Member E40

BHLHE41: Basic Helix-Loop-Helix Family Member E41

BMS-1 : PD-1/PD-L1 inhibitor 1

CBNPC : Cancer Bronchique Non-à-Petite Cellules

CD : Cluster de Différenciation

CLOCK: Clock Circadian Regulator

CRY1: Cryptochrome circadian regulator 1

CRY2: Cryptochrome circadian regulator 2

CTLA4 : Cytotoxic T-Lymphocyte Antigen 4

Durva : Durvalumab

EGFR : Epithelial Growth Factor Receptor

ESMO : European Society for Medical Oncology

HR : Hazard ratio

ICIs : Inhibiteurs de point de contrôle immunologique

IFN : Interferon

IL : Interleukine

Ipi : Ipilimumab

I<O : Indice de dichotomie

LAG-3 : Lymphocyte activation gene-3

NCI-CTCAE v4.0 : National Cancer Institute - Common Terminology Criteria for Adverse Events version 4.0.

DBP: D-Box Binding PAR BZIP Transcription Factor

Nivo : Nivolumab

NK : Natural Killer

NPAS2: Neuronal PAS domain protein 2

NR1D1: Nuclear Receptor Subfamily 1 Group D Member 1

NR1D2: Nuclear Receptor Subfamily 1 Group D Member 2

NSC : Noyaux suprachiasmatique

OMS : Organisation mondiale de la santé

PD-1 : Programmed cell Death-1

Pdcd-1 : Programmed Cell Death protein-1

PD-L1: Programmed Death-Ligand 1

Pembro : Pembrolizumab

PER: Period Circadian Regulator

PS : Performance status

RORA: Related orphan receptor A

ROS1 : Reactive oxygen species 1

SG : Survie globale

SIC : Society for Immunotherapy of Cancer

SSP : Survie sans progression

T4L : Tétra-iodothyronine libre

TIMELESS: Timeless circadian regulator

TSH : Thyroid Stimulating Hormone

ZT : Zeitgeber Time

RESUME

Responsable d'environ 10 millions de décès par an dans le monde, dont 157400 en France, le cancer reste la première cause de mortalité par maladie. Mieux comprendre les mécanismes physiopathologiques et mieux cibler les traitements, selon les caractéristiques de la personne et de sa tumeur, font l'objet de recherches intensives de par le monde, dans le cadre de la médecine cancérologique de précision. Celle-ci commence à intégrer les horloges circadiennes moléculaires, qui génèrent des oscillations périodiques d'environ 24h du métabolisme, de la prolifération et de la mort cellulaire, ainsi que des réponses aux substances toxiques ou agents thérapeutiques. Ces horloges moléculaires et les fonctions qu'elles contrôlent, sont coordonnées à l'échelle de l'organisme entier par les noyaux suprachiasmatiques de l'hypothalamus via la génération de rythmes physiologiques tels que le cycle veille/sommeil, les rythmes d'activité/repos, de la température corporelle, de la sécrétion de mélatonine et de cortisol et l'activité des gènes horloges. Ce système circadien est synchronisé essentiellement par l'alternance jour/nuit (ou lumière/obscurité). D'autres signaux périodiques de l'environnement peuvent également intervenir comme synchroniseurs tels que l'alternance bruit-silence, chaud-froid, activité-

repos et les impératifs horaires de la vie familiale et socio-professionelle. De nombreuses études expérimentales, cliniques et épidémiologiques ont montré le rôle important du système circadien dans toutes les étapes de la cancérogenèse, depuis l'initiation et la promotion jusqu'à la progression et la dissémination métastatique. La disruption circadienne provoquée au plan expérimental par la destruction des noyaux suprachiasmatiques, l'exposition à un décalage horaire chronique ou la mutation d'un ou plusieurs gènes de l'horloge, favorise la cancérogenèse. C'est aussi le cas de la disruption circadienne liée au travail de nuit, ainsi que l'a rapporté l'Agence Internationale pour la Recherche sur le Cancer (OMS). De même, la survenue d'une disruption circadienne chez les patients cancéreux a été associée à une réduction significative de la survie et à une moindre tolérance aux traitements. Le système circadien influence aussi la tolérance et l'efficacité des traitements anticancéreux tant dans les modèles expérimentaux que chez les patients. Il existe cependant une variabilité inter-patient de la phase et de la robustesse du système circadien nécessitant le développement de méthodes pour stratifier les patients selon le schéma de chronothérapie le mieux adapté. Constatant une apparente grande homogénéité des réponses temporelles à l'immunothérapie des cancers dans ma pratique clinique, j'ai décidé de rechercher le rôle du moment d'administration et des

rythmes circadiens dans l'efficacité des inhibiteurs de point de contrôle immunologique (ICIs). Cette approche novatrice de ma Thèse est confortée par le fait que cette dernière décade a vu émerger l'immunothérapie comme traitement de référence de plusieurs types de cancer. On constate cependant que tous les patients n'en bénéficient pas, et que la guérison ne concerne qu'un faible nombre d'entre eux.

Mon travail de thèse a pour objectif premier d'identifier le rôle de l'heure d'administration dans la journée des ICIs sur leur efficacité et leur tolérance chez des patients atteints de cancer, en identifiant les heures d'administration associées à une meilleure survie des patients cancéreux. En second lieu, l'objectif est de préciser la reproductibilité des résultats par une analyse exhaustive de la littérature, afin d'estimer l'amélioration de la survie liée à un horaire majoritaire d'administration. Ceci conduit à proposer les mécanismes circadiens principaux qui influencent l'efficacité de l'immunothérapie au cours de la journée. Enfin, le rôle de l'heure d'administration d'un ICI persiste-t-il en cas d'association avec une chimiothérapie immunomodulatrice ? Cet effet est-il récapitulé dans les quatre premiers cycles de traitement ?

Dans une première étude pilote sur 95 patients atteints de cancer bronchique non-à-petite cellules localement avancé ou métastatique, je montre un

quadruplement des médianes de survie globale (HR : 0,174 [IC à 95%, 0,082 – 0,370]), et de la survie sans progression (HR : 0,258 [0,115 – 0,580]) après administration majoritaire matinale plutôt que l'après-midi du nivolumab, un ICI anti-PD1 (Programmed cell Death 1), administré en monothérapie après échec d'une chimiothérapie (*Karaboué et al. Cancers 2022*). J'ai suivi une approche pragmatique dichotomisant la population de patients selon qu'ils aient reçu plus de la moitié des perfusions de nivolumab avant ou après la médiane située à 12h54. Ces différences d'efficacité hautement statistiquement significatives ne sont affectées par aucun des facteurs pronostiques connus comme l'âge, le sexe, l'indice de performance, le pourcentage d'expression des récepteurs PD-L1 (Programmed cell Death 1-ligand) sur la tumeur, le nombre de sites métastatiques, l'envahissement hépatique ou cérébral etc. Une étude de sensibilité confirme l'accroissement important et très significatif lié à l'administration matinale pour tous les paramètres d'efficacité.

Dans une méta-analyse sur données publiées, nous confirmons le rôle de l'heure d'administration des ICIs pour quatre agents différents (nivolumab, pembrolizumab, ipilimumab et atezolizumab) sur la survie globale (HR : 0,50 [0,42-0,58] ; P < 0,00001) et la survie sans progression (HR : 0,51 [0,42-0,61] ; P < 0,00001) dans 13 études rétrospectives publiées, portant sur un total de 1663

patients atteints de cancers métastatiques du poumon non-à-petites cellules , de mélanome, de carcinome rénal, urothélial ou de l'œsophage (*Landre*, Karaboué* et al. ESMO Open 2024*). Nous remarquons que le seuil horaire choisi pour dichotomiser les populations des patients en fonction des horaires de perfusion de l'immunothérapie se situe entre 12h00 et 14h00 pour six études, et entre 16h00 et 16h30 pour sept études. Pour ces seuils horaires, l'efficacité des traitements précoces semble améliorée en comparaison des administrations tardives.

Dans une revue visant à préciser les mécanismes potentiels en cause dans les différences d'efficacité liées à l'heure d'administration de l'immunothérapie (*Karaboué et al. Brit J Cancer 2024*), j'ai d'abord complété les résultats de la méta-analyse par ceux de cinq nouvelles études publiées depuis. Au total, 18 études portant sur 3250 patients atteints de mélanome, de cancer du poumon, du rein, de la vessie, de l'œsophage, de l'estomac ou du foie au stade avancé ou métastatique ont été réalisées dans 9 pays. Ces études de vie réelle ont montré chacune une amélioration très statistiquement significative ou proche de la significativité pour la survie globale et la survie sans progression chez les patients traités par ICIs le matin ou en début d'après-midi par rapport à la fin de l'après-midi ou la soirée. Une revue exhaustive de la littérature m'a permis d'identifier

les principaux mécanismes reliant l'horloge circadienne aux cycles de 24 heures de l'efficacité antitumorale des ICIs. En effet, il ressort que l'influence du rythme circadien sur le microenvironnement immunitaire de la tumeur constitue le mécanisme principal. Cependant, d'autres mécanismes peuvent être considés du fait de leurs interactions avec le système immunitaire et comprennent :

- La coordination par le système circadien de la physiologie des cellules, des tissus et de l'ensemble du corps sur une échelle de 24 heures
- Les rythmes circadiens générés au niveau cellulaire par un système d'horloge moléculaire qui implique 15 gènes d'horloge spécifiques
- La perturbation des rythmes circadiens pouvant déclencher ou accélérer la carcinogenèse et contribuer à l'échec des traitements anticancéreux par le biais d'une évasion immunitaire de la tumeur résultant d'un microenvironnement tumoral immunosuppressif.

Cette analyse des mécanismes potentiels de la chrono-immunothérapie chez les patients cancéreux, est étayée par les mécanismes expérimentaux de la chronoefficacité de l'immunothérapie récemment identifiés et rapportés.

L'hypothèse que l'importance de l'heure d'administration de l'immunothérapie pourrait être altérée voire supprimée en cas d'association à une chimiothérapie,

souvent immunosuppressive, est rejetée par une grande étude rétrospective portant sur 713 patients atteints de cancer de poumon avancé ou métastatique issus d'une cohorte française de 165 patients et d'une cohorte chinoise de 548 patients (*Huang*, Karaboué* et al. Soumis pour publication*). En effet l'administration d'au moins deux des quatre premières cures d'immuno-chimiothérapie débutant avant 11h30 améliore significativement la survie globale (HR : 0,47 [0,37 – 0,60] ; p < 0,0001) et la survie sans progression (HR : 0,54 [0,45 – 0,65] ; p < 0,0001), tant dans la population totale que dans chacune des deux cohortes. Ainsi les médianes de survie globale en cas de traitement majoritaire avant 11:30 en comparaison d'un traitement plus tardif sont de 33 mois vs 19,5 mois (p < 0,0001) pour la population globale, de 23,7 vs 9,3 mois (p=0,002) pour la Cohorte 1, et de 36 vs 20 mois (p<0,0001) pour la Cohorte 2. Les analyses de sensibilité et multivariées confirment l'indépendance du rôle de l'heure des traitements sur l'efficacité vis-à-vis de tous les facteurs pronostiques connus. Cette étude confirme aussi nos observations initiales dans la méta-analyse et le rôle déterminant des horaires des quatre premiers cycles de l'immunothérapie sur son efficacité.

En conclusion, les résultats de ma thèse conduisent à proposer une approche innovante de l'administration de l'immunothérapie pour optimiser son efficacité

qui consiste en une administration matinale plutôt que l'après-midi. Mes recherches soulèvent la question de la personnalisation de l'administration de l'immunothérapie selon les rythmes circadiens individuels des patients dans le but d'améliorer davantage encore son index thérapeutique, en prenant en compte conjointement chronoefficacité et chronotolérance. Dans cette perspective, j'ai contribué à l'élaboration d'un essai clinique randomisé multicentrique testant le rôle de l'heure d'administration de l'immunochimiothérapie des CBNPC, tout en examinant les méthodes d'optimisation personnalisée de la chronothérapie *(Annexe I : « IMMUNOTIME »*). Sa réalisation s'appuiera notamment sur mon expérience personnelle concernant les biomarqueurs circadiens, le chronotype des patients, les indicateurs de la coordination circadienne (rythmes d'activité-repos, de température corporelle et de la cortisolémie) et les rythmes des cellules immunitaires et du microenvironnement tumoral.

1 INTRODUCTION

Responsable d'environ 10 millions de décès par an dans le monde, dont 157400 en France, le cancer reste la première cause de mortalité par maladie(1). Mieux comprendre les mécanismes physiopathologiques et mieux cibler les traitements, selon les caractéristiques de la personne et de sa tumeur, font l'objet de recherches intensives de par le monde, dans le cadre de la médecine cancérologique de précision et personnalisée (2, 3). Celle-ci commence à intégrer les horloges circadiennes moléculaires, qui génèrent des oscillations périodiques d'environ 24h du métabolisme, de la prolifération et de la mort cellulaire, ainsi que des réponses aux substances toxiques ou agent thérapeutiques (4-7). Ces horloges moléculaires et les fonctions qu'elles contrôlent sont coordonnées à l'échelle de l'organisme entier par les noyaux suprachiasmatiques de l'hypothalamus via la génération de rythmes physiologiques tels que le cycle veille/sommeil, les rythmes d'activité/repos, de la température corporelle, de la sécrétion de mélatonine et de cortisol. L'ensemble de ces composants (horloges moleculaires, noyaux suprachiasmatiques et le rythmes physiologiques) constituent le système circadien (6).

1.1 LE SYSTEME CIRCADIEN

Les rythmes circadiens sont des oscillations biologiques endogènes avec une période d'environ 24 heures, qui caractérisent la plupart des paramètres physiologiques, contribuant ainsi au bien-être et à la santé (8-14). Chez les mammifères, les rythmes circadiens régulent l'éveil et le sommeil, l'activité et le repos, l'appétit, les performances musculaires et cognitives, la température corporelle, les sécrétions hormonales, le métabolisme, la prolifération et la mort cellulaire, ainsi que la production, les fonctions et la circulation des cellules immunitaires (15). Les rythmes circadiens présentent des périodes précises de 24 heures suite à leur synchronisation par les cycles environnementaux de 24 heures, en particulier l'alternance régulière de la lumière et de l'obscurité sur 24 heures (12-16). D'autres facteurs environnementaux influencent les rythmes circadiens, notamment les horaires d'activité physique et de repos, les interactions socioprofessionnelles et familiales et les habitudes alimentaires (17-20). La coordination circadienne et l'adaptation aux signaux environnementaux s'effectuent principalement par l'intermédiaire du noyaux suprachiasmatique (NSC), un « pacemaker » hypothalamique composé d'environ 50 000 neurones chez l'Homme, et situé au-dessus du chiasma optique (21). Chez la Souris ou le Rat, la plupart des rythmes circadiens sont supprimés à la suite d'une ablation

physique ou d'une altération fonctionnelle du NSC, par exemple en cas de décalage horaire chronique (22-25). Chez les mammifères, les signaux rythmiques générés par les NSC coordonnent les horloges moléculaires génétiques qui résident dans chaque cellule, réalisant un réseau d'horloges hiérarchisé qui constitue le système circadien (13, 26). Les cellules de mammifères sont dotées d'une horloge moléculaire impliquant au moins 15 gènes dits « de l'horloge » (NPAS2, PER2, DBP, ARNTL, PER3, NR1D1, CRY1, NR1D2, CLOCK, CRY2, BHLHE40, PER1, BHLHE41, RORA, TIMELESS) qui interagissent les uns avec les autres par le biais de trois boucles de régulation de la transcription/post-transcription, générant ainsi des oscillations circadiennes dans les cellules individuelles. Les dimères de protéines BMAL1::CLOCK (ou NPAS2) en particulier, jouent un rôle clé dans l'horloge moléculaire par l'activation de la transcription des gènes d'horloge *Per's, Cry's* et *Rev-erb's* (17, 27) (**Figure1**).

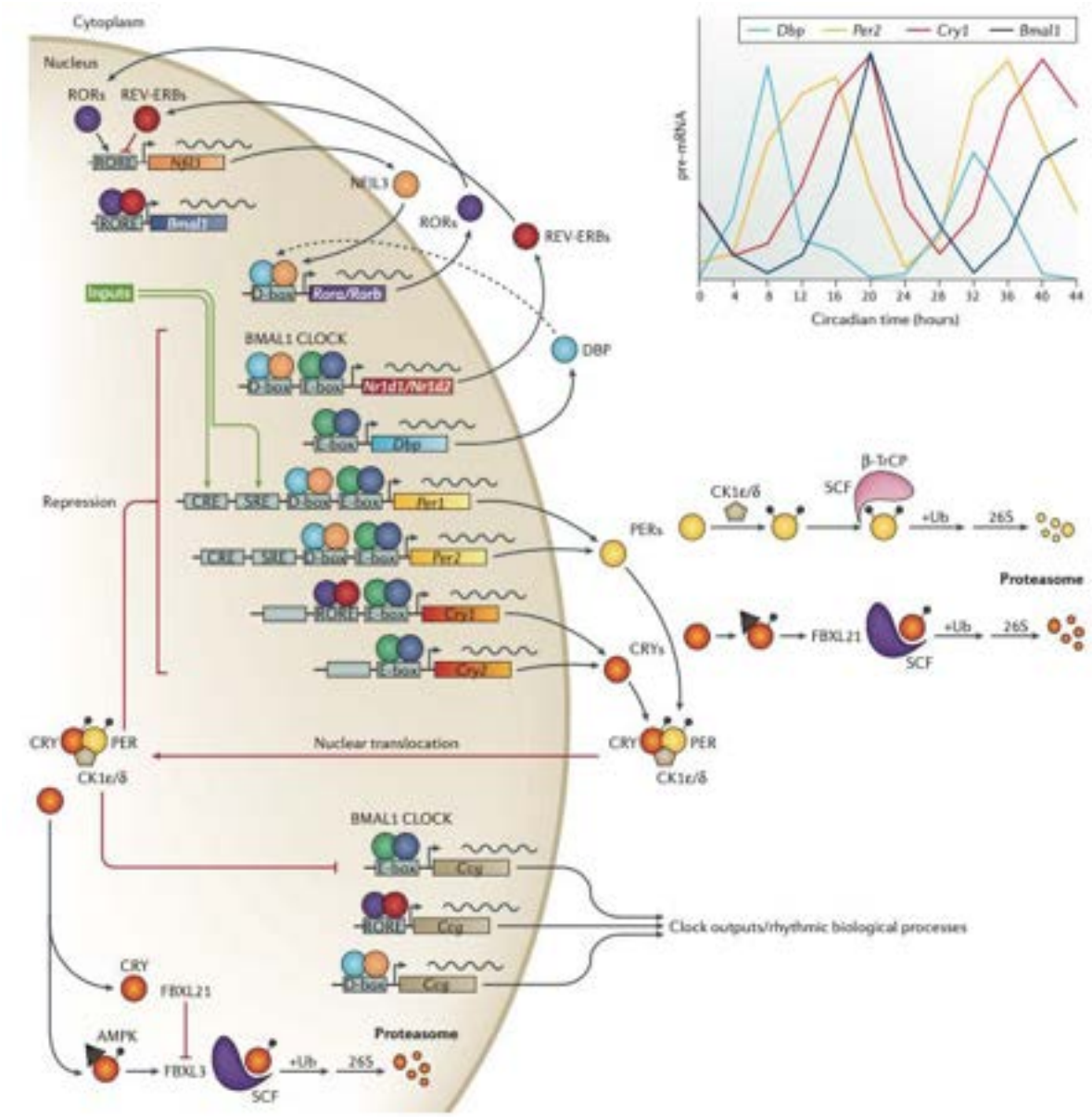

Figure 1: Organisation moléculaire de l'horloge circadienne chez les mammifères.

Au cœur du réseau, les facteurs de transcription bHLH (basic helix-loop-helix), PER, ARNT, SIM (PAS), CLOCK et BMAL1 activent les gènes Per1, Per2, Cry1 et Cry2 dont les produits protéiques interagissent et répriment leur propre transcription. La stabilité des protéines PER et CRY est régulée par des voies parallèles ubiquitines

E3-ligase. CLOCK et MAL1 régulent également les récepteurs nucléaires REV-ERBα et REV-ERBβ (codés respectivement par Nr1d1 et Nr1d2) qui répriment rythmiquement la transcription de Bmal1 et Nfil3 (qui code pour le facteur nucléaire régulé par l'interleukine-3) pilotée par les activateurs RORα (récepteur orphelin lié à l'acide rétinoïque) et RORβ (codés respectivement par Rora et Rorb). NFIL3 réprime à son tour le facteur PAR-bZip DBP (D-box binding protein) pour réguler le rythme des récepteurs nucléaires ROR. Ces boucles de rétroaction transcriptionnelles représentent les trois principaux régulateurs transcriptionnels de la majorité des gènes du cycle. Différentes combinaisons de ces facteurs génèrent différentes phases des rythmes transcriptionnels, comme l'illustre le graphique (en haut à droite) montrant les profils d'ARN de Dbp, Per2, Cry1 et Bmal1 dans le foie de la Souris. D'autres gènes de sortie rythmiques (appelés gènes contrôlés par l'horloge ou Ccgs) sont régulés sur le plan transcriptionnel par les trois boucles agissant sur E-box, RevDR2 et les liaisons ROR (ROREs) et D-box dans les régions régulatrices des gènes cibles.
AMPK = protéine kinase 5' AMP activée; CK1= caséine kinase 1 ; CRE=élément de réponse à l'AMPc ; FBX= protéine F-box ; SCF= protéine SKP1-cullin-F-box ; SRE= élément de réponse au sérum ; Ub= ubiquitine. (*Takahashi JS et al. Nat Rev Genet. 2017*)

Un couplage étroit entre l'horloge circadienne moléculaire et le cycle cellulaire a été demontré à l'echelle de la cellule individuelle dans des cultures de fibroblastes modifiés par l'insertion de rapporteurs fluorescents de *Rev-erbα* aux phases du cycle cellulaire. Ce couplage pourrait être altéré dans des cellules cancéreuses

individuelles (28). Dans les tissus sains de la Souris et/ou de l'Homme, les événements du cycle cellulaire, la réparation de l'ADN, l'apoptose et l'autophagie sont contrôlés par l'horloge circadienne (29).

1.2 LE SYSTEME CIRCADIEN IMMUNITAIRE

La découverte d'horloges circadiennes autonomes dans les cellules immunitaires (30) et les résultats récents de la chrono-immunopharmacologie ont établi le rôle majeur du système immunitaire circadien dans de nombreux effets chronopharmacologiques.

Des rythmes circadiens de grande amplitude caractérisent la prolifération, la circulation et les fonctions des lymphocytes T et B tant chez les rongeurs que chez l'Homme. Par exemple, la numération de lymphocytes circulants T $CD3^+$, T naïfs, effecteurs et mémoires $CD4^+$ et T naïfs $CD8^+$ était presque deux fois plus élevée à 2 heures du matin qu'à 8 heures chez les hommes en bonne santé. En revanche, des changements circadiens de moindre amplitude ont été observés pour les cellules T effectrices $CD8^+$ et les cellules tueuses naturelles (NK), avec les valeurs les plus élevées dans l'après-midi, et pour les cellules B circulantes avec un maximum vers 20 heures (31-34). Chez la Souris et l'Homme, les cellules de l'immunité innée ou adaptative [monocytes (35), macrophages (30), cellules

dendritiques (36), neutrophiles (37) et cellules T $CD4^+$ (38), T $CD8^+$ (39), NK (40) et B (36)] sont en outre dotées d'une horloge circadienne fonctionnelle. Le NSC produit des signaux rythmiques neuronaux et hormonaux, notamment par le biais du cortisol et des catécholamines qui agissent directement sur les cellules immunitaires pour réguler leur activité fonctionnelle au cours des 24h (15, 41-44) (**Figure 2**).

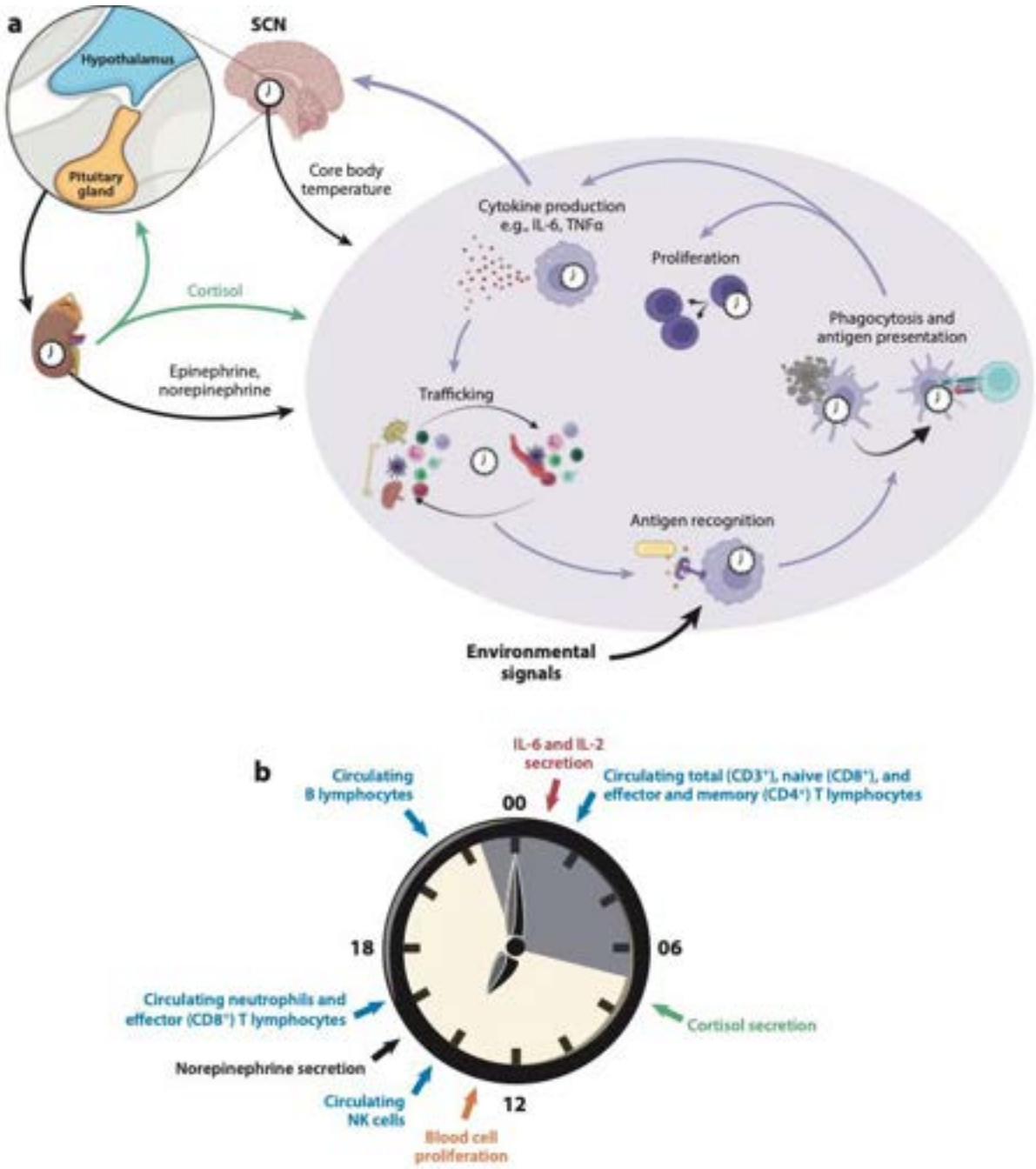

Figure 2: Interactions entre le système circadien et le système immunitaire

(Lévi et al. Annu Rev Pharmacol Toxicol. 2024)

(a) Les noyaux suprachiasmatiques génèrent des signaux hormonaux et neuronaux rythmiques qui ajustent les rythmes de la circulation et des fonctions

des cellules immunitaires au cours de la journée. Les horloges moléculaires intrinsèques aux cellules régulent les principales fonctions immunitaires, notamment les mécanismes de migration et de défense.

(b) Horaires des valeurs maximales des numérations sanguines des sous-populations de cellules immunocompétentes et d'étapes importantes dans les réponses immunitaires au cours des 24 heures. Les différents types de cellules immunitaires ont un comportement migratoire circadien différent selon leurs caractéristiques fonctionnelles. En général, les cellules effectrices et celles impliquées en première ligne dans les defences immunitaires (neutrophiles, cellules tueuses naturelles (NK) et cellules T $CD8^+$ sont plus abondantes dans le sang pendant la journée, avec un pic dans l'après-midi, tandis que les populations de cellules naïves ou non activées sont plus abondantes dans la circulation sanguine pendant la nuit.

1.3 IMMUNOTHERAPIE DES CANCERS

Le concept de l'immunothérapie du cancer apparaît dès la fin des années 1800(45). Les essais cliniques commencent principalement dans les années 1970 et impliquent d'abord des micro-organismes vivants ou inactivés, puis des anticorps polyclonaux ou monoclonaux, les interférons et les interleukines. La démonstration d'une efficacité antitumorale sans précédent des ICIs dans les tumeurs réfractaires à la chimiothérapie a permis de faire un bond en avant en termes d'efficacité (46). Les ICIs sont des anticorps monoclonaux humanisés qui

ciblent les récepteurs PD-1 (Programmed cell Death 1), CTLA4 (cytotoxic T-lymphocyte antigen 4), PD-L1 (programmed death-ligand 1) et LAG-3 (Lymphocyte activation gene-3). Le récepteur PD-1 est principalement exprimé à la surface des lymphocyte-T activés, des lymphocytes-B, des cellules NK (natural killer), les monocytes et les cellules souches mésenchymateuses (47). La protéine PD-L1 est aussi exprimée par de nombreux types de cellules tumorales et également de manière constitutive sur les cellules dendritiques, les macrophages, les cellules souches mésenchymateuses et les mastocytes dérivés de la moelle osseuse (48, 49). CTLA-4 (CD152) est principalement exprimé à la surface des lymphocytes-T (50). LAG-3 est exprimé par les lymphocytes T $CD4^+$ et $CD8^+$, les lymphocytes T régulateurs activés, les lymphocytes NK et NK invariants, ainsi que d'autres cellules immunitaires ou non immunitaires (51, 52). Le récepteur PD-1 est bloqué par le nivolumab, le pembrolizumab, le dostarlimab, le sintilimab et le cemiplimab, le récepteur CTLA4 par l'ipilimumab et le tremelimumab, le récepteur PD-L1 par l'atezolizumab, le durvalumab et l'avelumab, et le récepteur LAG-3 par le relatlimab. Le blocage d'un de ces récepteurs par un anticorps spécifique déclenche une réponse antitumorale, processus principalement orchestré par les lymphocytes-T cytotoxiques (53, 54). Par exemple, les cellules présentatrices d'antigènes telles que les cellules dendritiques, capturent les antigènes exprimés

par les cellules tumorales, puis migrent vers les ganglions lymphatiques qui drainent la tumeur, où les cellules T sont ensuite activées. En fin de compte, les lymphocytes-T activés infiltrent la tumeur primaire et participent à son éradication (**Figure 3**).

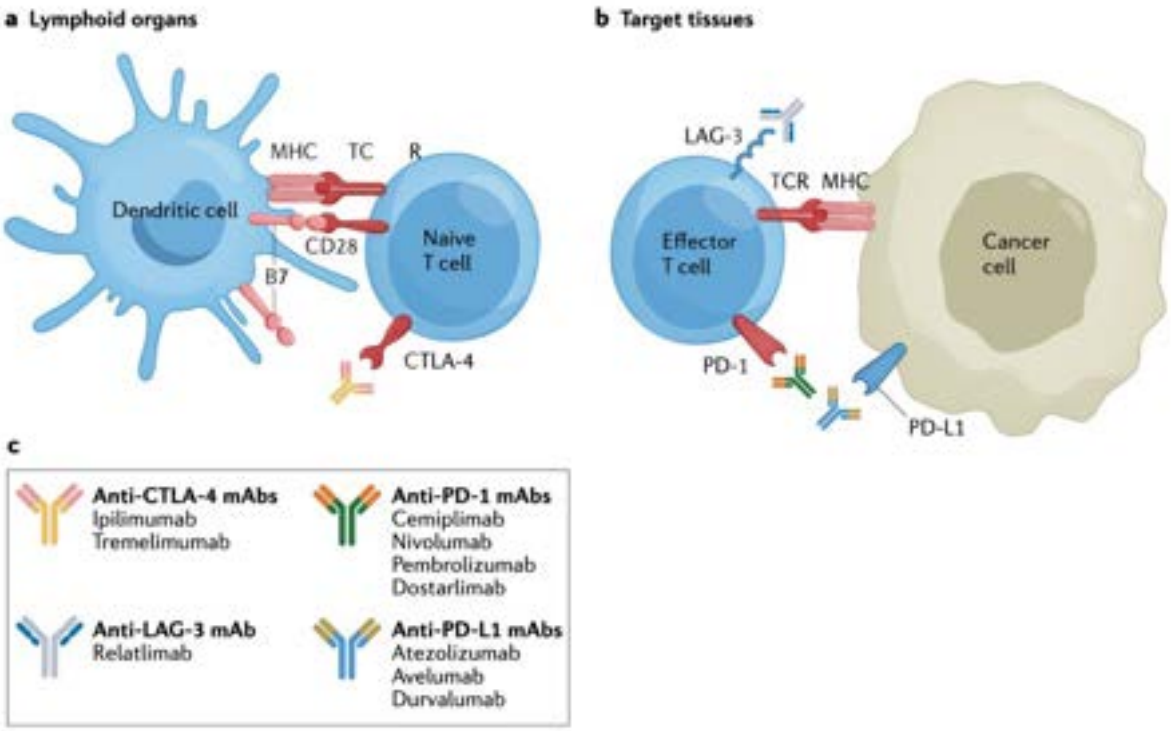

Figure 3: Mécanismes d'action des inhibiteurs de points de contrôle immunologique

(Tison A et al. Nat Rev Rheumatol. 2022)

(**a**)L'activation de cellules T nécessite deux signaux transmis par les cellules présentatrices d'antigènes. Le premier signal est la présentation d'un antigène par le complexe majeur d'histocompatibilité (CMH). Chaque antigène est reconnu

par un récepteur spécifique de cellule-T (TCR). Le second signal implique la liaison des molécules B7 (B7.1 (CD80) et B7.2 (CD86)) au récepteur CD28. La costimulation de CD28 induit la production de cytokines telles que l'IL-2 et l'expression du récepteur de l'IL-2 qui est essentiel à l'activation des lymphocytes et à la survie cellulaire via l'expression accrue de molécules anti-apoptotiques. CTLA-4 est l'equivalent de CD28 ; tous deux sont membres de la famille des immunoglobulines et partagent les ligands de B7, mais CTLA-4 régule négativement les fonctions des cellules- T.

(**b**) PD-1 appartient également à la famille des immunoglobulines et est régulé à la hausse sur le cellules-T effecteurs après la reconnaissance de l'antigène dans les tissus périphériques. Les ligands de PD-1 (PD-L1 et PD-L2) exercent un effet inhibiteur sur cellules-T exprimant PD-1. Ces ligands sont exprimés de manière constitutive sur les cellules immunitaires et certaines cellules tumorales, et leur expression peut également être induite en réponse à des signaux inflammatoires. LAG-3 est un autre point de contrôle immunitaire exprimé sur les cellules-T et agit en régulant négativement la prolifération et la fonction effectrice de cellules-T.

(**c**) Les ICIs actuellement disponibles sont des anticorps monoclonaux (mAbs) qui ciblent CTLA-4, PD-1, PD-L1 ou LAG-3.

Ces avancées en termes d'immunothérapie des cancers ont conduit à l'attribution du prix Nobel de physiologie ou de médecine 2018 à Tasuku Honjo et James Allison. Le professeur Honjo a été récompensé pour sa découverte de la molécule

PD-1 sur les lymphocytes-T. Le professeur Allison a découvert une autre molécule immunosuppressive importante, à savoir le CTLA-4 (55). Cependant, les données cliniques ont démontré des réponses hétérogènes chez les patients recevant des ICIs et la résistance au traitement primaire ou secondaire est fréquente (56). Ces différences d'efficacité des ICIs entre les patients pourraient résulter d'effets chronopharmacologiques qui feraient que les cellules immunitaires et les cellules cancéreuses réagiraient différemment à l'immunothérapie en fonction du moment de son administration sur un échelle de temps de 24 heures.

2 PROBLEMATIQUE ET OBJECTIFS

2.1 PROBLEMATIQUE

2.1.1 Le système circadien

Le système circadien dispose de mécanismes physiologiques et moléculaires responsables de la génération et de la coordination des rythmes d'environ 24 heures, ainsi que de leur entrainement par les cycles de l'environnement de période similaire appélés synchroniseurs. Le système circadien coordonne la plupart des fonctions cellulaires et organismiques sur une période d'environ 24 heures et ajuste leur phase en fonction des synchroniseurs environnementaux, par des mécanismes bien identifiés (57-59). Parfois, le système circadien peut connaitre des dysfonctionnements caractérisés par la perturbation ou l'abolition de plusieurs rythmes circadiens et/ou de leur coordination. Une telle disruption circadienne contribue au développement de pathologies diverses dont le cancer. En effet, plusieurs études expérimentales, cliniques et épidémiologiques ont montré le rôle important du dysfonctionnement du système circadien dans toutes les étapes de la cancérogenèse, depuis l'initiation et la promotion jusqu'à la progression et la dissémination métastatique (60-73). La disruption circadienne provoquée au plan expérimental par la destruction des noyaux suprachiasmatiques, l'exposition à un décalage horaire chronique ou la mutation

d'un ou plusieurs gènes de l'horloge, accélère ou déclenche la cancérogenèse (60, 66, 74-78). C'est aussi le cas de la disruption circadienne liée au travail de nuit (79-81), ainsi que l'a rapporté l'Organisation Mondiale de la Santé (OMS). De même la survenue d'une disruption circadienne chez les patients cancéreux a été associée à une réduction significative de la survie et à une moindre tolérance aux traitements (70, 82). Le système circadien influence aussi la tolérance et l'efficacité des traitements anticancéreux tant dans les modèles expérimentaux que chez les patients (4, 83, 84). Il a été également rapporté récemment une disruption circadienne chez les patients recevant une chimiothérapie immunomodulatrice (6, 85).

Il se pose alors la question du rôle du système circadien sur l'efficacité et la tolérance de l'immunothérapie seule ou en association avec la chimiothérapie chez les patients atteints de cancer, question à laquelle cette thèse vise à répondre.

2.1.2 La chronothérapeutique des cancers

Le système circadien contrôle le cycle cellulaire, l'apoptose, le métabolisme des médicaments dont les mécanismes de transport, de détoxification et de bioactivation dans les tissus sains (17, 86-91). En conséquence, la tolérance à la

chimiothérapie anticancéreuse varie grandement en fonction du moment circadien de l'administration du médicament dans les modèles expérimentaux. Les données de la littérature, de 1970 à nos jours, montrent que le système circadien peut modifier jusqu'à 5 fois l'efficacité et/ou la tolérance de plus 50 agents anticancéreux dans les modèles expérimentaux, et ce de façon statistiquement significative (89, 92-96). Par ailleurs, la meilleure efficacité antitumorale d'une chimiothérapie, en monothérapie ou en association correspond généralement à l'administration de médicaments anticancéreux à un moment où ils sont le mieux tolérés (92, 97-100). Les modèles mathématiques révèlent que cette coïncidence entre chronotolérance et chronoefficacité s'explique le mieux par les différences dans les dynamiques circadiennes du cycle cellulaire des cellules hôtes et cancéreuses, en particulier en ce qui concerne l'entraînement circadien et la variabilité du cycle cellulaire. En clinique, une grande amélioration de la tolérance a été démontrée dans des essais randomisés internationaux où des patients cancéreux ont reçu le même schéma de chronothérapie sinusoïdale sur 24 heures par rapport à une perfusion à débit constant ou à une chronothérapie décalée de 8 à 12 heures (89, 93-95, 101). Cependant, le sexe (102-105), la génétique (4, 106-108) et le mode de vie (109) peuvent influencer le schéma optimal de la chronothérapie, notamment les

heures optimales d'administration.

Le concept de chronothérapie s'applique aussi à l'administration de l'immunothérapie compte tenu des rythmes qui caractérisent le système immunitaire. Ces rythmes ont été identifiés chez la Souris depuis plusieurs décades. Une telle chrono-immunothérapie pourrait notamment réduire l'évasion immunitaire caractéristique de la cellule tumorale (110).

2.1.3 La chrono-immunothérapie

Bien que l'impact du rythme circadien sur le système immunitaire soit bien connu, la chrono-immunothérapie n'a pas encore été intégrée en oncologique clinique. Les premières études expérimentales de chrono-immunothérapie ont concerné la tolérance à l'interleukine-2 (IL2) et à l'interféron (IFN) chez la Souris ou le Rat dans les années 90 (111, 112). Chez des souris porteuses de mélanome B16, l'activité antitumorale maximale a été obtenue après administration à ZT4, au début de la période de repos diurne des souris, pour l'IFNα A/D humain recombinant et à ZT16 pour l'IFN-γ murin recombinant, c'est-à-dire peu avant le milieu de la période d'activité nocturne (111). L'expression de l'ARNm du récepteur de l'IFN était rythmique dans les cellules tumorales et atteignait son maximum de manière synchrone avec la proportion de cellules en phase S,

indiquant un lien entre le cycle cellulaire et la réponse immunologique des tumeurs. L'expression la plus élevée du récepteur de l'IFN avait lieu vers le milieu de la phase de repos des souris (112). La chronopharmacologie de l'IL-2 a été démontrée chez des rats porteurs d'hépatocarcinome. Les rats traités par une perfusion d'IL-2 à débit constant présentaient un taux de mortalité de 37,5 % et un taux de réponses tumorales de 25 %, alors que les animaux traités selon une perfusion chronomodulée d'IL-2 avec débits maxima de 15 à 21h n'ont présenté aucune mortalité et tous ont atteint une réponse tumorale objective (113).

Des essais cliniques de phase I et II ont montré une bonne tolérance d'administrations chronomodulées d'IFN 2b-α chez des patients atteints de mélanome ou d'IL-2 chez des patients atteint de cancer rénal malgré une augmentation significative de leur intensité de dose, en comparaison avec les résultats des perfusions continues standards (114, 115). La chronothérapie par IL-2 s'est également avérée sûre, modérément toxique et active chez les patients atteints de carcinome rénal métastatique (116).

Des rythmes circadiens ont été récemment mis en évidence dans les mécanismes impliqués de l'efficacité des ICIs chez la Souris. L'expression circadienne de l'ARN messager (ARNm) *Pdcd-1* a été trouvée dans les macrophages associés aux tumeurs, tandis que le nombre de macrophages associés aux tumeurs PD-1

positifs a également montré un rythme circadien significatif dans le mélanome B16/BL6 transplanté dans des souris C57BL/6J (117). *Pdcd- 1* a été caractérisé comme un gène contrôlé par l'horloge (117). Dans ce modèle de souris, le BMS-1, une petite molécule inhibitrice de PD-1/PD-L1, était significativement plus efficace après son administration à ZT18, vers le milieu de la période d'activité, par rapport à ZT6. Ces deux périodes circadiennes correspondent respectivement à l'augmentation et à la diminution de l'expression de PD-1 dans les macrophages associés aux tumeurs. Bien que la comparaison de deux points dans le temps ne permette pas d'identifier le moment optimal d'administration du BMS-1, les résultats suggèrent que l'expression circadienne de PD-1 à la surface des macrophages associés aux tumeurs, mais pas sur les monocytes circulants, pourrait aider à sélectionner le moment le plus approprié de la journée pour administrer les inhibiteurs de PD-1/PD-L1. Wang et al (118, 119) ont également démontré que la réponse immunitaire anti-tumorale variait au cours des 24 heures. Quatorze jours après la greffe sous-cutanée d'une charge fixe de cellules de mélanome, les volumes tumoraux étaient significativement plus importants chez les souris greffées à ZT21, la seconde moitié de la phase d'activité nocturne des souris, par rapport à celles greffées à ZT9, la seconde moitié de la phase de repos diurne des souris. Cette différence était abrogée chez les souris dépourvues

de cellules immunitaires adaptatives et innées. Les cellules T CD8, principales cibles des ICIs, étaient significativement plus nombreuses dans les tumeurs inoculées à ZT9 que dans celles inoculées à ZT21. Cette différence a été supprimée chez les souris dont l'expression de Bmal1 était supprimée spécifiquement dans les cellules dendritiques. Ces deux précédents articles révèlent que les ICIs pourraient être plus efficaces entre ZT9 et ZT18, soit entre la fin de la phase de repos et le milieu de la phase d'activité.

Ma thèse s'appuie aussi sur ces données expérimentales de chrono-immunothérapie pour étudier l'impact du système circadien sur l'efficacité des ICIs chez les patients atteints de cancer.

2.1.4 La chronothérapie personnalisée

Les principaux biomarqueurs physiologiques de l'hôte qui renseignent sur le fonctionnement du système circadien sur une période de 24 heures sont les rythmes d'activité-repos, de température corporelle, de fréquence cardiaque, ainsi que ceux qui modulent les sécrétions de cortisol et de mélatonine. Ces rythmes aident à coordonner les horloges périphériques sur 24 heures. Ils peuvent être influencés par le sexe, l'âge, ainsi que par le type et le stade du cancer (23, 120, 121). Chez les patients atteints de cancer métastatique, ces

biomarqueurs circadiens restent similaires à ceux des personnes en bonne santé, ou sont décalés, atténués ou complètement supprimés. Les effets chronopharmacologiques prévisibles nécessitent des horloges circadiennes bien coordonnées et fonctionnelles (13, 122). Des différences entre les patients ont été identifiées pour de nombreux biomarqueurs circadiens de l'hôte (83, 123, 124). En outre, les tumeurs de patients cancéreux peuvent présenter des expressions des gènes de l'horloge et/ou une fonctionnalité de l'horloge circadienne robustes, altérées ou supprimées (125, 126).

Le rythme d'activité-repos : C'est le premier biomarqueur physiologique qui a permis d'explorer le système circadien. Chez les souris dépourvues de noyau suprachiasmatique, porteuses de mutations de gènes de l'horloge, ou soumises à un décalage horaire chronique, on observe une disruption du rythme d'activité-repos associée à une accélération de la croissance tumorale et à des toxicités accrues de plusieurs anticancéreux. Chez les patients cancéreux, l'altération du rythme circadien d' activité-repos est associée à une moindre tolérance à la chimiothérapie, et à une moindre survie (82, 127-132) .

Le rythme de la température corporelle : celui-ci synchronise les horloges moléculaires des organes et peut influencer l'efficacité et/ou la tolérance de la chronothérapie (85, 131-133). Par exemple, Roche et al. ont montré que les

patients dont l'amplitude circadienne de la température était élevée avaient une survie globale deux fois plus longue que celle des patients dont l'amplitude circadienne thermique était faible (85).

Le sexe : Les récepteurs des hormones sexuelles s'expriment différemment dans les noyaux suprachiasmatiques des souris mâles ou femelles, suggérant un dimorphisme sexuel du système circadien (134). Les souris femelles présentent souvent des rythmes d'activité-repos, de température corporelle et de corticostérone d'amplitude plus élevée et un début d'activité plus précoce, en comparaison des souris mâles (134). Chez la Souris ou l'Homme, les niveaux moyens sur 24 heures et les amplitudes et/ou la phase circadienne pourraient présenter des profils différents d'expression de l'ARNm pour les gènes de l'horloge et les gènes contrôlés par l'horloge, ainsi que pour les activités qu'ils codent, en fonction du sexe (100, 135-137).

Le rôle du sexe a été également rapporté comme influençant l'efficacité de la chronothérapie (102, 103, 138). Par exemple, Giacchetti et al. ont montré dans une méta-analyse incluant 345 femmes et 497 hommes atteints de cancer colorectal métastatique que les hommes vivaient significativement plus longtemps sous un schéma fixe de chimiothérapie chronomodulée que sous chimiothérapie conventionnelle, mais que l'inverse était vrai chez les femmes

(102). D'autres travaux ont indiqué que le protocole de traitement anticancéreux chronomodulé optimal pourrait différer de 6 heures entre hommes et femmes.

On peut donc émettre l'hypothèse que les patients présentant des biomarqueurs rythmiques sur 24 heures pourraient bénéficier davantage de la chrono-immunothérapie, à condition d'en ajuster le schéma d'administration à la phase des rythmes du patient. Ainsi, une optimisation simultanée de la tolérance et de l'efficacité de l'immunothérapie gagnerait encore à une personnalisation de son administration circadienne.

2.2 HYPOTHESES ET OBJECTIFS

Ici, nous formulons les hypothèses suivantes :

1) L'heure d'administration de l'immunothérapie représente un paramètre essentiel de son efficacité chez des patients atteints de cancer du poumon non à petites cellules au stade métastatique ;
2) Comme cette chronoefficacité de l'immunothérapie est liée aux rythmes circadiens de l'hôte, et que ceux-ci sont conservés chez la majorité des patients de bon performance status (0 ou 1), les heures de meilleure efficacité antitumorale des ICIs sont semblables chez des patients atteints d'autres types de cancer « immunosensibles » ;

3) Les mécanismes de cette chronoefficacité sont liés au contrôle circadien du système immunitaire ;
4) L'heure d'administration des premiers cycles d'immunothérapie joue un rôle essentiel dans l'efficacité des ICIs ;
5) Le contrôle circadien de l'efficacité de l'immunothérapie persiste même lorsqu'elle est associée à la chimiothérapie.

Les objectifs généraux de cette thèse sont les suivants :

1) Identifier le rôle de l'heure d'administration dans la journée des ICIs sur leur efficacité et leur tolérance chez des patients atteints de cancer ;
2) Préciser les heures d'administration associées à une meilleure survie des patients cancéreux et leur reproductibilité, et estimer l'amélioration de la survie liée à un horaire majoritaire d'administration ;
3) Identifier les mécanismes circadiens potentiels qui influencent l'efficacité de l'immunothérapie au cours de la journée ;
4) Préciser l'importance des horaires des premiers cycles dans la chronoefficacité des ICIs et de l'immunochimiothérapie.

Les objectifs spécifiques incluent :

1) Identifier le rôle du moment d'administration de l'immunothérapie sur la survie des patients atteints de cancer du poumon ;
2) Identifier le rôle du moment d'administration des ICIs sur la survie et la survie sans progression des patients atteints d'autres cancers;
3) Identifier les mécanismes chronobiologiques qui expliquent l'efficacité de l'immunothérapie selon ses heures d'administration à travers une évaluation rigoureuse de la littérature traitant de la chrono-immunothérapie des cancers ;
4) Identifier le rôle du moment d'administration des premiers cycles de l'immunothérapie en association avec la chimiothérapie sur la survie des patients atteints de cancer du poumon et tous les paramètres d'efficacité.

3 CONDUITE DE LA THESE ET METHODOLOGIE GENERALE

J'ai centré mon projet de thèse sur l'implication du système circadien dans l'efficacité des ICIs et cherché à en comprendre les principaux mécanismes. J'ai d'abord cherché à préciser le rôle de l'heure d'administration de l'immunothérapie dans la survie des patients atteints de cancer du poumon métastatique. Ensuite, j'ai analysé le rôle de l'heure d'administration des ICIs sur la survie et la survie sans progression des patients atteints d'autres types de cancer, afin de préciser la cohérence ou la variabilité des résultats concernant les horaires d'administration associés à une meilleure efficacité. Afin de comprendre les mécanismes en cause, j'ai réalisé une évaluation rigoureuse de la littérature traitant de la chrono-immunothérapie des cancers. Comme l'association d'immunothérapie et de chimiothérapie est devenue le traitement recommandé pour la plupart des patients atteints de cancer du poumon non-petites cellules au stade métastatique, j'ai cherché à préciser si l'heure d'administration des ICIs jouait encore un rôle dans ce contexte. Plusieurs résultats expérimentaux et cliniques suggérant le rôle essentiel de l'horaire des premières administrations d' ICIs dans leur efficacité, j'ai choisi d'investiguer cette stratégie dans cette étude, d'autant que l'immunochimiothérapie est administrée pendant les 4 premiers

cycles dans les protocoles cliniques de référence.

3.1 LE MOMENT D'ADMINISTRATION DE L'IMMUNOTHERAPIE INFLUENCE-T-IL LA SURVIE DES PATIENTS TRAITES POUR CANCER DU POUMON ?

Pour répondre à cette question, j'ai réalisé une étude pilote qui a porté sur 95 patients atteints de cancer bronchique non-à-petites cellules (CBNPC) localement avancé ou métastatique traités par nivolumab. Cet ICI anti-PD1, a été administré en monothérapie comme traitement de deuxième ligne ou plus dans le Groupe Hospitalier Intercommunal Le Raincy Montfermeil entre septembre 2015 et novembre 2020.

Le nivolumab était administré par voie intraveineuse toutes les deux semaines en perfusion d'une heure à la dose de 3 mg/kg de septembre 2015 à septembre 2018, puis en perfusion de 30 minutes d'une dose fixe de 240 mg selon les nouvelles recommandations. Tous les patients ont reçu leur traitement dans l'unité d'hospitalisation de jour entre 09h27 et 17h14 à des horaires différents, attribués au hasard pour chaque cure par l'infirmière coordinatrice sur une base uniquement logistique. Cependant, il y avait une tendance à maintenir des créneaux horaires similaires pour chaque patient pour des raisons pratiques.

L'heure médiane d'administration du nivolumab a été calculée pour chaque patient, avec son coefficient de variation, lequel était toujours inférieur à 22%.

Les patients ont reçu des traitements de support pour les effets indésirables chaque fois que cela était indiqué. Les traitements ont été administrés jusqu'à la progression de la maladie, l'apparition de toxicités inacceptables ou de décès intercurrent.

Les données que j'ai recueillies comprenaient les caractéristiques initiales de chaque patient et de sa tumeur, la dose réelle de nivolumab administrée et la date et heure de début de la perfusion de nivolumab, ainsi que le nombre de cures administrées. L'indice de « performance status » était évalué selon la classification de l'OMS avant le début du traitement. Les réponses antitumorales ont été évaluées par scanner thoraco-abdominopelvien, imagerie par résonance magnétique cérébrale et/ou par tomographie à émission de positons réalisés toutes les quatre cures. Les réponses tumorales ont été catégorisées selon les critères iRECIST (139) comme réponse complète , réponse partielle, stabilité ou progression. La numération globulaire, les fonctions rénale et hépatique, les hormones thyroïdiennes plasmatiques (TSH et T4L) et les effets indésirables ont été évalués avant chaque cure et classés selon la terminologie NCI-CTCAE v4.0.

Les patients inclus dans cette étude pilote ont été répartis en deux groupes (« matin » et « après-midi ») en fonction de l'heure médiane de tous les traitements. Le groupe « matin » comprenait la moitié des patients qui ont reçu la majorité des administrations de nivolumab avant cette heure médiane, tandis que le groupe « après-midi » comprenait l'autre moitié des patients, pour lesquels la majorité des perfusions de nivolumab ont été administrées après l'heure médiane de tous les traitements. Les caractéristiques des patients, les toxicités et les taux de réponse tumorale ont été comparés à l'aide du test exact de Fisher ou du test du Khi-deux de Pearson. Le coefficient de variation intra-patient a été évalué par le rapport entre l'écart-type et la moyenne de tous les temps de perfusion pour chaque patient. Le coefficient de variation médian entre les patients des temps de perfusion du nivolumab a ensuite été calculé pour chaque groupe d'étude. La survie sans progression et la survie globale ont été calculées à partir de la date de la première administration de nivolumab jusqu'à la progression ou le décès, respectivement. Les courbes de survie sans progression et de survie globale ont été réalisées par de la méthode de Kaplan-Meier. Les groupes d'administration du nivolumab à des temps différents ont été comparés pour la survie sans progression et la survie globale à l'aide du test du Log-rank. Des analyses multivariées selon le modèle de Cox ont été utilisées pour identifier les principaux

facteurs prédictifs ou pronostiques de la survie sans progression et de la survie globale .

Dans le cadre d'une analyse de sensibilité, les patients ont été répartis en trois groupes afin de réduire le chevauchement pouvant résulter de la dichotomisation précédente : Le groupe 1 a reçu au moins 67% des perfusions avant l'heure médiane de tous les traitements, le groupe 2 a reçu au moins 33% des perfusions avant et 33% des perfusions après cette heure médiane, et le groupe 3 comprenait les patients ayant reçu au moins 67% des perfusions après cette heure médiane. Les courbes de survie sans progression et de survie globale des trois groupes ont été réalisées par de la méthode de Kaplan-Meier et comparées à l'aide du test du Log-rank. Toutes les analyses ont été réalisées avec le logiciel SPSS® v26.0 (Chicago, IL, USA). Une valeur $p < 0,05$ a été considérée comme statistiquement significative à l'aide de tests bilatéraux.

Les résultats de cette étude pilote présentés dans le Chapitre « Résultats / Etude n°1 », incitent à en rechercher confirmation dans des études similaires chez des patients atteints de cancer bronchique, mais aussi à examiner leur pertinence éventuelle chez des patients atteints d'autres types de cancer.

3.2 Le moment d'administration des inhibiteurs de point de controle immunologique influence-t-il la survie des patients atteints d'autres types de cancer ?

Pour répondre à cette question, j'ai mené, en collaboration avec le Dr Thierry Landré (Groupe Les hôpitaux Universitaires du GHU 93, Sevran, France), la première méta-analyse du rôle de l'heure d'administration des ICIs, fondée sur les données publiées de 13 études rétrospectives portant sur un total de 1663 patients. Nous nous sommes fixés comme objectif principal, l'impact de l'heure de la journée de la perfusion d'ICIs sur la survie globale des patients adultes atteints de tumeurs solides métastatiques. Les objectifs secondaires étaient la survie sans progression et le taux de réponse. Les bases de données PubMed et Cochrane ont été utilisées pour la recherche d'articles publiés avant le 15 août 2023, et complétées par une exploration manuelle des résumés soumis aux congrès annuels de l'« *American Society of Clinical Oncology* », de l'« *European Society for Medical Oncology* », de l'« *American Association for Cancer Research* » et de la « *Society for Immunotherapy of Cancer* » entre le 15 février 2020 et le 15 février 2023. Les termes de recherche utilisés étaient les suivants :

- *immunotherapy, Pembrolizumab, Nivolumab, Ipilimumab, Avelumab, Atezolizumab, Tislelizumab, Durvalumab, Dostarlimab, Tremelimumab,*

Sintilimab, Camrelizumab, Cemiplimab, Zimberelimab, immune checkpoint,

- *immunotherapy, anti-programmed cell death protein-1 (PD-1) and its ligand anti-programmed death-ligand 1 (PD-L1), or immune checkpoint inhibitor,*
- *cancer survival,*
- *time-of-day of infusion, circadian rhythm, circadian timing or chronotherapy.*

Lorsque les résultats d'une étude donnée ont été rapportés à la fois sous forme de résumé et d'article par la même équipe d'investigateurs, nous n'avons pris en compte que les données de l'article. Les informations suivantes ont été collectées:

- le nombre de patients,
- le type histologique du cancer et son stade évolutif
- le type d' ICI,
- l'horaire seuil de traitement utilisé pour différencier l'efficacité en fonction de l'heure la journée des perfusions de l'ICI,
- la proportion de perfusions jugée nécessaire pour réduire l'efficacité de l'ICI après un horaire seuil.

Les critères d'efficacité analysés étaient la survie globale, la survie sans progression et le taux de réponse, en fonction de la dichotomisation des

populations de patients selon chaque horaire seuil spécifique à l'étude. L'analyse principale a été réalisée sur tous les types de cancer métastatique. L'analyse secondaire portait spécifiquement sur l'effet du moment d'administration de l'ICI pour le cancer du poumon non-à-petites cellules, le carcinome rénal et le mélanome, étant donné qu'au moins deux rapports indépendants étaient disponibles pour chaque population de patients. Les analyses ont été réalisées selon la méthode Cochrane pour les méta-analyses et calculées avec le logiciel Review Manager (*RevMan version 5.4 ; Cochrane, Oxford, UK*). La statistique d'hétérogénéité a été évaluée à l'aide des tests c2 et des statistiques I2, un test c2, $p < 0,05$ définissant la présomption d'hétérogénéité. L'I2 évalue l'hétérogénéité entre les études. L'hétérogénéité était classée de l'homogénéité parfaite (0 %) à l'hétérogénéité modérée (30-60 %) jusqu'à l'hétérogénéité totale (100 %). Les diagrammes en entonnoir de Begg ont été utilisés pour visualiser l'hétérogénéité entre les études. Un modèle à effets fixes a été utilisé pour calculer le risque relatif cumulé lorsque l'hétérogénéité entre les études était faible. Lorsque cette hétérogénéité était élevée, un modèle randomisé a été appliqué. Les résultats de la méta-analyse pour la survie globale et la survie sans progression ont été présentés sous forme de rapport de risque (« hazard ratio ») avec un intervalle de confiance à 95 %. Tous les tests étaient bilatéraux, $p < 0,05$

définissant la signification statistique.

Devant la cohérence des résultats de cette méta-analyse concernant l'impact de l'heure d'administration des ICIs dans 5 cancers différents, rapportée dans le chapitre « Résultats/Etude n°2 », j'ai analysé les mécanismes potentiels qui pourraient expliquer la chronoefficacité apparente de l'immunothérapie

3.3 QUELS SONT LES MECANISMES CIRCADIENS POTENTIELS QUI INFLUENCENT L'EFFICACITE DE L'IMMUNOTHERAPIE AU COURS DE LA JOURNEE ?

Pour répondre à cette question, j'ai d'abord complété les données cliniques de la méta-analyse, par les résultats de 5 nouvelles études rapportées depuis la parution de notre article. J'ai ensuite analysé les mécanismes par lesquels les rythmes circadiens peuvent influencer les résultats des thérapies par ICIs. Ainsi, j'ai synthétisé les données de vie réelle issues de 18 études rétrospectives comparant la survie globale et la survie sans progression de groupes de patients selon que les perfusions d'ICIs aient eu lieu en début ou en fin de journée. Ces études ont porté sur un total de 3250 patients atteints de mélanome métastatique, de cancer du poumon, du rein, de la vessie, de l'œsophage, de l'estomac ou du foie dans 9 pays de 4 continents.

Dans un second temps, j'ai examiné, à partir d'une analyse exhaustive de la

littérature, comment les troubles du rythme circadien peuvent affecter le microenvironnement immunitaire de la tumeur, en tant que mécanisme principal reliant l'horloge circadienne aux cycles de 24 heures de l'efficacité antitumorale des ICIs.

Cette revue a concerné également :

- La coordination par le système circadien de la physiologie des cellules, des tissus et de l'ensemble du corps sur une échelle de 24 heures.
- Les rythmes circadiens générés au niveau cellulaire par un système d'horloge moléculaire qui implique 15 gènes d'horloge spécifiques.
- La perturbation des rythmes circadiens pouvant déclencher ou accélérer la carcinogenèse et contribuer à l'échec des traitements anticancéreux par le biais d'une évasion immunitaire de la tumeur résultant d'un microenvironnement tumoral immunosuppressif.

Cette revue critique de la littérature est parue dans le « British Journal of Cancer, 2024 » et est rapportée dans le chapitre « Résultats/Etude n°3 ».

Au vu de ces résultats, et d'une possible disruption circadienne et d'une immunosuppression engendrée par la chimiothérapie, se pose la question de l'altération possible du rôle du moment d'administration de l'immunothérapie en association avec la chimiothérapie, en particulier au cours des quatre premiers

cycles de leur combinaison.

3.4 QUEL EST LE ROLE DU MOMENT D'ADMINISTRATION DES PREMIERS CYCLES DE L'IMMUNOTHERAPIE EN ASSOCIATION AVEC LA CHIMIOTHERAPIE SUR LA SURVIE DES PATIENTS ATTEINTS DE CANCER DU POUMON ?

Pour répondre à cette question, j'ai réalisé en collaboration avec le Professeur Zhe Huang, chef du département d'oncologie de l'hôpital « Hunan Cancer Hospital », Changsha, Chine, une étude rétrospective internationale basée sur des données individuelles pour identifier la pertinence de l'heure d'administration dans la journée des quatre premières cures de l'association d'un ICI anti-PD-1 et d'une chimiothérapie, en première ligne de traitement pour cancer bronchique non-à-petites cellules avancé ou métastatique.

Cette large étude rétrospective internationale s'appuie sur les données individuelles anonymisées de 713 patients enregistrés dans une cohorte française ou une cohorte chinoise. La cohorte française comprenait 165 patients atteints de CBNPC métastatique et traités à l'hôpital Le-Raincy Montfermeil à Montfermeil ou à l'hôpital universitaire Avicenne à Bobigny. La cohorte chinoise comprenait 548 patients atteints de CBNPC localement avancé ou métastatique traités à l'hôpital de Hunan à Changsha.

Les principaux critères d'inclusion dans l'étude étaient :

- CBNPC histologiquement prouvé
- Stade avancé ou métastatique
- Indice de statut de performance selon l'OMS de 0, 1 ou 2
- Absence de mutation tumorale de l'EGFR (Epithelial Growth Factor Receptor), de translocation ROS1 (Reactive oxygen species 1) ou d'anomalie du gène ALK (Anaplasic Lymphoma Kinase)
- Administration d'au moins une cure de protocole standard de première ligne associant un ICI anti-PD-1 suivi d'une chimiothérapie par pemetrexed-carboplatine (ou cisplatine), ou par paclitaxel-carboplatine.

Les patients présentant des métastases cérébrales ne nécessitant pas de traitement par glucocorticoïdes ont été inclus.

N'étaient pas inclus dans l'étude:

- Les patients recevant des doses quotidiennes de glucocorticoïdes >10 mg/jour pour une quelconque condition médicale.
- Les patients pour lesquels il manquait des informations sur le moment de la perfusion des ICIs au cours des quatre premiers cycles.

Les créneaux horaires des cures de l'association ICIs et chimiothérapie étaient attribués au hasard pour chaque cure par le coordinateur de l'hôpital de jour sur

une base logistique et ont été documentés avec précision. Pour des raisons logistiques, les créneaux horaires de perfusion des ICIs ultérieurs sont restés à peu près similaires pour chaque patient. Les traitements ont été administrés jusqu'à la progression de la maladie, l'apparition de toxicités inacceptables ou de décès intercurrent.

Les données anonymisées issues des dossiers médicaux recueillis concernaient *a priori* :

- Les caractéristiques initiales des patients et de leur tumeur, y compris l'état de performance selon l'OMS
- Le type d'ICI
- L'heure quotidienne du début de l'administration de l'ICI pour chacune des quatre perfusions initiales (date et heure d'horloge)
- Le nombre de cures administrées.
- Les réponses antitumorales évaluées par tomodensitométrie, imagerie par résonance magnétique et/ou tomographie par émission de positons, toutes les deux ou trois perfusions. Les réponses ont été classées selon les critères iRECIST (139) comme complètes, partielles, stables ou progressives.

Les résultats des analyses sanguines et les effets indésirables n'ont pas été

recueillis dans le cadre de cette étude.

L’objectif principal était la survie, définie comme le temps entre la première administration d'ICIs et le décès toutes causes confondues ou la dernière visite de suivi pour les patients ayant été vus en vie pour la dernière fois.

Les objectifs secondaires étaient la survie sans progression et le taux de meilleure réponse tumorale. La survie sans progression a été définie comme le temps écoulé entre la première administration de l'ICI et le décès ou la progression de la maladie ou jusqu'à la dernière visite de suivi pour les patients vus pour la dernière fois en vie et n'ayant pas connu de progression de la maladie. La réponse objective a été définie comme le pourcentage de patients dont la meilleure réponse tumorale a été catégorisée comme réponse partielle ou réponse complète.

Les variables catégorielles ont été présentées sous forme de fréquences et de pourcentages et les variables continues sous forme de moyenne et d'écart-type ou de médiane et d'intervalle interquartile. L'hypothèse de normalité a été évaluée graphiquement. La médiane du suivi a été estimée à l'aide de la méthode inverse de Kaplan-Meier. Pour chaque patient, la médiane de l’heure d’administration de la journée a été définie sur la base de l'heure de début d'administration des 4 premières cures d'immunochimiothérapie. Le coefficient

de variation intra-patient a été évalué par le rapport entre l'écart-type et la moyenne des quatre temps de perfusion pour chaque patient. Le coefficient de variation médian entre les patients des temps de perfusion de l'ICI, ainsi que son interquartile et son intervalle, ont ensuite été calculés pour chaque groupe d'étude.

Différents seuils horaires ont été étudiés, de 10h30 à 13h00, avec des intervalles de 30 minutes. Pour chaque seuil, la population a été classée comme « Avant » ou « Après », en fonction de la médiane des heures d'administration de chaque patient. Les caractéristiques des patients des groupes « Avant » et « Après » ont été comparées à l'aide du test t-Student ou du test non paramétrique de Wilcoxon pour les variables quantitatives, du test du Khi-deux ou du test exact de Fisher pour les variables catégorielles non ordonnées et du test de Cochran-Armitage pour les variables catégorielles ordonnées (état de performance et expression de PDL-1).

La survie globale et la survie sans progression ont été estimées dans chaque groupe à l'aide de la méthode de Kaplan-Meier et comparées à l'aide du test du logarithme. Les associations entre le seuil de l'heure d'administration de la journée et la survie globale ou la survie sans progression ont été évaluées à l'aide du modèle de Cox en prenant en compte le pays, le sexe, l'âge, l'état de

performance, le stade, l'expression de PDL-1, le nombre de sites métastasiques, du type d'immunothérapie et de chimiothérapie.

Des modèles de Cox non ajustés ont également été réalisés au sein de différents sous-groupes afin d'explorer la cohérence de l'effet du seuil de l'heure d'administration dans la journée sur la survie globale et la survie sans progression. L'association entre le seuil de l'heure d'administration et le taux de réponse a été évaluée à l'aide d'un modèle de régression logistique prenant en compte les mêmes facteurs.

L'analyse principale a été réalisée sur les données individuelles des deux cohortes. Des analyses de sensibilité ont également été réalisées à des fins exploratoires dans chaque cohorte individuelle. Les mêmes horaires seuils ont servi pour dichotomiser les patients. La survie globale et la survie sans progression ont été estimés dans chaque groupe « Avant » et « Après » et comparées par le test du Log-rank. L'association entre le seuil de l'heure d'administration de la journée et la survie globale ou la survie sans progression a été évaluée par le modèle de Cox non ajusté.

Le seuil de significativité de la valeur p a été fixée *a priori* à 0,05 pour toutes les analyses et les intervalles de confiance à 95 % des estimations ont été calculés. Tous les tests étaient bilatéraux. Les analyses ont été réalisées à l'aide de SPSS®

v26.0 (Chicago, IL, USA) et de R® v4.3.0.

Les résultats sont rapportés dans le chapitre « Résultats/Etude n°4 ».

4 RESULTATS

4.1 ROLE DU MOMENT D'ADMINISTRATION DANS L'EFFICACITE ANTICANCEREUSE DES INHIBITEURS DE POINT DE CONTROLE IMMUNOLOGIQUE

4.1.1 Etude princeps

Article n°1, *Cancers (Basel), 11 Février 2022*

(Google Scholar: 42 citations; top 5% of all research outputs ever tracked by Altmetric)

Efficacité de l'inhibiteur du point de contrôle Nivolumab selon l'heure d'administration : résultats d'une étude pilote chez des patients atteints d'un cancer du poumon non-à-petites cellules au stade métastatique.

Karaboué A, *Collon T , Pavese I, Bodiguel V , Cucherousset J , Zakine E , Innominato P , Bouchahda M , Adam A , Lévi F.*

Plusieurs observations cliniques initales nous ont révélé un suivi remarquablement plus long des patients atteints de cancer du poumon non-à-petites cellules métastatique recevant des perfusions de nivolumab principalement le matin, par rapport à ceux traités l'après-midi. Des études

expérimentales et cliniques antérieures ont démontré la distribution temporelle de la prolifération, de la circulation, de la reconnaissance et de la destruction des antigènes par les cellules immunitaires au cours des 24 heures. Ici, nous avons émis l'hypothèse que le rythme circadien pourrait jouer un rôle important dans l'efficacité du nivolumab, comme cela a déjà été démontré pour la toxicité et/ou l'efficacité de la chimiothérapie chronomodulée chez les patients atteints de cancer colorectal ou broncho-pulmonaire. Après validation du projet par un comité scientifique interne, les heures d'administration de chacune des 1818 perfusions de nivolumab administrées à 95 patients consécutifs dans le cadre d'un traitement standard du cancer du poumon non-à-petites cellules métastatique ont été extraites des dossiers de l'hôpital de jour. Les événements indésirables et les réponses tumorales radiologiquement documentées ont été relevés et analysés à partir des dossiers cliniques des patients. Les patients ont été répartis dans les groupes « Matin » (N = 48 patients) ou « Après-midi » (N = 47 patients), selon qu'ils avaient reçu la majorité des perfusions de nivolumab avant ou après 12:54, c'est-à-dire l'heure médiane de toutes les perfusions. L'administration majoritaire de nivolumab le matin a presque quadruplé la médiane de survie sans progression et la survie globale par rapport à l'administration de nivolumab l'après-midi. Le nivolumab administré le matin était

significativement plus efficace, indépendamment de l'âge, du sexe, du statut de performance, des traitements antérieurs, de l'histologie de la tumeur ou de l'expression de PD-L1. En revanche, la résistance primaire au nivolumab a été le plus souvent observée après le traitement de l'après-midi. En conclusion, des essais randomisés sont justifiés à la fois pour mieux identifier le moment optimal d'administration des ICIs chez les patients atteints de cancer et pour identifier les principaux mécanismes qui déterminent précisément l'efficacité et la résistance de l'immunothérapie au long de l'échelle de temps circadienne.

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Article

Time-Dependent Efficacy of Checkpoint Inhibitor Nivolumab: Results from a Pilot Study in Patients with Metastatic Non-Small-Cell Lung Cancer

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Simple Summary: Initial clinical observations revealed strikingly longer follow-up for metastatic non-small-cell lung cancer (NSCLC) patients receiving nivolumab infusions predominantly in the morning as compared to those treated in the afternoon. Prior experimental and human studies have demonstrated the temporal distributions of immune cells' proliferation, trafficking, and antigen recognition and destruction over the 24 h. Here, we hypothesized that circadian timing could play an important role in nivolumab's efficacy, as previously shown for the toxicity and/or efficacy of chronomodulated chemotherapy in colorectal and lung cancer patients. Following project validation by an internal scientific review board, the dosing times of each of the 1818 nivolumab infusions given to 95 consecutive patients as a standard treatment for metastatic NSCLC were retrieved from the day-hospital records. Adverse events and radiologically documented tumor responses were retrieved and reviewed from patients' clinical charts. Patients were allocated to 'morning' (N = 48 patients) or 'afternoon' (N = 47 patients) groups, according to whether they had received the majority of nivolumab infusions before or after 12:54, i.e., the median time of all infusions, respectively. 'Morning' nivolumab dosing nearly quadrupled median progression-free and overall survival as compared to 'afternoon' dosing. 'Morning' nivolumab was significantly more effective irrespective of age, sex, performance status, prior treatments, tumor histology, or PD-L1 expression. In contrast, nivolumab primary resistance was most often observed following 'afternoon' dosing. Randomized trials are warranted both to further identify the optimal timing of checkpoint inhibitors in individual cancer patients, and to determine the main mechanisms that precisely drive immunotherapy efficacy and resistance along the circadian timescale.

Abstract: Hypothesis: Prior experimental and human studies have demonstrated the circadian organization of immune cells' proliferation, trafficking, and antigen recognition and destruction. Nivolumab targets T(CD8) cells, the functions, and trafficking of which are regulated by circadian clocks, hence suggesting possible daily changes in nivolumab's efficacy. Worse progression-free survival (PFS), and overall survival (OS) were reported for malignant melanoma patients receiving more than 20% of their immune checkpoint inhibitor infusions after 16:30 as compared to earlier in the

day. Methods: Consecutive metastatic non-small-cell cancer (NSCLC) patients received nivolumab (240 mg iv q 2 weeks) at a daily time that was 'randomly' allocated for each course on a logistical basis by the day-hospital coordinators. The median time of all nivolumab administrations was computed for each patient. The study population was split into two timing groups based upon the median value of the median treatment times of all patients. CTCAE-toxicity rates, iRECIST-tumor responses, PFS and OS were computed according to nivolumab timing. PFS and OS curves were compared and hazard ratios (HR) were computed for all major categories of characteristics. Multivariable and sensitivity analyses were also performed. Results: The study accrued 95 stage-IV NSCLC patients (PS 0–1, 96%), aged 41–83 years. The majority of nivolumab administrations occurred between 9:27 and 12:54 for 48 patients ('morning' group) and between 12:55 and 17:14 for the other 47 ('afternoon' group). Median PFS (95% CL) was 11.3 months (5.5–17.1) for the 'morning' group and 3.1 months (1.5–4.6) for the 'afternoon' one ($p < 0.001$). Median OS was 34.2 months (15.1–53.3) and 9.6 months (4.9–14.4) for the 'morning' group and the 'afternoon' one, respectively ($p < 0.001$). Multivariable analyses identified 'morning' timing as a significant predictor of longer PFS and OS, with respective HR values of 0.26 (0.11–0.58) and 0.17 (0.08–0.37). The timing effect was consistent across all patient subgroups tested. Conclusions: Nivolumab was nearly four times as effective following 'morning' as compared to 'afternoon' dosing in this cohort of NSCLC patients. Prospective timing-studies are needed to minimize the risk of resistance and to maximize the benefits from immune checkpoint inhibitors.

Keywords: non-small-cell lung cancer; checkpoint inhibitors; nivolumab; circadian timing; overall survival

1. Introduction

The recent advent of immunotherapy represents a major milestone for the improvement of the outcomes of patients with advanced or metastatic non-small-cell lung cancer (NSCLC) [1–3]. Thus, several randomized controlled trials have demonstrated large and statistically significant prolongation of both progression-free survival (PFS) and overall survival (OS) with antibodies directed against programmed death 1 (PD-1) or programmed death-ligand 1 (PD-L1) as compared to cytotoxic chemotherapy in lung cancer patients [1,4–6]. As a result, nivolumab has become the first immunotherapeutic agent approved as the standard of care for patients with metastatic or advanced NSCLC who progressed during or after treatment with platinum-based chemotherapy [7,8].

Nivolumab is a fully human immunoglobulin G4 monoclonal antibody that binds to programmed death-1 (PD-1) receptors and blocks their interaction with programmed death-ligand 1 (PD-L1) and programmed death-ligand 2 (PD-L2), thereby preventing activation of the PD-1 pathway [9]. PD-1 is highly expressed on the surface of tumor-specific T (CD8) lymphocytes, as well as on other activated T, natural killer and B lymphocytes, macrophages, dendritic cells and monocytes [9]. Nivolumab mainly enhances the activity of T cells against tumor cells. It has long been known that both the functional activity and trafficking of immune cells are regulated over the 24 h by molecular circadian clocks expressed by these cells [10–17]. This is notably the case for T(CD8) lymphocytes, which represent the main target cells of nivolumab [18]. Based on several experimental and human studies, our team earlier hypothesized a circadian coordination of various immune functions, notably with the highest proliferation of immune cells in the early afternoon, increased blood circulation in the late evening for B-cells and near the middle of the night for T-cells, and antigen recognition and destruction processes predominant in the early morning hours [19]. These and the large body of more recent data [10–17] suggest a possible impact of dosing time of nivolumab on its efficacy. Several studies have mapped circadian rhythms in NSCLC patients, including those in circulating lymphocyte subsets, and others have shown the relevance of circadian timing for chemotherapy tolerability [20–25]. Finally, recent data highlighted the clinical relevance of the timing of administration of immune

checkpoint inhibitors (ipilimumab, nivolumab, or pembrolizumab, or a combination of these) in metastatic melanoma patients [26].

The present study was based on an initial clinical observation of a strikingly longer follow up of metastatic NSCLC on second-line nivolumab treatment administered in the morning as compared to the afternoon. We then hypothesized that circadian timing could play an important role in nivolumab's efficacy, based on our prior trials of chronomodulated chemotherapy [27–29]. Strikingly, PFS and OS differences were found in an initial retrospective evaluation of efficacy as a function of morning vs. afternoon nivolumab administration in a first cohort of 57 patients [30]. We then prospectively assessed the nivolumab timing efficacy in a further 38 additional NSCLC patients in order to increase the sample size and provide independent confirmation of the initial findings. Here, we present the results of the whole study, which highlight a striking impact of nivolumab administration's timing on its efficacy in 95 consecutive patients with metastatic NSCLC.

2. Patients and Methods

2.1. Patient Selection

For this study, we collected data from consecutive patients with histologically or cytologically documented locally advanced or metastatic NSCLC, treated with single agent nivolumab as second- or later-line treatment, at a single institution from September 2015 to November 2020. This retrospective evaluation of patients' data according to nivolumab timing was approved by the local Institutional Review Board (#202028).

2.2. Nivolumab Treatment and Timing

Nivolumab was delivered intravenously every second week as a 1-h infusion at 3 mg/kg from September to 2015 to September 2018, then as a 30-min infusion of a fixed-dose of 240 mg, as recommended. All the patients started their treatment in the "day hospital" unit between 09:27 and 17:14. Nivolumab timing slots were randomly allocated for each course by the day-hospital coordinator on a logistical basis and accurately recorded. As a result, actual nivolumab timing could vary between courses for each patient, yet with a tendency to maintain similar time-slots for each patient for practical reasons. Thus, both the median time of nivolumab administration and its coefficient of variation were computed for each patient.

Patients received supportive care medications for immune-mediated adverse effects whenever indicated. Treatments were administered until disease progression, occurrence of unacceptable toxicities, or intercurrent death.

2.3. Assessments

The data collected included patient and tumor baseline characteristics, nivolumab's actual dose and time of administration (date and clock hour), and the number of courses. WHO performance status was rated before the start of treatment. Anti-tumor responses were documented using thoraco-abdominopelvic computed tomography scans, cerebral magnetic resonance imaging and/or positron emission tomography with 18-fluorodeoxyglucose, which were performed after every four treatment courses. Responses were categorized according to iRECIST [31] criteria as complete (CR), partial (PR), stable (SD) or progressive disease (PD). Blood cell counts and differentials, renal and hepatic serum biochemistry, plasma thyroid hormones (TSH and fT4), and adverse events were assessed before each treatment course and graded according to NCI-CTCAE v4.0. All data were independently reviewed by two clinicians, alongside the lead oncologist for each patient, to ensure quality of data for consistency and plausibility.

2.4. Statistical Considerations

First, patients included in the study were dichotomized into two nivolumab timing groups relative to the median clock hour of all treatment courses. The 'morning' group included half of the patients who received the majority of the nivolumab administrations

before this median clock hour, whereas the 'afternoon' group included the other half of the patients, for whom most of the nivolumab infusions were administered after the median time of all the courses. Patient characteristics, toxicities, and tumor response rates were compared using Fisher exact or Pearson χ^2 tests. The intra-patient coefficient of variation was assessed by the ratio of the standard deviation to the mean of all infusion times for each individual patient. The median between-patients coefficient of variation of the nivolumab infusion times was then computed for each study group. PFS and OS were calculated from the date of the first administration of nivolumab until documented progression or death, respectively, and their curves were generated using the Kaplan–Meier method. The association between the nivolumab timing groups and PFS or OS was compared with the log-rank test.

Landmark PFS and OS analyses were performed, using 2 months for the base case analysis, then 4 months as representing the best balance allowing enough treatment exposure for responses to occur, whilst providing enough follow-up data to model survival beyond the landmark point. A landmark of 6 months was further explored for sensitivity analysis. Finally, multivariate Cox proportional hazards regression models were used to identify the main predictive or prognostic factors of PFS and OS with a minimum of 10 events per factor.

In a second sensitivity analysis, patients were also trichotomized in order to reduce the overlap that could result from the previous dichotomization: Group 1 received at least 67% of the infusions before the median clock hour of all treatment courses, Group 2 received at least 33% of the infusions before, and 33% of the infusions after this median time, and Group 3 involved those patients receiving at least 67% of the infusions after this median time. PFS and OS curves of the three groups were generated using the Kaplan–Meier method and compared with the log-rank test.

All analyses were performed with SPSS® v26.0 software (Chicago, IL, USA). A p-value < 0.05 was considered statistically significant using bilateral tests.

3. Results

3.1. Patient Characteristics

Ninety-five consecutive patients were included in the study and categorized into two nivolumab timing groups according to the overall median treatment clock hour (Table 1). Thus, the majority of nivolumab administrations occurred between 9:27 and 12:54 for 48 patients ('morning' group) and between 12:55 and 17:14 for the 47 other patients ('afternoon' group) (Figure 1). The median nivolumab intra-patient timing coefficient of variation was 10%, ranging from 2% to 21%. The systematic determination of tumor molecular markers was recommended for routine oncology practice in 2020. Thus, mutations or rearrangements were searched for ALK1, ROS1 and EGFR, and PD-L1 expression was determined in the primary tumors of 43, 15, 32 and 72 patients, respectively. No mutation was found for ROS1. Mutations were identified for ALK1 in a single patient in the morning group, and in four patients for EGFR, including one with Exon 19 mutation and one with Exon 21 mutation in each group. Tumor PD-L1 expression exceeded 1% for 39 of the 72 patients tested (54.2%), with a similar distribution between the two nivolumab timing groups. A single patient who declined chemotherapy received nivolumab as a first-line treatment. Nivolumab was administered as a second-line treatment for 75.8% of the patients, and as third or later line for 23.2% of them, without any imbalance between the timing groups. Prior chemotherapy regimens included carboplatin (87.4% of the patients), cisplatin (17.9%), paclitaxel (69.5%), pemetrexed (33.7%), gemcitabine (20%), bevacizumab (10.5%), docetaxel (7.4%), erlotinib (4.2%), vinorelbine (2.1%), etoposide (1.1%) and osimertinib (1.1%). As compared to the patients in the 'morning' group, those in the afternoon group were slightly older (p = 0.012), more frequently female (p = 0.025), and tended to have a performance status of 1 rather than 0 (p = 0.061). No imbalance was apparent for any other patient or tumor characteristics (Table 1). A total of 1818 nivolumab treatment courses were administered with a median number of 9 courses per patient (range: 3 to 133). The median

number of treatment courses was 19 (3 to 133) for the 'morning' group and 6 (3 to 30) for the 'afternoon' group ($p < 0.001$). The major reasons for treatment discontinuation irrespective of nivolumab timing were disease progression (74.7%), toxicity (7.4%), loss to follow-up (4.2%) and pulmonary sepsis (2.1%).

Table 1. Main baseline characteristics of whole study population, and according to nivolumab (NIV) timing groups.

Characteristics	All Patients (N = 95)	'Morning' Group (n = 48)	'Afternoon' Group (n = 47)	*p*-Value
Timing of >50% of infusions		**09:27 to 12:54**	**12:55 to 17:14**	
Actual NIV [a] timing				
Median time, hh:min	12:54	11:55	13:34	**<0.001**
(range)	(9:27–17:14)	(9:27–16:52)	(10:10–17:14)	
(IQR)	(11:55–13:34)	(11:11–12:26)	(13:10–14:44)	
Median intra-patient CV [b]	10%	11%	8%	
(range)	(2%–21%)	(2%–21%)	(2%–19%)	
Age, years				
Median (range)	66.9 (41.2–82.5)	66.2 (41.2–80.8)	69.2 (48.8–82.5)	**0.012**
Sex				
Female	16 (16.8%)	4 (8.3%)	12 (25.5%)	**0.025**
Male	79 (83.2%)	44 (91.7%)	35 (74.5%)	
Tobacco-induced COPD [c]	74 (77.9%)	37 (77.1%)	37 (78.7%)	0.847
Histological type				
Adenocarcinoma	55 (57.9%)	26 (54.2%)	29 (61.7%)	
Squamous cell carcinoma	37 (38.9%)	20 (41.7%)	17 (36.2%)	0.696
NSCLC unspecified	3 (3.2%)	2 (4.2%)	1 (2.1%)	
Tumor PD-L1 expression				
≥1%	39 (41.1%)	23 (47.9%)	16 (34.0%)	0.098
<1%	33 (34.7%)	13 (27.1%)	20 (42.6%)	
Not assessed	23 (24.2%)	12 (25.0%)	11 (23.4%)	
Primary resected	15 (15.8%)	9 (18.8%)	6 (12.8%)	0.424
Prior adjuvant chemo.	11 (11.6%)	7 (14.6%)	4 (8.5%)	0.355
Prior radiotherapy	57 (60.0%)	27 (56.3%)	30 (63.8%)	0.451
N of prior chemo. lines				
0	1 (1.1%)	1 (2.1%)	0 (0.0%)	
1	72 (75.8%)	36 (75.0%)	36 (76.6%)	0.955
2–5	22 (23.2%)	11 (22.9%)	11 (23.4%)	
Number of sites involved				
Median (range)	4 (1–8)	4 (1–7)	3 (2–8)	0.854
Site of metastases				
Brain	23 (24.2%)	12 (25.0%)	11 (23.4%)	0.856
Pleura	39 (41.1%)	16 (33.3%)	23 (48.9%)	0.122
Bone	49 (51.6%)	21 (43.8%)	28 (59.6%)	0.123
Liver	24 (25.3%)	15 (31.3%)	9 (19.1%)	0.175
Adrenal gland	19 (20.0%)	10 (20.8%)	9 (19.1%)	0.837
Pericardium	7 (7.4%)	4 (8.3%)	3 (6.4%)	1
WHO performance status				
0	35 (36.8%)	23 (47.9%)	12 (25.5%)	
1	56 (58.9%)	23 (47.9%)	33 (70.2%)	0.061
2	4 (4.2%)	2 (4.2%)	2 (4.3%)	

[a] *Nivolumab;* [b] *coefficient of variation;* [c] *chronic obstructive pulmonary disease.*

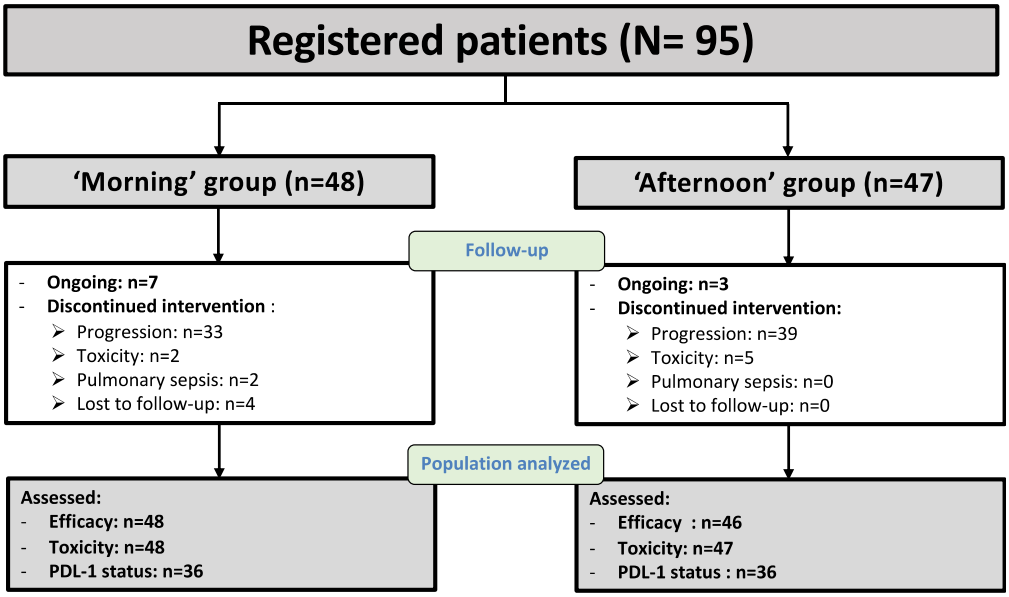

Figure 1. Study flow diagram.

3.2. Treatment Safety

No toxic death was encountered. Fatigue was the most frequent grade 3 or 4 adverse event. It occurred in 10.5% of the patients, including three patients in the 'morning' group (6.3%) and seven patients in the 'afternoon' group (14.9%) ($p = 0.024$) (Table 2). In contrast, grade 2–3 skin toxicities occurred more frequently in the 'morning' group (31.9% vs. 12.7%, $p = 0.049$). Toxicities were otherwise infrequent and similar in both treatment timing groups. Intercurrent events with questionable relation to nivolumab treatment were also observed, including pulmonary sepsis (38 patients, 40%), pulmonary embolism (3 patients, 3.2%) and thrombosis (6 patients, 6.4%). The frequency of these intercurrent events was similar in both groups except for pulmonary sepsis ('morning' and 'afternoon' groups, 17.9% and 7.4%, respectively, $p = 0.049$).

Table 2. Toxicity grades in all 95 patients and separately in 'morning' and 'afternoon groups.

Toxicities	Toxicity Grade	All (n = 95)	Morning' Group (n = 48)	Afternoon' Group (n = 47)	*p*-Value
Fatigue	*Grade 0*	43 (45.3%)	20 (41.7%)	23 (48.9%)	**0.024**
	Grade 1	13 (13.7%)	11 (22.9%)	2 (4.3%)	
	Grade 2	29 (30.5%)	14 (29.2%)	15 (31.9%)	
	Grade 3	9 (9.5%)	2 (4.2%)	7 (14.9%)	
	Grade 4	1 (1.1%)	1 (2.1%)	0 (0.0%)	
Skin toxicity	*Grade 0*	70 (74.5%)	30 (63.8%)	40 (85.1%)	**0.049**
	Grade 1	3 (3.2%)	2 (4.3%)	1 (2.1%)	
	Grade 2	19 (20.2%)	14 (29.8%)	5 (10.6%)	
	Grade 3	2 (2.1%)	1 (2.1%)	1 (2.1%)	
Anorexia	*Grade 0*	71 (74.7%)	36 (75.0%)	35 (74.5%)	0.856
	Grade 1	11 (11.6%)	6 (12.5%)	5 (10.6%)	
	Grade 2	9 (9.5%)	5 (10.4%)	4 (8.5%)	
	Grade 3	4 (4.2%)	1 (2.1%)	3 (6.4%)	

Table 2. *Cont.*

Toxicities	Toxicity Grade	All (n = 95)	Morning' Group (n = 48)	Afternoon' Group (n = 47)	*p*-Value
Anemia	*Grade 0*	72 (75.8%)	39 (81.3%)	33 (70.2%)	0.193
	Grade 1	6 (6.3%)	1 (2.1%)	5 (10.6%)	
	Grade 2	16 (16.8%)	7 (14.6%)	9 (19.1%)	
	Grade 3	1 (1.1%)	1 (2.1%)	0 (0%)	
Nausea	*Grade 0*	75 (78.9%)	40 (83.3%)	35 (74.5%)	0.633
	Grade 1	15 (15.8%)	6 (12.5%)	9 (19.1%)	
	Grade 2	4 (4.2%)	2 (4.2%)	2 (4.3%)	
	Grade 4	1 (1.1%)	0 (0.0%)	1 (2.1%)	
Hepatitis	*Grade 0*	80 (84.2%)	39 (81.3%)	41 (87.2%)	0.828
	Grade 1	13 (13.7%)	7 (14.6%)	6 (12.8%)	
	Grade 2	1 (1.1%)	1 (2.1%)	0 (0%)	
	Grade 3	1 (1.1%)	1 (2.1%)	0 (0.0%)	
Renal failure	*Grade 0*	80 (84.2%)	38 (79.2%)	42 (89.4%)	0.173
	Grade 1	15 (15.8%)	10 (20.8%)	5 (10.6%)	
Vomiting	*Grade 0*	81 (85.3%)	42 (87.5%)	39 (83.0%)	0.75
	Grade 1	9 (9.5%)	4 (8.3%)	5 (10.6%)	
	Grade 2	3 (3.2%)	1 (2.1%)	2 (4.3%)	
	Grade 3	1 (1.1%)	1 (2.1%)	0 (0.0%)	
	Grade 4	1 (1.1%)	0 (0.0%)	1 (2.1%)	
Hypothyroidism	*Grade 0*	83 (87.4%)	40 (83.3%)	43 (91.5%)	0.353
	Grade 1	8 (8.4%)	6 (12.5%)	2 (4.3%)	
	Grade 2	4 (4.2%)	2 (4.2%)	2 (4.3%)	
Muscle toxicity	*Grade 0*	83 (87.4%)	41 (85.4%)	42 (89.4%)	0.102
	Grade 1	4 (4.2%)	4 (8.3%)	0 (0.0%)	
	Grade 2	6 (6.3%)	3 (6.3%)	3 (6.4%)	
	Grade 3	2 (2.1%)	0 (0.0%)	2 (4.3%)	
Diarrhea	*Grade 0*	84 (88.4%)	43 (89.6%)	41 (87.2%)	0.197
	Grade 1	6 (6.3%)	4 (8.3%)	2 (4.3%)	
	Grade 2	3 (3.2%)	0 (0.0%)	3 (6.4%)	
	Grade 3	1 (1.1%)	1 (2.1%)	0 (0.0%)	
	Grade 4	1 (1.1%)	0 (0.0%)	1 (2.1%)	
Abdominal pain	*Grade 0*	87 (91.6%)	43 (89.6%)	44 (93.6%)	0.24
	Grade 1	3 (3.2%)	2 (4.2%)	1 (2.1%)	
	Grade 2	3 (3.2%)	3 (6.3%)	0 (0.0%)	
	Grade 3	2 (2.1%)	0 (0.0%)	2 (4.3%)	

3.3. Tumor Responses

The rates of objective responses (ORR) and disease control (DCR) were 27.7% (95% CL: 18.7 to 36.7) and 63.8% (54.1 to 73.5), respectively, for the 95 patients. ORR and DCR were significantly larger in the 'morning' group as compared to the 'afternoon' group (ORR: 37.5% vs. 14.4%, $p = 0.029$; DCR: 77.1% vs. 50.0%, $p = 0.006$).

3.4. Progression-Free Survival (PFS) and Overall Survival According to Nivolumab Timing

In the 95 patients, median PFS and OS (95% CL) were 3.9 months (2.1–5.8) and 14.0 months (9.5–18.4), respectively. Strikingly, median PFS and median OS were both nearly four times as long following 'morning' rather than 'afternoon' nivolumab dosing (Figure 2). Median PFS varied from 11.3 months (5.5–17.1) for the 'morning' group down to 3.1 months (1.5–4.6) for the 'afternoon' group ($p < 0.001$) (Figure 2a). Corresponding median OS values were 34.2 months (15.1–53.3) and 9.6 months (4.9–14.4), respectively ($p < 0.001$ (Figure 2b).

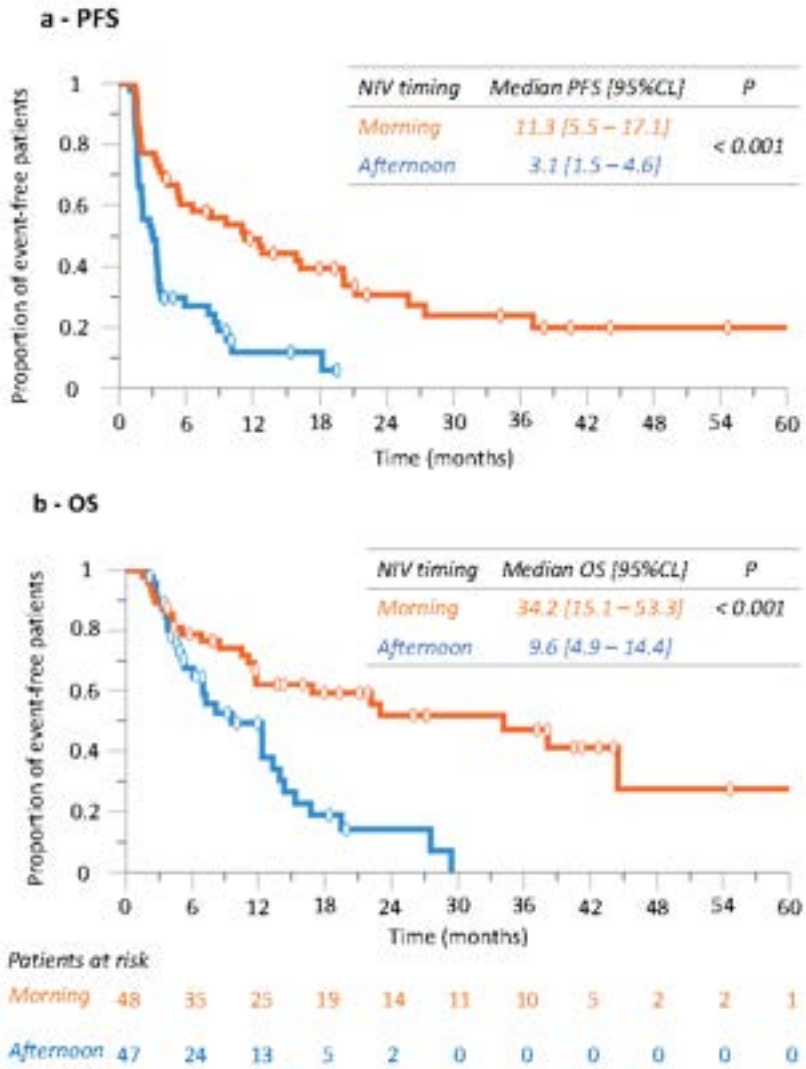

Figure 2. Kaplan–Meier curves of progression-free survival (PFS, (**a**)) and overall survival (OS, (**b**)) according to nivolumab timing group in whole study population.

Landmark analysis revealed that both PFS and OS were significantly longer for the 'morning' vs. 'afternoon' groups already at 2 months. Thus, the median PFS in the 42 patients in the 'morning' group exposed to nivolumab at the 2-month landmark was 15.8 months (6.2–25.5), whereas it was 3.5 months (3.2–3.9) for the 31 patients in the 'afternoon' group with the same nivolumab exposure ($p < 0.001$) (Figure 3). Similar results were obtained at the 4-month and 6-month landmark analyses (Figure 3). These findings indicated that nivolumab timing was critical for efficacy from the very beginning of treatment administration, and that the duration of tumor control by immunotherapy was critical for OS.

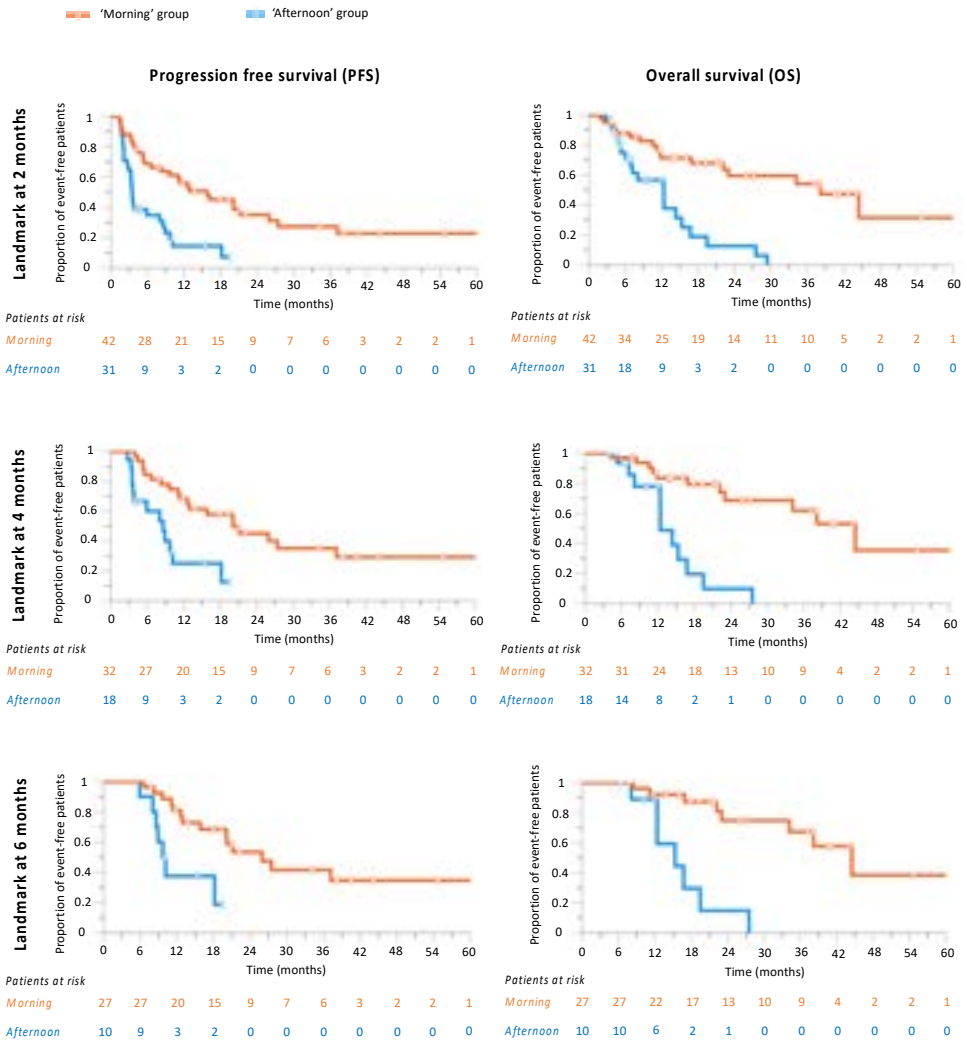

Figure 3. Progression-free survival (PFS) and overall survival (OS) landmark analysis by nivolumab timing group at 2, 4 and 6 months of exposure to nivolumab.

3.5. Did Patient Performance Status Influence Nivolumab Timing-Dependent Efficacy?

Median PFS was significantly longer in the 35 patients with a PS of 0 (11.1 months (2.2–20.0)) as compared to that in the 56 patients with PS = 1 (3.3 months (2.4–4.2)) (p = 0.020). A similar trend was found for OS according to PS, although non-significantly so (PS = 0: 19.5 months (8.2–30.8); PS = 1: 11.8 months (9.4–14.2); p = 0.13). Notwithstanding, 'morning' nivolumab significantly improved both PFS and OS in each PS category in comparison with 'afternoon' administration (Figure 4a,b).

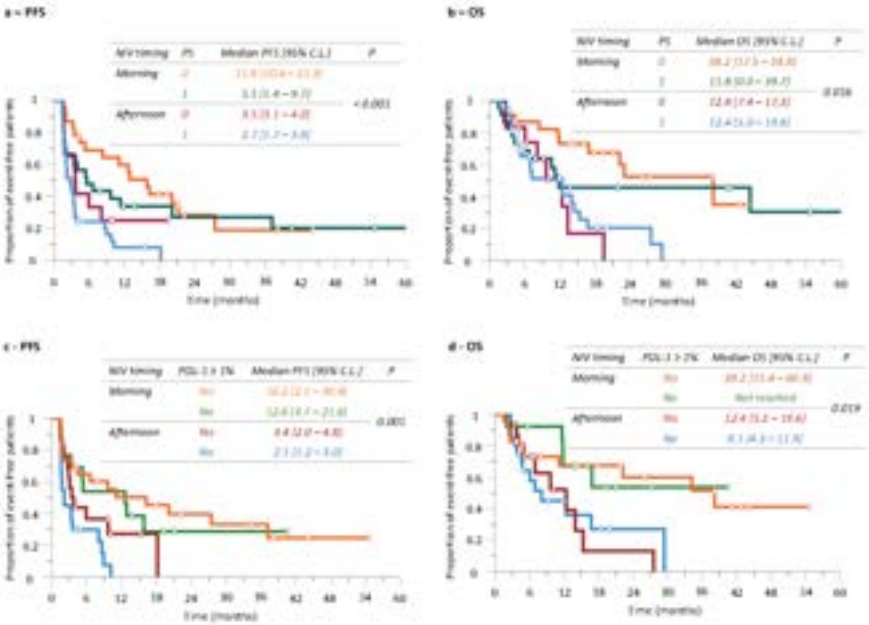

Figure 4. Kaplan–Meier curves of progression-free survival (PFS) and overall survival (OS) according to nivolumab timing group and WHO PS (**a**,**b**) or PD-L1 status (**c**,**d**).

3.6. Did Tumor PD-L1 Expression Influence Nivolumab Timing-Dependent Efficacy?

The 39 patients with PD-L1-positive (+) NSCLC had longer PFS and OS as compared to the 33 patients with PD-L1-negative (-) tumors. However, the differences were not statistically significant either for PFS (PD-L1(+): 9.6 months (2.4–16.8); PD-L1(-): 3.4 months (0.0–6.8); $p = 0.071$) or for OS (PD-L1(+): 22.2 months (7.0–37.4); PD-L1(-): 12.4 months (5.5–19.3); $p = 0.57$).

Most importantly though, 'morning' nivolumab nearly quadrupled both median PFS and median OS as compared to 'afternoon' dosing for patients in each tumor PD-L1 category (Figure 4c,d).

3.7. Univariate and Multivariate Cox Model Analyses of PFS and OS

'Morning' nivolumab consistently reduced the respective risks of an earlier progression or death as compared to 'afternoon' dosing in each prognostic category according to the Cox proportional univariate hazard ratios (Figure 5). Multivariate analyses further confirmed nivolumab timing as an independent prognostic factor for both PFS and OS (Table 3). The best outcomes resulted from 'morning' administration, with hazard ratios (HR) of 0.258 (0.115–0.580) for PFS and of 0.174 (0.082–0.370) for OS ($p < 0.001$ for both). Another joint significant prognostic factor was treatment line, with second-line nivolumab being associated with better PFS and OS as compared to third or later line. No other prognostic factors were identified for PFS. For OS, PS, histological type as well as bone and liver metastases were also statistically significant, with improved OS being predicted by a PS of 0, by the primary tumor being an adenocarcinoma rather than a squamous cell carcinoma, and by the lack of bone or liver metastases.

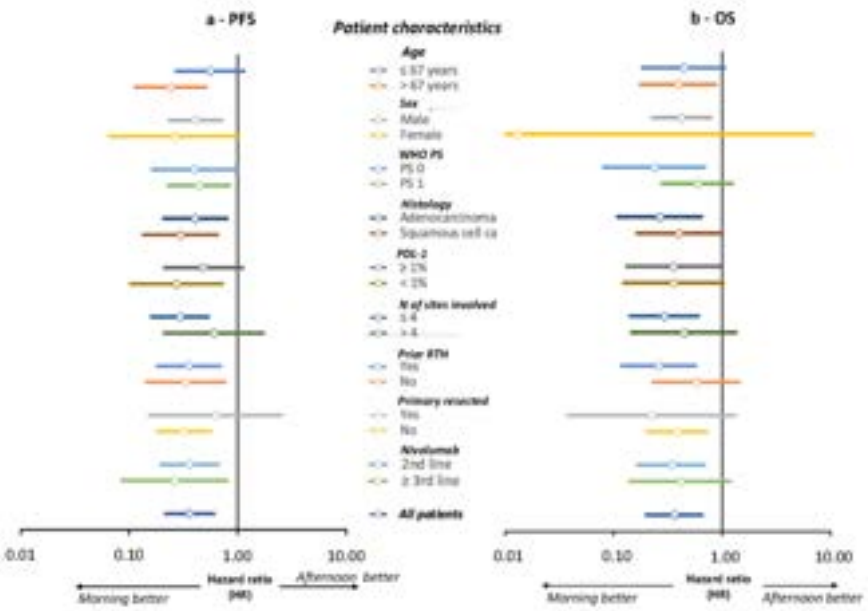

Figure 5. Forest plots of progression-free survival (PFS, (**a**)) and overall survival (OS, (**b**)), according to categorized patient characteristics. Hazard ratios and 95% CL of an earlier progression or an earlier death for 'morning' vs. 'afternoon' nivolumab groups.

Table 3. Prognostic factors of PFS and OS.

		PFS		
			95% CL of hazard ratio	
Prognostic factors of PFS	***p*-values**	**Hazard ratio**	**CL inf**	**CL sup**
NIV dosing time	0.001	0.258	0.115	0.580
2nd line NIV	0.009	0.324	0.140	0.752
		OS		
			95% CL of hazard ratio	
Prognostic factors of OS	***p*-value**	**Hazard ratio**	**CL inf**	**CL sup**
NIV dosing time	<0.001	0.174	0.082	0.370
2nd line NIV	0.004	0.390	0.204	0.746
Adenocarcinoma	<0.014	0.460	0.248	0.854
Bone not involved	0.005	0.420	0.230	0.765
Liver not involved	<0.001	0.300	0.153	0.588
WHO PS 0	0.048	2.005	1.007	3.993

3.8. Nivolumab's Efficacy According to Infusion Time following Trichotomization of Patients

There were 36 patients receiving at least 67% of the infusion before 12:54 (Group 1), 24 patients receiving at least 33% of the infusion before 12:54 and 33% after 12:54 (Group 2) and 35 patients receiving at least 67% of the infusion after 12:54 (Group 3) (Table S1). Patient characteristics did not differ significantly between groups, except for liver metastases, which were predominant in group 1 ($p = 0.010$).

Efficacy results revealed consistent and statistically significant differences for both PFS and OS among the three groups ($p = 0.002$ and $p = 0.003$, respectively) (Figure 6). Most importantly, consistent trends were found with Group 1 displaying longest median values for PFS (11.1 (0.0–25.5) and for OS (34.2 (NR, NR)), Group 2 having intermediate median values (PFS: 5.9 (0.0–13.3); OS: 15.3 (8.0–22.7)) and Group 3 experiencing the worst

outcomes (median PFS: 3.1 (1.6–4.5); median OS: 12.4 (4.0–20.7)). These results supported the relevance of morning nivolumab dosing, and further highlighted that poor efficacy was associated with more infusions being delivered after 12:54.

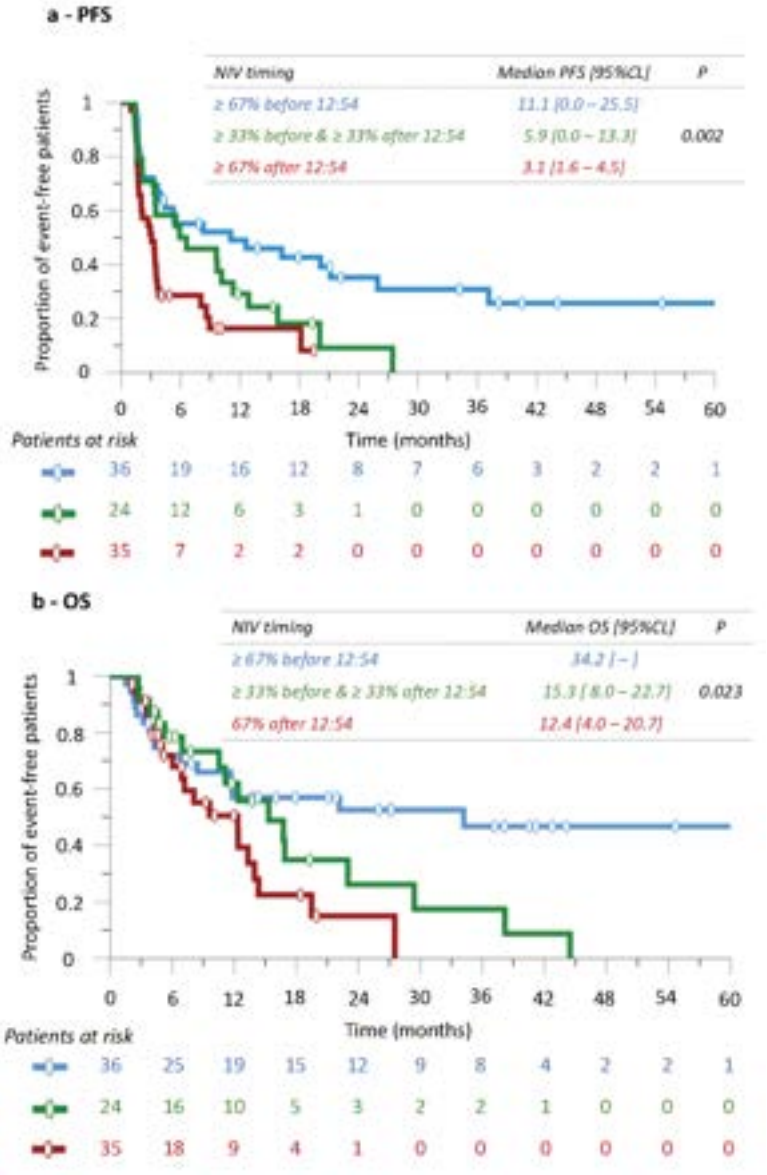

Figure 6. Kaplan–Meier curves of progression-free survival (**a**) and overall survival (**b**), according to three timing groups. Group 1 received at least 67% of the infusions before 12:54, Group 2 received at least 33% of the infusions before 12:54 and 33% of the infusions after 12:54, and Group 3 involved those patients receiving at least 67% of the infusions after 12:54.

At the 2-month landmark, 73 patients (76.8%) were exposed to nivolumab treatment, including 42 in the 'morning' group and 31 in the 'afternoon' group. The four-month landmark concerned 50 patients (52.6%), 32 and 18 in the 'morning' and the 'afternoon' groups, respectively. Finally, 37 patients were exposed at the 6-month landmark point, 27 patients in the 'morning' group and 10 patients in the 'afternoon' group.

Median PFS at the 2-, 4- and 6-month landmark was 15.8 months (6.2–25.5), 20.1 (9.7–30.6) and 25.9 (16.8–35.0) in the 'morning' group, respectively, and 8.6 (3.2–3.9), 8.6'(5.0–12.2) and 9.7 (8.0–11.4) in the 'afternoon' group, respectively. PFS was statistically different at each of the three landmark points with respective *p*-values of <0.001, 0.001 and 0.005.

Median OS at the 2-, 4- and 6-month landmark was 38.2 months (22.4–53.9), 44.5 (31.8–57.1) and 44.5 (32.9–56.1), respectively, in the 'morning' group and 12.4 (7.4–17.3), 12.4 (10.4–14.4) and 15.3 (8.0–22.7), respectively, in the 'afternoon' group. *p*-values were < 0.001 at each of the three landmark points.

4. Discussion

To the best of our knowledge, no study has yet explored whether the daily timing of anti-PD-1/PD-L1 immune checkpoint inhibitor drugs could moderate therapeutic efficacy in NSCLC patients. Our current investigation revealed for the first time that both median PFS and median OS were nearly quadrupled in metastatic NSCLC receiving the majority of nivolumab administrations between 9:27 and 12:54, rather than between 12:55 and 17:14. The large difference in both endpoints was consistent in all patient subgroups, with similar hazard ratios for the reduction in an earlier progression or an earlier death. Additionally, nivolumab's timing of administration was confirmed as an independent prognostic indicator of both PFS and OS, with hazard ratios of 0.258 and 0.174 in respective multivariate analyses. These results were consistent in all the subgroup analyses performed. Moreover, the trichotomization of the patient population further showed that the best outcomes resulted from delivering 67% of nivolumab infusion before 12:54. Taken together, the results strongly support a dramatic increase in nivolumab's efficacy following morning dosing.

The main limitation of our study is related to its retrospective nature and to the lack of statistical randomization between 'morning' and 'afternoon' nivolumab administrations. However, nivolumab timing was allocated for each course in each patient by the day-hospital coordinator, on a strictly logistical basis, thus reducing the risk of an uncontrolled bias. Moreover, both PFS and OS in our whole study population of 95 consecutive patients were comparable to the landmark trials with NSCLC patients receiving time-unspecified nivolumab [32–35]. Some studies have demonstrated improved efficacy of nivolumab in NSCLC patients whose tumors expressed PD-L1 [4,36,37]. We confirmed such a trend for PFS, but not for OS, in our study patients, possibly because of the limited size of our study population, using the standard cut-off of 1% of PD-L1-positive cells for considering a tumor as being PD-L1(+) [4]. Strikingly however, nivolumab's timing of administration significantly influenced PFS and OS both in the patients with PD-L1(+) tumors and in those with PD-L1(-) tumors. Similarly, 'morning' nivolumab also significantly improved PFS and OS in patients with a PS of 0 and in those with a PS of 1 in comparison with 'afternoon' dosing.

Skin toxicities were reported to predict for improved PFS and OS on nivolumab [38]. We confirmed this finding here as well, with 29.8% patients with grade 2 skin toxicities on morning dosing as compared to 10.6% of those on afternoon dosing. Consistent, with other reports [39,40], grade 3–4 fatigue was the main adverse event, yet it occurred in 6.3% of the patients on 'morning' nivolumab as compared to 14.9% following 'afternoon' dosing.

The landmark analyses of PFS and OS according to nivolumab timing further revealed that the timing-related differences were detected from the first 2 months on treatment, thus indicating that nivolumab's effects might be impaired in the 'afternoon' from the very beginning of such immunotherapy. Indeed, the major cause of treatment discontinuation was progressive disease, which occurred very early in the 'afternoon' group, resulting in fewer courses being given to the patients treated predominantly later in the day.

Our results are in rather good agreement with those recently reported in patients with metastatic malignant melanoma by Qian DC et al. [38], although they categorized their patients differently. More precisely, they used 16:30 as a temporal cut-off, and showed that the administration of 20% or more of ICI's infusions later than 16:30 increased the relative risk of an earlier death with an HR of 2.04 (1.04–4.0) (p = 0.038) in a propensity score-matched analysis involving 146 patients.

Taken together, both study results indicate that the biological mechanisms of nivolumab and other ICIs could differ largely according to their dosing time. Indeed, differences in time-dependent tolerability and/or efficacy have been shown for nearly 50 anticancer drugs in experimental models and/or cancer patients, with the main mechanisms involving circadian changes in pharmacokinetics and pharmacodynamics [21,28,41–43]. Understanding the main mechanisms at work for chronotherapy with nivolumab and other ICIs represents an important challenge. The average half-life of nivolumab of 20 days, with a reported sustained mean occupancy of >70% of PD-1 molecules on circulating T cells for two months or more following infusion [44], indeed suggests a limited role for circadian time-dependent pharmacokinetics as the main mechanism of its chrono-efficacy.

However, we believe that our understanding of nivolumab's binding dynamics, likely tested at a single time of day, is probably not correct. Rather, our data and those of Qian et al. invite a more fundamental scientific look at the rhythmic binding over 24 h, as well as the trafficking of T-cells into the tumor-draining lymph nodes. Thus, whilst PD-L1 expression on tumor cells has been considered as a critical mechanism of nivolumab's anti-tumor activity, this factor was not proven as an essential determinant of clinical efficacy in NSCLC. Although the selection of high tumor PD-L1 expression subsets enriched the response to anti–PD-1 therapy, more than half of these patients lacked durable responses to PD-1 blockade [45]. Such results concurred with the lack of survival prediction by PD-L1 positivity in our study.

Large amplitude circadian rhythms in T- and B-lymphocytes trafficking and functions have long been known, both in rodents and in humans. For instance, the counts of circulating total T lymphocytes and their subsets were nearly twice as high in healthy people at 08:00 in the morning as compared to midnight or 04:00 at night [15,46,47]. An experimental study further revealed a precise circadian control of lymphocyte trafficking from tissues to blood and vice-versa [46], which might be relevant here. Most importantly, no homing of T-lymphocytes from blood to lymph nodes was demonstrated in the second half of the nocturnal activity span of mice [48], which would correspond to afternoon and evening in humans, and might account for the observed timing-related differences in nivolumab's efficacy. Furthermore, a circadian rhythm characterized the in vivo T-(CD8+) cell response to immunization of mice with dendritic cells loaded with ovalbumin antigen [14,49]. In humans, BCG vaccination in the morning induced stronger trained immunity and adaptive responses compared with evening vaccination [12]. Increased antibody responses were also reported for morning versus afternoon vaccinations against influenza virus [50]. The reduced activity of afternoon nivolumab could involve the exacerbation of resistance mechanisms in this temporal window, including defective antigen presentation processes, multiple immune checkpoint interactions, transient refractoriness of the tumoral immune microenvironment, activation of oncogenic pathways, and epigenetic changes of key proteins in tumors, among other factors [51,52]. Nivolumab could also become inactivated shortly after its infusion through binding to circulating proteins or extracellular vesicles [53,54], thus accounting for consistent resistance in the 'afternoon' group in the landmark analyses. In general, the relevance of dosing time for cancer treatments has been underestimated in oncology, with temporal logistics organization of hospitals predominating over temporal changes in biological functions. Education of the medical community to circadian clocks and their relevance for health and treatment effects has been largely lacking. Finally, the drug companies that have developed immune checkpoint inhibitors have not investigated whether timing could impact efficacy (or have not reported their results on this issue). The lack of mandatory investigations on drug timing before drug registration

(or in postmarketing studies) is also a critical consideration that lets people believe that immunotherapy timing would have no real effect. Our results, and those of Qian et al. [26], clearly highlight that this bottleneck can and needs to be further circumvented through the undertaking of randomized clinical trials.

5. Conclusions

In conclusion, we feel that the fourfold difference in overall survival found in NSCLC patients according to nivolumab circadian timing supports the morning administration of this checkpoint inhibitor at arguably no cost. Our results, however, highlight the need for prospective randomized-controlled timing studies of nivolumab as well as of other anticancer immunotherapies. Carefully conducted translational investigations are mandatory to precisely identify the clinico-biological mechanisms underlying the major circadian dependencies in nivolumab's efficacy, so as to optimize individual patient benefits on immunotherapy.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers14040896/s1, Table S1: Main baseline characteristics of whole study population, and according to nivolumab (NIV) timing groups.

Author Contributions: Conceptualization and methodology: A.K., F.L. and R.A.; Validation: A.K., T.C., I.P. and V.B.; Formal analysis: A.K., F.L., R.A. and P.F.I.; Investigation: A.K., T.C., I.P., P.F.I. and M.B.; Resources: A.K., T.C., I.P., V.B., J.C. and E.Z.; Data Curation: A.K. and T.C.; Writing—original draft: A.K. and F.L.; Writing—review and editing: T.C., I.P., V.B., J.C., E.Z., P.F.I., M.B. and R.A.; Supervision: F.L. and R.A.; Project administration: A.K., F.L. and T.C. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The retrospective study project was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of Paris-Saclay university on 28 April 2020.

Informed Consent Statement: Patient consent was waived due to the lack of research-driven assignment of treatment timing, and the deidentification of data.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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4.1.2 Première méta-analyse évaluant le rôle de l'heure d'administration des inhibiteurs de point de contrôle immunologique sur la survie des patients cancéreux

***Article n°2**, ESMO Open, 16 Janvier 2024.*

Effet de l'heure de perfusion de l'immunothérapie sur la survie des patients atteints de cancers avancés : une méta-analyse au niveau des études

*Landré T, **Karaboué A**, Buchwald ZS, Innominato P, Qian DC, Assié JB, Chouaïd C, Lévi F, Duchemann B*

(Google Scholar: 5 citations)

Contexte : Les ICIs sont devenus les traitements de référence de nombreuses tumeurs malignes. De nouvelles données suggèrent que l'heure d'administration des ICIs pourrait avoir un impact sur les résultats des patients atteints de cancer. La pertinence des effets de l'heure d'administration sur l'efficacité des ICIs nécessite une première évaluation quant à l'importance et la cohérence des résultats.

Matériel et méthodes : Cette méta-analyse intègre les données de survie sans progression et de survie globale provenant d'études avec définition d'un seuil horaire d'administration discriminant l'efficacité antitumorale. Les Hazard ratios (HR) [intervalle de confiance à 95 % (IC)] d'une progression ou d'un décès précoce en fonction d'une administration « précoce » ou « tardive » dans la journée des ICIs ont été recueillis dans chaque étude et poolés.

Résultats : Treize études ont porté sur 1663 patients (statut de performance 0-1, 83 % ; hommes, 67% ; femmes, 33 %) atteints de cancer du poumon non-à-petites

cellules (47 %), de carcinome rénal (24 %), de mélanome (20 %), de cancer urothélial (5 %) ou de carcinome de l'œsophage (4 %). La plupart des patients ont reçu un anti-PD-1 ou un anti-PD-L1 (98 %), et une petite proportion a également reçu un anti-CTLA-4 (18 %). Les seuils discriminants de l'heure d'administration se situaient entre 12h00 et 14h00 pour six études et entre 16h00 et 16h30 pour sept études. Les analyses des données « poolées » ont révélé que les groupes traités précocement avaient une meilleure survie globale (HR : 0,50 [0,42-0,58] ; p<0,00001) et une meilleure survie sans progression (HR : 0,51 [0,42-0,61] ; p<0,00001) par rapport aux groupes traités tardivement.

Conclusions : Les patients atteints de divers cancers métastatiques semblent bénéficier largement des perfusions précoces d'ICIs, ce qui est cohérent avec les mécanismes circadiens des fonctions et de la circulation des cellules immunitaires. Des essais prospectifs randomisés sont nécessaires pour établir des recommandations sur le schéma circadien optimal des thérapies anticancéreuses à base d'ICIs.

Pour ce travail, j'ai identifié et sélectionné les articles pertinents, et contribué à l'analyse et l'interpretation des résultats, ainsi qu'à la rédaction et la version finale du manuscrit.

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ORIGINAL RESEARCH

Effect of immunotherapy-infusion time of day on survival of patients with advanced cancers: a study-level meta-analysis

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Background: Immune checkpoint inhibitors (ICIs) have become the standard of care for numerous malignancies. Emerging evidence suggests that the time of day (ToD) of ICI administration could impact the outcomes of patients with cancer. The consistency of ToD effects on ICI efficacy awaits initial evaluation.

Materials and methods: This meta-analysis integrates progression-free survival (PFS) and overall survival (OS) data from studies with a defined 'cut-off' ToD. Hazard ratios (HRs) [95% confidence interval (CI)] of an earlier progression or death according to 'early' or 'late' ToD of ICIs were collected from each report and pooled.

Results: Thirteen studies involved 1663 patients (Eastern Cooperative Oncology Group performance status 0-1, 83%; males/females, 67%/33%) with non-small-cell lung cancer (47%), renal cell carcinoma (24%), melanoma (20%), urothelial cancer (5%), or esophageal carcinoma (4%). Most patients received anti-programmed cell death protein 1 or anti-programmed death-ligand 1 (98%), and a small proportion also received anti-cytotoxic T-lymphocyte-associated protein 4 (anti-CTLA-4) (18%). ToD cut-offs were 13:00 or 14:00 (i.e. ICI median infusion time), for six studies, and 16:00 or 16:30 (i.e. reported threshold for weaker vaccination responses) for seven studies. Pooled analyses revealed that the early ToD groups had longer OS (HR 0.50, 95% CI 0.42-0.58; $P < 0.00001$) and PFS (HR 0.51, 95% CI 0.42-0.61; $P < 0.00001$) compared with the late ToD groups.

Conclusions: Patients with selected metastatic cancers seemed to largely benefit from early ToD ICI infusions, which is consistent with circadian mechanisms in immune-cell functions and trafficking. Prospective randomized trials are needed to establish recommendations for optimal circadian timing of ICI-based cancer therapies.

Key words: immunotherapy, chronotherapy, immune checkpoint inhibitors, meta-analysis, circadian rhythms

INTRODUCTION

The time of day (ToD) of administration has not been specified nor evaluated in clinical trials assessing the efficacy and adverse events of checkpoint inhibitors (ICIs), but is anticipated to have occurred mostly in the morning.[1,2] However, findings in several retrospective clinical studies have indicated that ICI infusion ToD is usually spread between 08:00 and 18:00, and could affect treatment efficacy. Such results are in line with those from prior randomized chronotherapy trials involving chemotherapy and radiation therapy, as well as recent meta-analyses and systematic reviews.[3,4] Because of the accumulating evidence of ToD effects on the efficacy of ICIs in retrospective clinical studies, we carried out the first meta-analysis for accurately assessing and quantifying the impact of ToD of ICI infusions on their effectiveness in patients with different advanced cancers.

Indeed, circadian rhythms have been shown to rhythmically regulate organismic and cellular functions along the 24-h scale. Modifications of these rhythms have been associated with the development or the worsening of numerous illnesses, including cancer, cardiovascular, neurological and psychiatric diseases, metabolic syndrome, and immune and inflammatory disorders.[5-8]

Circadian rhythms are endogenous and genetically based biological variations with an ∼24-h period. They are generated by molecular clocks that consist in feedback

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transcription-post-transcription and post-translation regulatory loops involving 15 clock genes within each cell.[9] The discovery of the fine molecular mechanisms responsible for the ticking of molecular circadian clocks across species by Jeffrey Hall, Michael Rosbasch, and Michael Young was acknowledged by the 2017 Nobel Prize in Physiology or Medicine Award.[10] Amazingly, 1 year later, James Allison and Tasuku Honjo won the 2018 Nobel Prize for Physiology or Medicine for discoveries leading to new approaches in harnessing the immune system to fight cancer, such as ICIs.[10]

Circadian rhythms help cells and whole organisms to adapt to cyclical changes in their environment, including the alternation of light and darkness over the 24 h in particular.[11] The suprachiasmatic nucleus, the central circadian pacemaker in the hypothalamus, receives and integrates photoperiodic information, and coordinates the molecular clocks in the genome of each cell through the generation of rhythmic physiological and hormonal signals, such as those in core body temperature and cortisol secretion. The circadian timing system rhythmically controls drug absorption, distribution, metabolism, and elimination processes at both tissue and whole organism levels, as well as cellular proliferation and death in healthy cells.[12] Moreover, a one-to-one coupling of the circadian clock with the cell cycle has been demonstrated at the single-cell level for proliferating fibroblasts.[13] Such coupling may be altered in cancer cells,[14] whose molecular clock and/or its regulatory functions can be disrupted.[4-8] Yet, host circadian rhythms can both regulate cancer cell proliferation and their metastatic potential which seems to be greater at night in patients with breast cancer.[8,15-18]

An autonomous molecular circadian clock has also been identified in most immune cells, whose function and trafficking have long been known to display prominent circadian rhythms in rodents and humans.[19] For instance, in humans, proinflammatory cytokines [e.g. interleukin-1 (IL-1) and tumor necrosis factor-α] circulate at higher levels during the rest span at night, whereas anti-inflammatory cytokines (e.g. IL-4 and IL-10) do so during active periods at daytime.[20,21] Furthermore, the release of glucocorticoids, potent immunosuppressors, peaks at the beginning of the day and reaches its lowest level during the first half of the night.[22] Concerning cellular immunity, T lymphocytes (CD8+), a main cellular target of ICIs, are subjected to circadian variations, with notably more intense proliferation during the early afternoon; circulation of B-lymphocytes in the blood is enhanced at the end of the evening, while circulating T and T(CD4) lymphocytes peak near the middle of the night, and processes of recognition and destruction of antigens predominate in the very early morning.[16-18,23,24] The relevance of such circadian orchestration of the immune system for the efficacy of ICIs is emerging through numerous and recent retrospective clinical studies, whose consistency of results is evaluated here in a comprehensive meta-analysis of available evidence.

MATERIALS AND METHODS

This meta-analysis of published data evaluated the impact of the ToD of ICI infusion on the overall survival (OS) of adult patients with metastatic solid tumors, as a primary endpoint. The secondary endpoints were progression-free survival (PFS) and response rate. Incidence of immune-related adverse events was not included as an endpoint, because their rate was inconsistently reported in the studies, thus preventing any meaningful meta-analysis. PubMed and Cochrane databases were used for the search of published articles (15 August 2023), complemented by a manual exploration of abstracts submitted to the annual meetings (including site-specific ones) of the American Society of Clinical Oncology, the European Society for Medical Oncology, the American Association for Cancer Research, and the Society for Immunotherapy of Cancer from 15 February 2020 to 15 February 2023. The search terms used were 'immunotherapy, Pembrolizumab, Nivolumab, Ipilimumab, Avelumab, Atezolizumab, Tislelizumab, Durvalumab, Dostarlimab, Tremelimumab, Sintilimab, Camrelizumab, Cemiplimab, Zimberelimab, immune checkpoint', 'immunotherapy, anti-programmed cell death protein-1 (PD-1) and its ligand anti-programmed death-ligand 1 (PD-L1), or immune checkpoint inhibitor', 'cancer survival', and 'time-of-day of infusion, circadian rhythm, circadian timing or chronotherapy' (Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2023.102220).

All the study data were extracted independently by two readers (TL and FL). Disagreements were resolved by discussion with a third reader (PFI). Whenever results in a given study population were reported both in abstract form and as an article by the same investigator team, we only considered the data in the article. The following information was collected: the number of patients, the histological type of cancer, the type of ICIs administered, the daily cut-off time applied for differentiating efficacy according to the ToD of ICI infusions, and the proportion of infusions deemed to be needed for weakening ICI efficacy. The efficacy criteria analyzed were OS, PFS, and response rate, as a function of dichotomization of the patient populations according to each study-specific cut-off time and proportion of infusions with late ToD. The main analysis included all types of metastatic cancers, while a secondary analysis specifically addressed ICI timing effect for non-small-cell lung cancer (NSCLC), renal cell carcinoma, and melanoma since at least two independent reports were available for each patient population.

The analyses were conducted according to the Cochrane method for meta-analyses and computed with Review Manager software (RevMan version 5.4; Cochrane, Oxford, UK). The heterogeneity statistic was evaluated with χ^2 tests and I^2 statistics, with a χ^2 test $P < 0.05$ defining the presence of heterogeneity. I^2 assesses heterogeneity among studies, with heterogeneity ranking from perfect homogeneity (0%) to moderate heterogeneity (30%-60%) up to total heterogeneity (100%). Begg's funnel plots were used to visualize the heterogeneity among studies. A fixed-effects model was used to calculate the cumulative relative risk when interstudy heterogeneity was low; when such heterogeneity was high, a randomized model was applied. The meta-analysis results for OS and PFS are reported as hazard

ratio (HR) [95% confidence interval (CI)]. All tests were bilateral, with $P < 0.05$ defining statistical significance.

RESULTS

The search found 16 studies, including 8 articles in peer-reviewed international journals[25-32] and 8 congress abstracts, which were presented as poster communications at major international congresses.[33-40] Three abstracts were discarded because the data[40] were updated in a subsequently published article,[26] or detailed HR data were not reported.[34,35] All 13 retained study reports consisted of retrospective evaluations of ToD ICI administration effects. They involved patients receiving ICIs for metastatic lung cancer,[26-28,36,39] malignant melanoma,[25,30,32] urothelial cancer,[33] renal cell carcinoma,[29,37,38] and esophageal carcinoma.[31] ICIs were anti-PD-1 and/or anti-PD-L1, which were combined with anti-cytotoxic T-lymphocyte-associated protein 4 (anti-CTLA-4) ipilimumab for some patients in five studies[25,29,30,32,37] (Supplementary Figure S1, available at https://doi.org/10.1016/j.esmoop.2023.102220).

The cut-off time defining early or late ToD ICI infusion was given in each study report, along with a threshold value expressed as the proportion of ICI infusions received before or after that 'cut-off' time. A difference of up to 4.5 h characterized the defined cut-off times that discriminated early versus late ToD ICI groups among the 13 studies. The percentage of infusions administered after this threshold that was associated with a weak efficacy of ICIs also differed from 20% to 75% between studies. Most studies considered all the immunotherapy cycles,[25-29,31,33,36-38] but a few studies only considered the initial ones.[30,31,39]

A pragmatic approach was used in five studies which selected 12:00,[39] 12:54,[26] 13:00,[30,31,37] or 14:00[32] as cut-off points corresponding to the median time of the per-patient median times of reported ICI infusions. The percentage of courses associated with a weaker ICI efficacy after the ToD 'cut-off' was 50% in one study[38] and 25% in another.[37] In two studies, ToD groups differed as a function of whether patients received one to four of the initial four ICI infusions before 12:00 or 13:00 versus all four initial infusions after 12:00 or 13:00.[30,39]

The ToD 'cut-off' was 16:00 for one study[36] and 16:30 for six others.[25,27-29,33,38] For those seven studies, the early ToD groups consisted of patients receiving fewer than 20% of their ICI infusions after the cut-off time. The patients in the late ToD groups received at least 20% of their ICI infusions after that cut-off time. The cut-off times and proportions in all the studies included in this meta-analysis are summarized in Figure 1.

The current meta-analysis involved 1663 patients, with 1037 patients (62.4%) and 626 patients (37.6%) being allocated to the early and late ToD infusion groups, respectively. The main characteristics of the 13 retained studies are listed in Table 1 and the main patients' characteristics are listed in Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2023.102220. Most importantly, a statistically significant difference favored early ToD administration for OS, with an HR of 0.50 (95% CI 0.42-0.58; $P < 0.00001$), based on available data from all 13 studies (Figure 2A). The analyses further revealed significant benefits in both secondary endpoints, whenever such data were available. This was the case for PFS, with early ToD infusions being associated with an HR of 0.51 (95% CI 0.42-0.61; $P < 0.00001$) from available data reported for seven studies[26-28,30,31,33,39] (Figure 2B). Similarly, response rates were largely improved in the early ToD groups from the six studies where this endpoint was reported (Supplementary Figure S2, available at https://doi.org/10.1016/j.esmoop.2023.102220).

Results from the analyses restricted to metastatic NSCLCs also supported early ToD ICI infusions for prolonging both OS [HR 0.53 (95% CI 0.34-0.80); $P = 0.003$] and PFS [HR 0.55 (95% CI 0.44-0.69); $P < 0.00001$; Figure 3A and B]. Analyses restricted to metastatic renal cell carcinoma or melanoma also revealed statistically significant differences in OS that favored early ToD ICI infusion groups (Figure 4).

A subgroup analysis of OS was carried out according to the ToD 'cut-off' of the studies. The benefit of early ToD ICI infusions for studies with a cut-off between 12:00 and 14:00 (HR 0.42, 95% CI 0.33-0.53; $P < 0.00001$) was higher than that of studies with a cut-off between 16:00 and 16:30 (HR 0.58, 95% CI 0.47-0.72; $P < 0.00001$; subgroup differences: $P = 0.05$; Supplementary Figure S3, available at https://doi.org/10.1016/j.esmoop.2023.102220).

Assessment of heterogeneity bias among studies showed that a single study[26] fell outside the 95% CI (Supplementary Figure S4, available at https://doi.org/10.1016/j.esmoop.2023.102220), with a major effect in favor of the administration of ≥50% ICI infusions in the morning. The exclusion of this study from the meta-analysis did not alter the survival improvement from early ToD ICI infusions, as shown with an HR of 0.52 (95% CI 0.44-0.61; $P < 0.00001$; Supplementary Figure S5, available at https://doi.org/10.1016/j.esmoop.2023.102220).

DISCUSSION

This meta-analysis of 13 clinical studies reporting the effects of ICI daily timing for OS revealed consistent and statistically significant prolongations of OS (primary endpoint) as well as PFS and objective response rate (secondary endpoints) in those patients with cancer receiving most ICI administrations in the early rather than in the late part of the day. The near doubling of ICI efficacy through early ToD dosing, based on our three endpoints, stood out quite robustly because the patients were receiving single-agent or combined ICIs, as first- or second-line pharmacotherapy, for different cancer types, and in nine countries from four continents. Moreover, all the studies included in this meta-analysis were reported recently and quickly after the first reports (i.e. within the past 2 years).

However, all study designs involved the retrospective assessment of ToD of ICI administration, each constrained by the availability of slots in the treatment delivery day unit, patient preference, interaction with clinical review, and

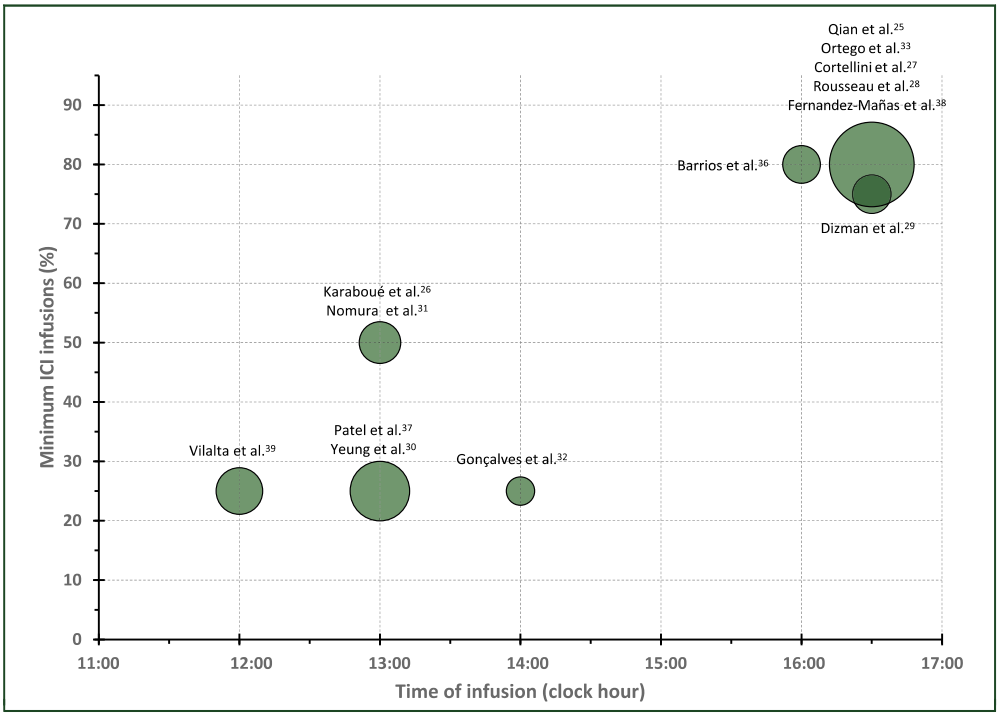

Figure 1. Two-dimensional and bubble plots illustrating the sample sizes of studies according to both the daily cut-off time selected for differentiating early versus late ToD groups (abscissa) and the minimum percentage of ICI infusions administered before this cut-off time (ordinate). The diameter of each bubble is proportional to the number of patients included in a single study, or in an aggregate of studies that chose the same criteria for timing cut-off and proportion of ICI infusions before the cut-off time. Corresponding study reference(s) are shown above or below the relevant bubble. Note the occurrence of two clusters of six and seven studies with respective cut-off times at 12:00-14:00 and 16:00-16:30.
ICI, immune checkpoint inhibitor; ToD, time of day.

unplanned deferrals or further investigations required before safe administration. Thus, within-patient differences were found for actual ToD of ICI administration along the course of their immunotherapy. Two main different cut-off times were used to differentiate early versus late ToD groups, that is, 16:00-16:30 or 12:00-14:00. Worse efficacy was associated with the administration of ≥20% infusion cycles after 16:00-16:30 on the one hand, or ≥25%-75% infusion cycles after 12:00-14:00 on the other hand. These respective ToD cut-offs were based on results from a vaccination study that revealed less antibody formation in healthy people vaccinated after 16:00-16:30,[24,25] while the 12:00-12:30 cut-off corresponded to the median cut-off of the individual median timing of ICI infusions in the study population, and to a lesser pharmacologic response in some vaccination studies.[41] In our study, results of an exploratory subgroup analysis suggest better efficacy for infusion before 14:00 rather than before 16:00.

Regarding sensitivity analysis, several authors have analyzed the prognostic influence of the timing cut-off[25] or of the proportion of infusions beyond the cut-off time,[26,38] as well as the critical role of ICI timing during the initial treatment months.

The prognosis was all the worse as the proportion of patients treated after the cut-off increased. For the timing cut-off, the prognosis impact was variable between 16:00 and 17:00 with a more significant association for a cut-off at 16:15.[25]

Karaboué et al.[26] included a sensitivity study to examine that criterion by analyzing OS and PFS for three patient groups: at least 67% of infusions given before 12:54, at least 33% of the infusions administered before 12:54 and 33% after that time, and at least 67% of administrations after 12:54. Large and statistically significant differences characterized both OS and PFS of these three groups, that is, median OS, 34.2 (not reached—not reached), 15.3 (8.0-22.7), and 12.4 (4.0-20.7) months, respectively ($P = 0.003$); and median PFS, 11.1 (0.0-25.5), 5.9 (0.0-13.3), and 3.1 (1.6-4.5) months, respectively ($P = 0.002$). All sensitivity analyses concurred in showing progressively worse efficacy outcomes with late ToD ICI administration, and improved OS through the administration of more courses before the

Table 1. The main characteristics of the studies included in the meta-analysis

Study Country Method Period	Metastatic cancer	ICI	Infusion cut-off time	Number of patients (B/A)	Outcome measure
Ortego et al.[33] Spain and Italy Multicenter, retrospective 2016-2021	Urothelial	Anti-PD-1 Anti-PD-L1	16:30	88 (62/26)	OS PFS
Qian et al.[25] United States Longitudinal 2012-2021	Melanoma	Anti-PD-1 Anti-CTLA-4	16:30	146 (73/73)	OS
Gonçalves et al.[32] Portugal Retrospective 2016-2022	Melanoma	Anti-PD-1 Anti-CTLA-4	14:00	73 (48/25)	OS
Yeung et al.[30] Canada Retrospective 2015-2021	Melanoma	Anti-PD-1 Anti-CTLA-4	13:00	121 (98/23)	OS PFS
Karaboué et al.[26] France Retrospective 2015-2020	NSCLC	Anti-PD-1	12:54	95 (48/47)	OS PFS
Cortellini et al.[27] United States, United Kingdom, and Italy Multicenter cohort 2018-2022	NSCLC	Anti-PD-1	16:30	180 (136/44)	OS PFS
Barrios et al.[36] Brazil Real-world database 2018-2021	NSCLC	Anti-PD-1 Anti-PD-L1	16:00	129 (86/43)	OS
Vilalta et al.[39] Spain Retrospective 2015-2020	NSCLC	Anti-PD-1	12:00	197 (104/93)	OS PFS
Rousseau et al.[28] France Retrospective 2014-2021	NSCLC	Anti-PD-1 Anti-PD-L1	16:30	180 (115/65)	OS PFS
Dizman et al.[29] United States Retrospective 2018-2022	RCC	Anti-PD-1 Anti-CTLA-4	16:30	135 (89/46)	OS
Fernandez-Mañas et al.[38] Spain Retrospective 2014-2022	RCC	Anti-PD-1 Anti-PD-L1	16:30	56 (32/24)	OS
Patel et al.[37] United States Retrospective 2015-2020	RCC	Anti-PD-1 Anti-CTLA-4	13:00	201 (119/82)	OS
Nomura et al.[31] Japan Retrospective 2017-2022	Esophagus	Anti-PD-1	13:00	62 (27/35)	OS PFS

CTLA-4, cytotoxic T-lymphocyte-associated protein 4; ICI, immune checkpoint inhibitor; NSCLC, non-small-cell lung cancer; OS, overall survival; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; PFS, progression-free survival; RCC, renal cell carcinoma. A indicates the number of patients receiving infusion after the cut-off, while B indicates the number of patients receiving infusion before the cut-off.

selected ToD cut-off time. Strikingly, two studies have revealed that early ToD ICIs over the initial 2 or 3 treatment months could be most critical for improving efficacy.[30,31]

Moreover, despite the retrospective nature of the 13 study reports, it is striking to note that all the results showing an impact of ToD consistently favored early injections and that not a single study showed improved outcomes through late ToD administration. Finally, for one of the studies,[36] survival data were immature, but also favored early ToD.

Nonpharmacological factors, potentially accounting for the observed consistent differences in outcomes according to ICI ToD administration, have been raised. These include social factors, related to a preference for morning day

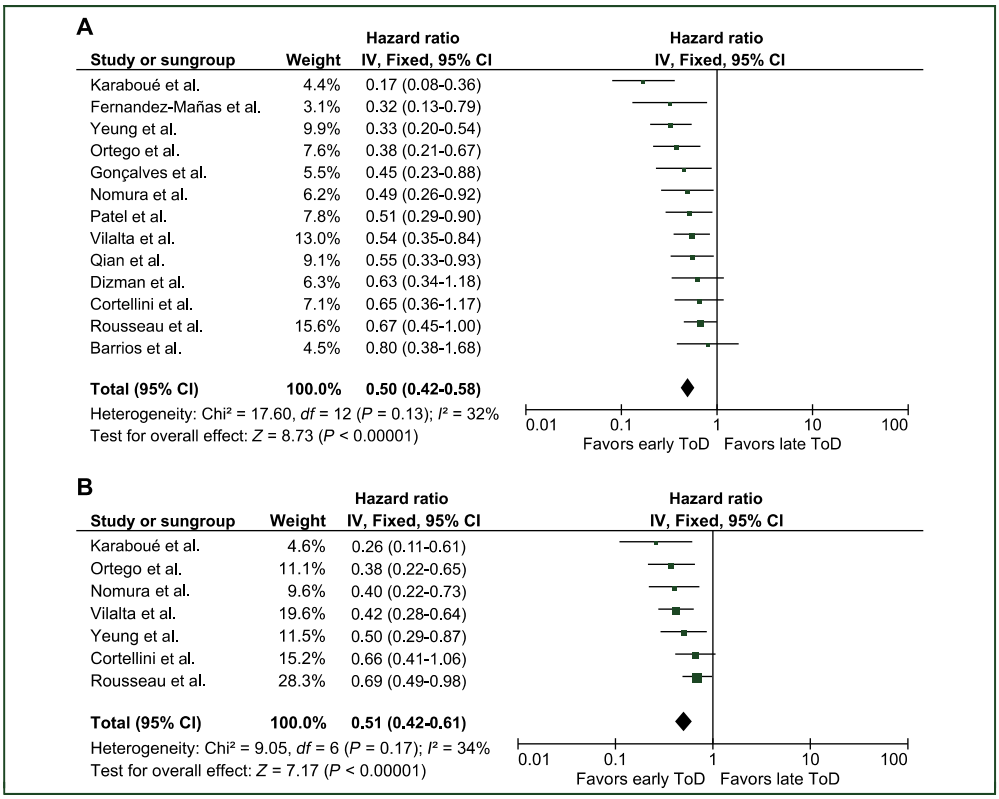

Figure 2. Meta-analysis results for (A) overall survival and (B) progression-free survival according to the early or late ToD group of ICI infusions.
CI, confidence interval; ICI, immune checkpoint inhibitor; ToD, time of day; IV, inverse variance.

hospital visits in wealthier patients, intrinsically expected to have better outcomes,[42] and a potential associative bias with the number of administered cycles, with patients having an intrinsically immunotherapy-resistant disease more likely to receive a smaller number of administrations, thus more likely to receive a higher proportion of later ToD infusions.[27] In the former case, although an imbalance in sociodemographic factors could not be entirely excluded, it would be unlikely to have impacted OS, PFS, or relative risk.[43]

The mechanisms at work for anti-PD-1 ICI efficacy largely involve T(CD8)- and dendritic cell-based immune responses, the same as those involved in vaccination responses which have been shown to depend on ToD both in mice[44] and in humans.[41] According to a randomized study involving people aged >65 years, BCG vaccination yielded significantly better humoral responses following dosing in the morning, between 9:00 and 11:00, compared with vaccination in the afternoon, between 15:00 and 17:00.[45] People vaccinated in the morning also experienced a better response to influenza or severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccines compared with those vaccinated in the afternoon/evening in observational studies.[45-47] Different criteria defined improved vaccination responses in these three studies, that is, greater antibody response[45,46]; higher production of interferon-γ, tumor necrosis factor-α, and IL-1β, supporting increased short-term immunity[46,48]; or increased long-term immunity with increased frequencies of memory B cells.[46] Morning vaccination also produced stronger B-cell, T-cell, monocytes, and dendritic cell responses.[41]

Experimental studies have revealed that cancer growth was rhythmically regulated by circadian waves of tumor infiltration with tumor-associated macrophages and dendritic cells, while PD-1 expression was also rhythmically controlled by the molecular clock in these cells.[5,49,50] Moreover the trafficking of circulating lymphocytes from blood to lymph nodes was restricted to the rest span of mice at daytime, which would correspond to the rest span at night in humans.[50] Taken together, these and other results support the enhanced efficacy of anti-PD-1 ICIs following their administration in the morning, when

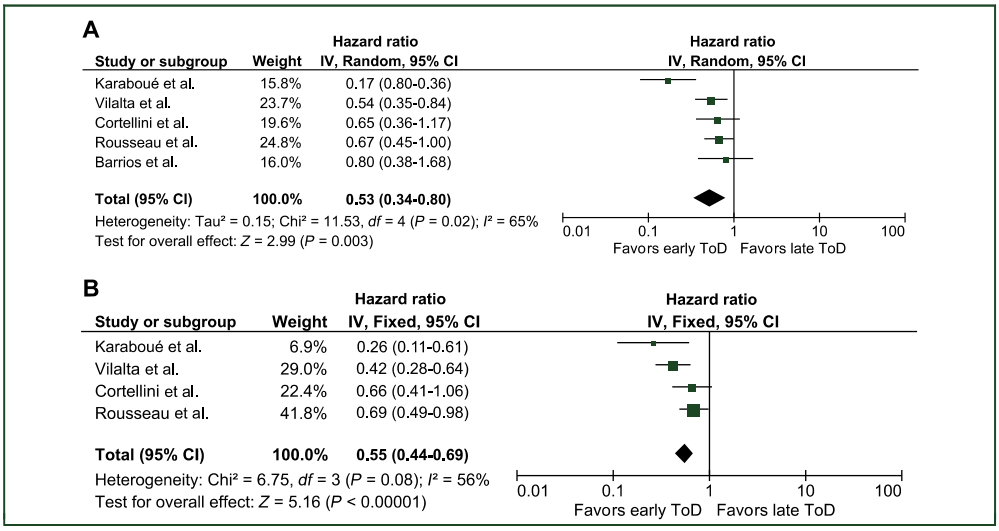

Figure 3. Meta-analysis results restricted to patients with metastatic non-small-cell lung cancer for (A) overall survival and (B) progression-free survival according to the early or late ToD group of ICI infusions.
CI, confidence interval; ICI, immune checkpoint inhibitor; ToD, time of day; IV, inverse variance.

susceptible immune cells are primarily located in the tumor and its draining lymph nodes. This may explain why ICI daily timing of administration could matter so much for efficacy, despite nivolumab and pembrolizumab, for instance, having plasma half-lives of 2—3 weeks,[51] in agreement with comments highlighting the minimal expected impact of drug pharmacokinetics on these circadian-based findings.[52] Finally, there were not enough data available to allow for an exploration of possible interactions between sex and ICI timing effects. It will be crucial to determine further the relevance of sex, along with hormonal and biological parameters in prospective trials.

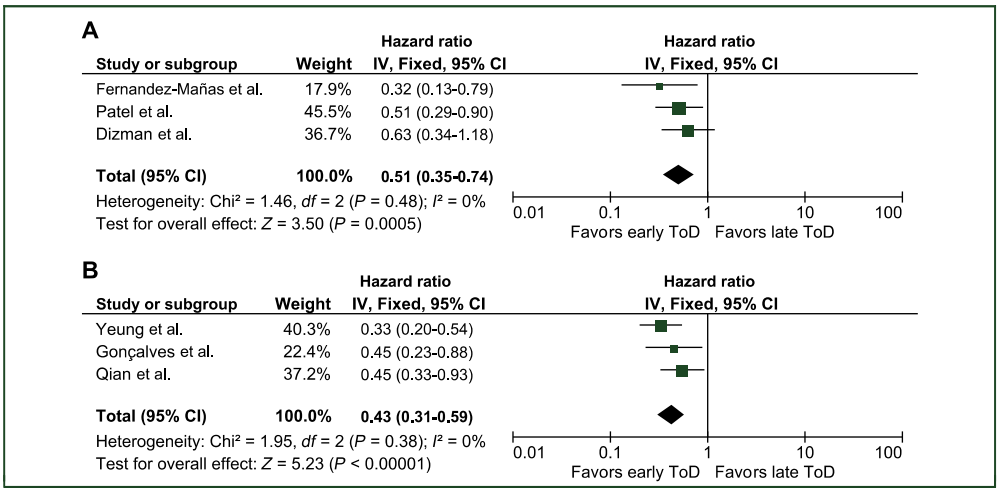

Figure 4. Meta-analysis results restricted to patients with (A) metastatic renal cell carcinoma and (B) melanoma for overall survival according to the early or late ToD group of ICI infusions.
CI, confidence interval; ICI, immune checkpoint inhibitor; ToD, time of day; IV, inverse variance.

Conclusion

Notwithstanding the fact that morning administrations of ICIs could already be advised at basically no cost and arguably no risk, further randomized clinical and translational studies are needed to conclusively establish recommendations for personalized chronotherapy of ICI-based treatments, to enhance efficacy through patient-specific ToD administration.

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4.2 MECANISMES IMPLIQUES : ROLE DES RYTHMES CIRCADIENS DANS LA CHRONOEFFICACITE ANTI TUMORALE DES INHIBITEURS DE POINT DE CONTROLE IMMUNOLOGIQUE

4.2.1 Revue des mécanismes impliqués dans la chronoefficacité des inhibiteurs de point de immunologique

Article n°3, *Brit Journal of Cancer, 4 Juin 2024*

Pourquoi le rythme circadien de l'administration est-il important pour l'efficacité des inhibiteurs de points de contrôle immunitaire?

Karaboué A, *Innominato FP, Wreglesworth NI, Duchemann B, Adam R, Lévi F.*

Contexte : La tolérance et l'efficacité antitumorale de la chimiothérapie et de la radiothérapie peuvent varier considérablement en fonction de l'heure d'administration sur l'échelle de 24 heures, en raison de la modulation de leurs mécanismes moléculaires et cellulaires par les rythmes circadiens. Des données cliniques récentes ont mis en évidence un rôle majeur de l'heure d'administration dans l'efficacité de l'immunothérapie des cancers, ce qui nécessite une évaluation critique.

Méthodes: Nous examinons ici les données cliniques et analysons les

mécanismes par lesquels les rythmes circadiens peuvent influencer les résultats des thérapies par ICIs. Nous examinons comment les troubles du rythme circadien peuvent affecter le microenvironnement immunitaire de la tumeur, en tant que mécanisme principal reliant l'horloge circadienne aux cycles de 24 heures dans l'efficacité antitumorale des ICIs.

Résultats : Les données en vie réelle de 18 études rétrospectives ont révélé que la perfusion précoce dans la journée d'ICIs pouvait améliorer la survie sans progression et/ou globale jusqu'à quatre fois par rapport à l'administration tardive (en fin d'après-midi). Les études ont porté sur un total de 3250 patients atteints de mélanome métastatique, de cancer avancé ou métastatique du poumon, du rein, de la vessie, de l'œsophage, de l'estomac ou du foie dans 9 pays. Des différences aussi importantes et cohérentes de l'effet du moment de la journée du traitement sur les résultats ne peuvent résulter que d'un mécanisme chronobiologique robuste jusqu'ici ignoré. Le système circadien coordonne la physiologie des cellules, des tissus et de l'ensemble du corps sur une échelle de 24 heures. Les rythmes circadiens sont générés au niveau cellulaire par un système d'horloges moléculaires qui impliquent 15 gènes circadiens spécifiques. La perturbation des rythmes circadiens peut déclencher ou accélérer la carcinogenèse et contribuer à l'échec des traitements anticancéreux, peut-être

par le biais d'une évasion immunitaire de la tumeur résultant d'un microenvironnement tumoral immunosuppressif.

Conclusion et perspectives : Cette compréhension émergente de la régulation de l'immunité antitumorale par les rythmes circadiens nécessite maintenant la réalisation d' essais cliniques randomisés afin de préciser l'impact des rythmes circadiens pour l'efficacité et la tolérance des ICIs actuels et en développment, personnaliser la chrono-immunothérapie en fonction des biomarqueurs circadiens des patients individuels.

British Journal of Cancer www.nature.com/bjc

REVIEW ARTICLE OPEN

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Translational Therapeutics

Why does circadian timing of administration matter for immune checkpoint inhibitors' efficacy?

Abdoulaye Karaboué[1,2], Pasquale F. Innominato[3,4], Nicholas I. Wreglesworth[3,5], Boris Duchemann[1,6], René Adam[1,7] and Francis A. Lévi[1,8,9]✉

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BACKGROUND: Tolerability and antitumour efficacy of chemotherapy and radiation therapy can vary largely according to their time of administration along the 24-h time scale, due to the moderation of their molecular and cellular mechanisms by circadian rhythms. Recent clinical data have highlighted a striking role of dosing time for cancer immunotherapy, thus calling for a critical evaluation.

METHODS: Here, we review the clinical data and we analyse the mechanisms through which circadian rhythms can influence outcomes on ICI therapies. We examine how circadian rhythm disorders can affect tumour immune microenvironment, as a main mechanism linking the circadian clock to the 24-h cycles in ICIs antitumour efficacy.

RESULTS: Real-life data from 18 retrospective studies have revealed that early time-of-day (ToD) infusion of immune checkpoint inhibitors (ICIs) could enhance progression-free and/or overall survival up to fourfold compared to late ToD dosing. The studies involved a total of 3250 patients with metastatic melanoma, lung, kidney, bladder, oesophageal, stomach or liver cancer from 9 countries. Such large and consistent differences in ToD effects on outcomes could only result from a previously ignored robust chronobiological mechanism. The circadian timing system coordinates cellular, tissue and whole-body physiology along the 24-h timescale. Circadian rhythms are generated at the cellular level by a molecular clock system that involves 15 specific clock genes. The disruption of circadian rhythms can trigger or accelerate carcinogenesis, and contribute to cancer treatment failure, possibly through tumour immune evasion resulting from immunosuppressive tumour microenvironment.

CONCLUSIONS AND PERSPECTIVE: Such emerging understanding of circadian rhythms regulation of antitumour immunity now calls for randomised clinical trials of ICIs timing to establish recommendations for personalised chrono-immunotherapies with current and forthcoming drugs.

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INTRODUCTION

Cancer immunotherapy has developed with ups and downs since the late 1800s [1], with clinical trials mainly starting in the 1970s, and involving first live or inactivated microorganisms, then polyclonal or monoclonal antibodies, interferons, and interleukins. A leap forward in efficacy was achieved with the demonstration of unprecedented antitumor efficacy using immune-checkpoint inhibitors (ICIs) in chemo-refractory tumors [2]. ICIs are humanized monoclonal antibodies that target inhibitory receptors programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte antigen 4 (CTLA4) and programmed death-ligand 1 (PD-L1). PD-1 receptor is mainly expressed in activated T-cells, B-cells, natural killer cells, monocytes, and mesenchymal stem cells [3]. PD-L1 is expressed by many types of tumor cells and also constitutively expressed on dendritic cells, macrophages, mesenchymal stem cells, and bone marrow-derived mast cells [4, 5]. CTLA-4 (CD152) is predominantly expressed by T cells [6].

PD-1 is targeted by nivolumab, pembrolizumab, dostarlimab, sintilimab and cemiplimab, CTLA4 by ipilimumab and tremelimumab, and PD-L1 by atezolizumab, durvalumab, and avelumab. Receptor blockade by the specific antibody elicits an anti-tumor response, a process mostly orchestrated by cytotoxic T-cells [7, 8]. Specifically, antigen-presenting cells, e.g., dendritic cells, capture tumor antigens in the primary tumor tissues, then migrate to the tumor-draining lymph nodes, where T-cells are subsequently primed. Ultimately,

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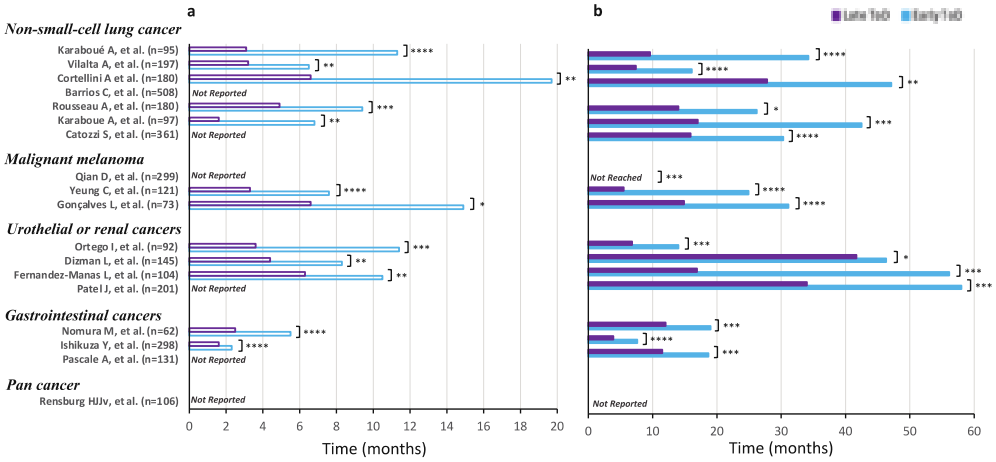

Fig. 1 Bar graph of efficacy endpoints of immune checkpoint inhibitors in compared timing groups from 18 studies involving a total of 3250 patients with different cancer types. a Median progression-free survival data. **b** Median overall survival data. Violet bars correspond to late time-of-day ICI dosing. Blue bars correspond to early time-of-day of ICI administration. The main characteristics of these studies are summarized in Table S1. Note: Qian et al. study results not shown despite statistically significant differences in overall survival in favor of the early time-of-day group, because median values were not reached Reported p values from timing groups comparisons of PFS or survival curves: *$P > 0.20$; ** $0.05 < p \leq 0.20$; *** $0.01 < p \leq 0.05$; ****$0.001 < p \leq 0.01$.

primed T-cells infiltrate into the primary tumor sites and mediate tumor eradication.

These scientific advances led to the award of the 2018 Nobel Prize in Physiology or Medicine to Tasuku Honjo and James Allison for their discoveries in cancer immunology. Professor Honjo was awarded due to his discovery of the programmed cell death molecule-1 (PD-1) on T cells. Professor Allison discovered another important immunosuppressive molecule, i.e the cytotoxic T-lymphocyte antigen-4 (CTLA-4) [9]. However, clinical evidence has demonstrated heterogeneous responses in patients receiving ICIs, and primary or secondary therapy resistance is common [10]. Such between-patients differences in ICIs efficacy could result from chronopharmacologic effects, that would make immune cells and cancer cells respond differently to immunotherapy as a function of its administration timing along the 24 h time scale. Here, we first summarize the main results from fourteen retrospective studies, which have reported large and consistent differences in efficacy outcomes as a function of the Time of Day (ToD) of ICI infusions over the past 2 years. We then outline the physiologic and molecular mechanisms at work within the circadian timing system (CTS), which rhythmically regulates immune cell functions and trafficking, besides cellular metabolism and proliferation over the 24-h. Thus, circadian time dependencies characterize both the tolerability and efficacy of chemotherapeutic and immunotherapeutic agents, thus supporting their optimization according to circadian rhythms, so-called chronotherapy [11, 12], as well as its personalization based upon circadian biomarkers metrics.

IMPROVED ICI'S EFFICACY THROUGH EARLY TIME OF DAY INFUSIONS

In seminal clinical observations, one of us (AK) first noticed a strikingly longer follow-up for those patients who received most nivolumab infusions in the morning as compared to those treated mostly in the afternoon for metastatic non-small cell lung cancer (mNSCLC) [13]. The results suggested a possible role for the CTS on immunotherapy effects, similar to that found for chronomodulated chemotherapy [14–16]. Seventeen of eighteen retrospective studies have now reported prolonged progression-free and/or OS through early ToD of ICI infusions in five different cancer types (Fig. 1, Table S1).

ToD of immunotherapy for metastatic non-small-cell lung cancer patients

In 95 mNSCLC patients on second-line nivolumab, both PFS and OS were 3 to 4 times as large in those receiving the majority of nivolumab infusions before 12:54, i.e. the median value of the per-patient median timing of infusions, with respective Hazard Ratios of an earlier progression or an earlier death of 0.258 [95% CL, 0.115–0.580] and 0.174 [0.082–0.370] [11]. Predominantly morning administration of nivolumab was significantly more effective irrespective of performance status and tumor PD-L1 expression, as shown in multivariable analyses (PFS, $p = 0.001$; OS, $p < 0.001$). The larger the proportion of infusions given before 12:54, the better the PFS and OS outcomes. Thus, median OS was 34.2 months for those patients receiving at least 67% of ICIS infusion before 12:54, 12.4 months for those given at least 67% of ICI infusions after 12:54 and 15.3 months for those receiving at least 33% of ICIs before and 33% after 12:54 ($p = 0.023$).

Two studies investigated whether ToD of infusions influenced the efficacy of single-agent pembrolizumab as 1st or 2nd line in 180 mNSCLC patients each [17, 18]. The early ToD group comprised patients who received <20% infusions after 16:30 whilst the late ToD group involved patients who received >20% infusions after 16:30, according to the initial methodology proposed by Qian et al. [12]. As compared to the late ToD group, the early ToD groups displayed longer PFS in both studies, i.e. 19.7 vs 6.6 months, Log Rank, $p = 0.056$ [17], and 9.4 vs 4.9 months, $p = 0.020$ [18]. Median OS was also largely prolonged in the early vs late ToD group, yet not significantly so (Fig. 1). The relevance of the ToD of the initial 4 ICI infusions was shown in a study involving 197 mNSCLC [19], using 12:00 noon as a time cut-off. Their early ToD group consisted of patients receiving at least one of the initial

4 ICI infusions before noon, whilst their late ToD group involved those receiving all initial four infusions after 12:00 noon. Median PFS was 6.5 months in the early ToD group and 3.2 months in the late group ($p = 0.066$). Median OS were 16.1 and 7.4 months, respectively ($p = 0.003$). In a multivariate analysis, HR was 0.42 [0.27–0.63] ($p < 0.001$) for PFS and 0.54 [0.35–0.84] ($p = 0.007$) for OS.

The critical relevance of early ToD of infusions over the initial 4 treatment courses was confirmed in 97 mNSCLC patients receiving 1st line pembrolizumab as a single agent ($N = 41$) or combined with chemotherapy ($N = 56$) [20]. Thus OS was significantly prolonged through the administration of three or four of the 4 initial pembrolizumab infusions before 11:45 (early ToD group) as compared to one or two (late ToD). Thus, respective 2-year survival rates were 65% and 38% ($p = 0.010$). Multivariable analysis confirmed that the delivery of a majority of the four initial pembrolizumab-based courses in the morning was an independent predictor of a longer OS (HR = 0.28 [0.13–0.64], $p = 0.003$).

In a cohort of 361 patients with metastatic NSCLC, Catozzi et al. also found that early ToD infusions of pembrolizumab, nivolumab, atezolizumab, durvalumab, or avelumab significantly prolonged overall survival. They identified 11:37 as the best discriminant cut-off time. As a result, median overall survival times were 30.3 months for the patients whose majority of ICI infusions occurred before 11:37as compared to 15.9 months for those treated later in the day ($p = 0.0024$) [21].

All the above studies also showed that the early ToD groups received significantly more ICI infusions as compared to the late ToD groups since efficacy was best on early ToD treatment. Such an explanation is consistent with all the results where an ICI-based treatment arm proved more effective in randomized trials. Thus ICIs withdrawal is mostly related to disease progression [22]. As a result, time to treatment failure was also found to be longer following early vs late ToD administration of nivolumab, pembrolizumab or atezolizumab in 129 mNSCLC patients, with respective median times of 14 months (CI 95%, 8.9-23.4) vs 4.9 months (2.8-13.5) [22]. There were not enough events for any comparative survival assessment at the time of the report.

ToD of immunotherapy for metastatic malignant melanoma patients

In 299 patients with stage IV malignant melanoma in the MEMOIR Cohort Study, Qian et al. [12] first reported that more frequent early ToD dosing of nivolumab, pembrolizumab and/or ipilimumab nearly doubled OS as compared with late ToD administrations. The early or late ToD groups involved the patients who had received less than 20% of ICIs infusions before 16:30 or 20% or more infusions after 16:30, respectively. In a propensity score-matched (PSM) analysis of 146 patients, the relative risks of earlier disease progression or death were nearly twice as low in the early vs late ToD group. Median OS was 4·8 years [3·9–not estimable] for the late ToD group, and was not reached for the early ToD group (HR = 2·04 [1·04–4·00]; $p = 0·038$). ICI timing was an independent prognostic factor for OS in multivariable analyses and remained unaltered using different types of ICIs, or prior corticosteroids or brain radiotherapy.

The above ToD effects were confirmed in a cohort of 121 patients with advanced melanoma [23]. The patients receiving all initial four infusions in the afternoon (after 12:00– noon) displayed worse PFS (3.3 vs 7.6 months; $p = 0.009$) and worse OS (5.5 vs 24.9 months; $p < 0.001$) compared to those given at least one of the initial four infusions in the morning. The results were confirmed using multivariable analyses.

Another retrospective study involving 73 patients with stage IV melanoma further showed that those patients receiving more than 75% of infusions of nivolumab, pembrolizumab, or nivolumab + ipilimumab in the afternoon had a shorter median survival as compared with those receiving more morning infusions (14.9 vs. 38.1 months; HR = 0.45 [0.23–0.86]; $p < 0.01$) [24].

ToD of immunotherapy for metastatic urothelial or renal cancers

A significant association between early ToD of ICI infusions and improved patient outcomes has been reported for metastatic urothelial or renal cancers in four studies.

In 92 patients receiving single-agent ICIs for metastatic urothelial cancer in Spain or Italy, Ortega et al. [25] showed that those receiving fewer than 20% ICI infusions before 16:30, had longer PFS and OS, compared to those receiving 20% or more ICI infusions after 16:30. Median PFS ranged from 11.4 to 3.6 months (HR = 2.66 [1.53–4.63] ($p = 0.001$) and median OS varying from 14.0 to 6.8 months (HR = 2.62 [1.48–4.63] ($p = 0.001$). Early ToD of infusions also improved tumor response rate (59.3% vs 16.0%). In 201 patients receiving pembrolizumab (8% of the patients), nivolumab (61%), or dual nivolumab/ipilimumab (31%) for stage IV renal cell carcinoma (RCC), Patel et al. [26] observed that the patients receiving 25% or more of their ICI infusions in the morning (before 13:00) had significantly longer OS compared to those receiving more than 25% of infusions after 13:00. Median OS were 58 and 34 months, respectively (HR = 0.51, [0.29–0.89] ($p = 0.017$). Early ToD was prognostic of longer survival, independently of age, sex, tumor histology, liver or brain metastases, pre-treatment LDH, and initial ICI. Similar results were found in a study involving 56 patients receiving anti-PD-1/PDL-1 as 1st or 2nd line treatment for metastatic RCC [27]. Those patients receiving 20% or more ICI infusions after 16:30 experienced worse OS (HR = 3.1 [1.27–7.36] ($p = 0.01$), shorter time on treatment (HR = 2.5 [1.21–4.99] ($p = 0.013$) and shorter time to next treatment line (HR = 1.9, [0.96–3.71] ($p = 0.067$) as compared to those who received less than 20% of the infusions after 16:30. In 145 patients treated with nivolumab alone, or in combination with ipilimumab as first- or second-line treatment for metastatic RCC, Dizman et al. [28] showed consistent trends towards improved response rate, prolonged time-to-treatment failure, and OS among those patients receiving less than 25% of ICI infusions after 16:30.

ToD of immunotherapy for metastatic gastrointestinal cancers

In 62 patients treated with nivolumab for recurrent or metastatic squamous cell carcinoma of the esophagus, significantly superior response rate, PFS, and OS resulted from the administration of the first infusion of nivolumab before 13:00 compared to its administration after 13:00 [29]. Response rate and PFS were also significantly larger in those patients who received the majority of infusions before 13:00 during the first 3 months of treatment. In contrast, no significant differences were found in response rate, PFS, and OS as a function of whether the majority of all nivolumab infusions was given before or after 13:00.

In 248 patients receiving nivolumab monotherapy for metastatic gastric cancer, Ishizuka et al. observed significant difference in response rate, PFS and OS according to whether patients received more or fewer than 70% of ICI infusions before 14:00. The respective efficacy data were: ORR, 16.9% vs 3.3%, ($P = 0.01$); PFS, 2.3 vs 1.6 months (HR = 0.65; $P < 0.01$); OS, 7.6 vs 3.9 months, (HR = 0.64; $P < 0.01$) [30].

In 131 patients receiving atezolizumab ± bevacizumab as first-line treatment for advanced hepatocellular carcinoma, Pascale et al. showed that the administration of the first two treatment courses after 13:00 was associated with a significant reduction of median OS as compared to the administration of at least one of the two first courses before 13:00 (11.5 months vs 18.7 months), $p = 0.015$). This ToD-related difference in OS outcome was supported by multivariate analysis [31].

A meta-analysis evaluated the consistency of ToD-related efficacy findings, using published data from 13 studies involving 1663 patients with advanced non-small-cell lung cancer (47%), renal cell carcinoma (24%), melanoma (20%), urothelial cancer (5%), or esophageal carcinoma (4%). The patients had received anti-programmed cell death protein 1, or anti-programmed

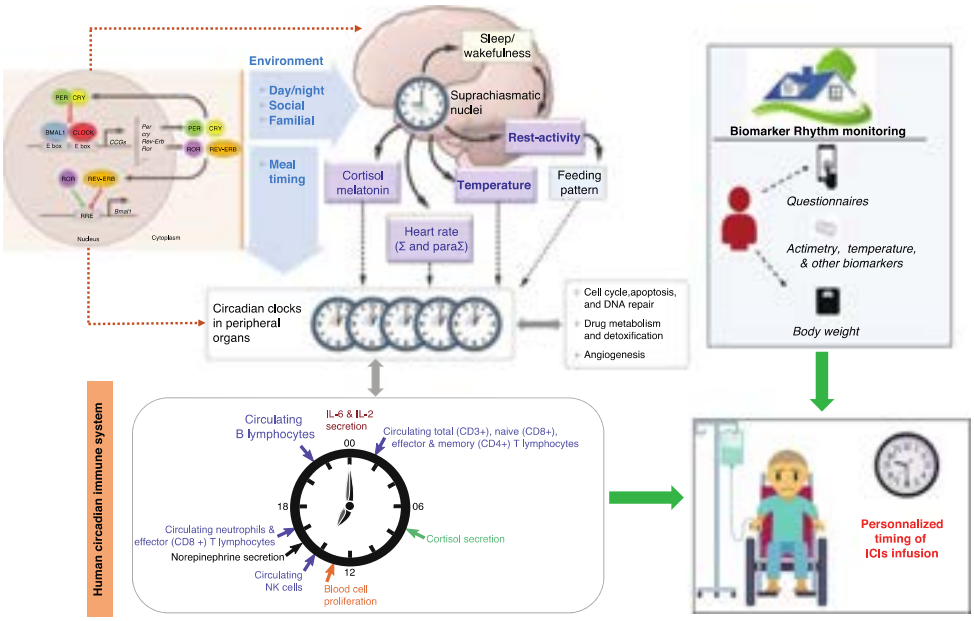

Fig. 2 Interactions between the circadian timing and the immune systems. Σ and paraΣ = sympathetic and parasympathetic systems.

death-ligand 1 (98%) and/or anti-cytotoxic T-lymphocyte- associated protein 4 (anti-CTLA-4) (18%). The early ToD groups had nearly twice as long OS and PFS, as compared to late ToD-treated patients. Respective overall HRs were 0.50 [95% CI, 0.42–0.58]; $P < 0.00001$) for OS, and 0.51 [0.42–0.61]; $P < 0.00001$) for PFS [32].

A single study involving 106 patients with more than six different types of solid tumours found no difference in PFS or OS between early and late ToD infusion of pembrolizumab. No role was found for cut-off points at 12:00 noon or at 15:06 (median) or at 16:30, nor for the proportion of cycles given after this cut-off point (20% or 50%). The authors ascribed this negative result to the large heterogeneity of their patient population [33].

Thus, 17 of the 18 above study reports, involving a total of 3144 metastatic cancer patients from nine countries in 4 continents, have consistently indicated improved patient outcomes following predominant early ToD administrations of ICIs (Fig. 1a, b). However, the definition of the threshold for early ToD varies from 11:37 to 16:30, whilst the definition of the proportion of late ToD infusions associated with poor ICIs efficacy ranges from 20% to 75%.. Four studies further highlighted the critical relevance of early ToD over the initial 2-months of ICI treatment. Thus, there is a clear need both for improving precision regarding the determination of optimal timing and for assessing the current evidence for the circadian mechanisms at work for cancer chronotherapy.

FROM CIRCADIAN RHYTHMS TO CANCER CHRONOTHERAPY

The circadian timing system

Circadian rhythms consist of endogenous biological oscillations with a period of about 24 h, which characterize most physiological parameters, thus contributing to well-being and health [34–40]. In mammals, circadian rhythms regulate sleep-wakefulness, rest-activity, appetite, muscular and cognitive performance, body temperature, hormonal secretions, cellular metabolism, proliferation, and death, as well as immune cells production, functions and trafficking [41]. Circadian rhythms display precise 24-h periods, following their synchronization with 24-h environmental cycles, and especially the regular alternation of light and darkness over the 24 h [38, 42]. Other environmental cues that influence circadian rhythms include timed physical activity and rest, socio-professional and familial interactions, and feeding patterns [43–46]. The circadian coordination and adjustment to environmental cues mostly operate through the suprachiasmatic nuclei (SCN), a hypothalamic pacemaker located above the optic chiasm [47]. Most circadian rhythms are suppressed following SCN physical ablation or functional alteration, through chronic jet lag for instance [48–51]. SCN-dependent rhythmic signals coordinate genetic molecular clocks that reside within each mammalian cell, resulting in a hierarchical clocks network that constitutes the circadian timing system (CTS) [38, 52]. Indeed, mammalian cells are endowed with a molecular clock involving 15 clock genes (NPAS2, PER2, DBP, ARNTL, PER3, NR1D1, CRY1, NR1D2, CLOCK, CRY2, BHLHE40, PER1, BHLHE41, RORA, and TIMELESS), that interact with each other through three transcription/post-transcription regulatory loops, thus generating circadian oscillations in individual mammalian cells. The BMAL1::CLOCK (or NPAS2) protein dimers, in particular, play a key role in the molecular clock through the activation of the transcription of clock genes *Per's*, *Cry's and Rev-erb's* [44, 53] (Fig. 2). Dynamic single-cell reporter studies have revealed the tight coupling between the molecular circadian clock and the cell cycle, in individual non-malignant cells, whilst such coupling might be altered in individual cancer cells [54]. In healthy mouse and/or human tissues, cell cycle events, DNA repair, apoptosis and autophagy are controlled by the circadian clock [55]. Experimental studies have further highlighted the essential roles of tissue-specific regulation and intercellular signaling in clock synchronization.

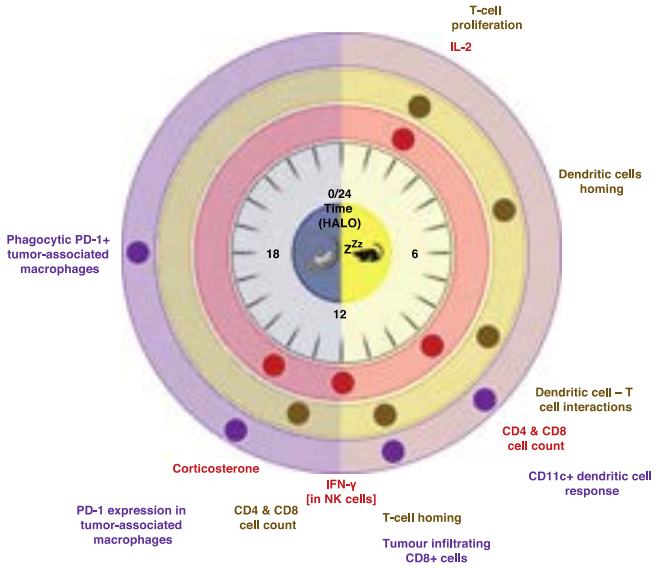

Fig. 3 Schematic polar representation of the distribution of peak times of selected rhythmic immune functions involved in antitumor immunity in rodent models over 24 h. Peak time data are shown in three key compartments, i.e. bloodstream [red], lymph nodes [green], and tumor immune microenvironment [purple]). The circle in the center depicts the light-dark synchronization schedule of nocturnally active mice or rats. HALO Hours After Light Onset.

Chronopharmacology and chronotherapy

Comprehensive reviews have summarized the evidence of circadian time-dependencies in drug effects, i.e. chronopharmacology, and the main mechanisms that determine chronopharmacokinetics and chronopharmacodynamics [38, 44, 56–59]. Drug exposure is moderated by the circadian regulation of Absorption, Distribution, Metabolism, Elimination and Toxicities (ADMET) mechanisms at molecular, tissue and organ levels. Circadian rhythms characterize most bioactivation, detoxification and elimination processes at the transcription, protein and enzymatic levels in the liver, the chief drug-metabolizing organ, as well as in the intestine, kidney, lung, etc [60]. Recently, sex dimorphism has emerged for circadian drug responses and their molecular mechanisms, thus supporting sex-specificities in optimal circadian-based treatments, i.e. chronotherapy [61, 62].

Circadian rhythms in cancer biology

The occurrence of circadian patterns in clock gene expressions has been shown for several but not all rodent or human cancer cell lines in synchronized cell culture studies [63–66]. Large between-tumor variations characterize both the extent of clock gene expressions and their circadian patterns in cancer tissues from rodents or humans. A metric for molecular circadian clock functionality and timing has recently been developed using Time Teller, an artificial intelligence algorithm, that proved able to model the molecular clock from a single tissue biopsy transcriptome or RNAseq [67]. Time Teller application to human oral mucosa and breast cancer omics data reveal a molecular clock disruption in nearly half of the patients' tumors. TimeTeller has further revealed that the survival of 209 patients with primary breast cancer treated with neoadjuvant chemotherapy was significantly less when circadian molecular clocks in cancer tissue were functional, probably due to the reduced effectiveness of chemotherapy on cancer cells with functional clocks [67]. Interestingly, the expressions of common oncogenic drivers or inhibitors such as RAS, c-MYC, NOTCH and TP53 are rhythmically controlled by the circadian clock, and also interact with it, thus supporting an overarching regulatory role of the circadian clock in cancer processes and precision cancer treatments.

Other algorithms that estimate molecular circadian clock functionality and timing have been developed such as Time-Signature [68], ZeitZeiger [69], and CYCLOPS [70]. However, the key point differentiating TimeTeller from the other algorithms is that, apart from identifying timing deviations, these do not provide any other assessments of clock functionality or other quality controls on the individual timing assessments [67].

Cancer chronotherapy

Cancer chronotherapy aims at the delivery of anticancer agents at a time (on the 24-h scale) when efficacy can be maximized and/or toxicity can be minimized. As a result, the circadian dosing time can improve or increase the extent of treatment toxicities by up to fivefold, as shown for more than 50 agents in experimental models, and 12 of them in cancer patients [62]. Most importantly, the dosing time associated with least toxicity has achieved similar or improved efficacy both in rodents and in patients [71, 72]. Clinical trials have been conducted from the evidence provided by experimental studies in nocturnal rodents, and more recently in synchronized cell cultures as well [38, 56, 65]. Overall, the clinical relevance of daily timing has been investigated for anticancer agents in human colorectal, lung, breast, pancreas, kidney, bladder, endometrium, ovary, head and neck or hematologic cancers [62]. The discovery of oxaliplatin efficacy in colorectal cancers resulted from its chronopharmacologic administration, from Phase I to large international Phase III trials [73–75]. An individual patient-level meta-analysis involving data from 345 females and 497 males with metastatic colorectal cancer revealed that males lived significantly longer on chronomodulated

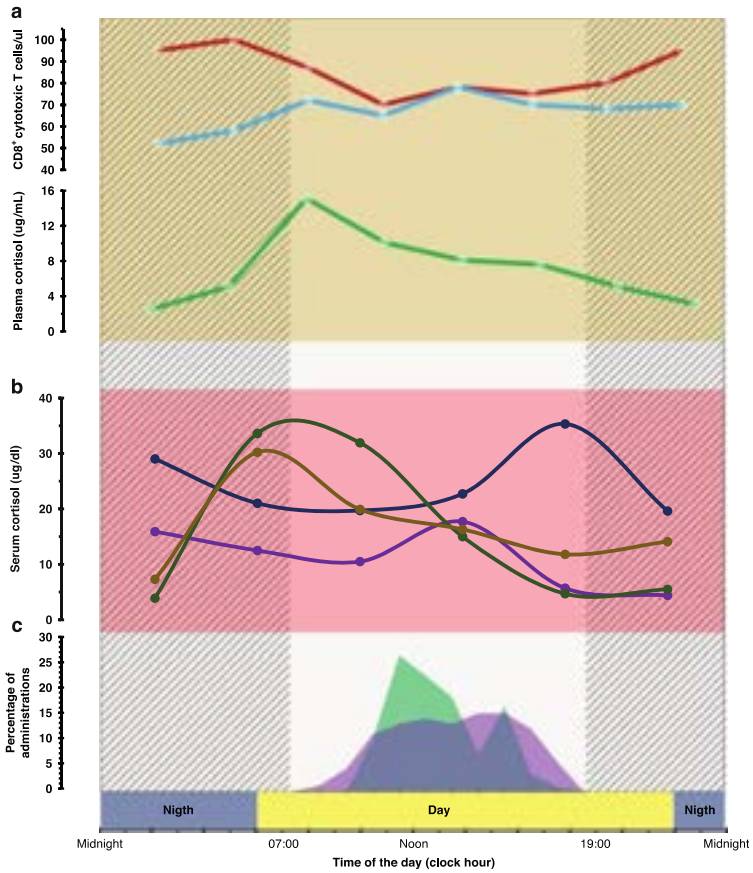

Fig. 4 Spectrum in peak times of selected human circulating immunological factors with a proven 24-h rhythm relevant to cancer immunotherapy. a Healthy (male) subjects. CD8 cytotoxic Central memory [red], effector [blue], plasma cortisol [green]. **b** Salivary cortisol in four individual patients with advanced cancer. **c** Distribution of daily timings of ICI administration in two studies: Karaboué et al. Cancers [11] [green] and Qian et al. Lancet Oncol. [12] [purple].

oxaliplatin-5-fluouracil-leucovorin rather than on conventional chemotherapy, whilst the opposite was observed for women [76]. Further experimental and clinical data support an about 6-h delay in the optimal timing of oxaliplatin, 5-fluorouracil and irinotecan, both in mice and in cancer patients [62]. Altogether, a recent systematic review involving 18 randomized, controlled studies including a total of 2547 patients concluded that most studies provided evidence of the reduction of toxicity resulting from chronomodulated chemotherapy, while efficacy was maintained or improved [77].

MECHANISMS AT WORK FOR CANCER CHRONOIMMUNOTHERAPY

The circadian immune system

Oscillating molecular circadian clocks have been identified in all the cells that participate in immune system functions [78–80]. Immune cells express circadian clock genes and display 24-h mRNA and/or protein rhythms for a wide array of functional genes. As a result, circadian rhythms characterize (i) the synthesis and release of cytokines, chemokines and cytolytic factors, (ii) the daily gating of the immune response occurring through pattern recognition receptors, (iii) phagocytosis, (iv) migration to inflamed or infected tissue and immune cells trafficking, (v) cytolytic activity, and (vi) proliferative response to antigens [81]. Consequently, alterations of circadian rhythms can lead to profound disturbances in immune responses.

In experimental models, the encounter of an immune challenge given during the middle of the light span, i.e. at Zeitgeber 7 (ZT7) 7 h after light onset, when mice are resting, has been associated with an enhancement of immune responses compared to exposure in the middle of the night (ZT19) when mice are most active [41, 82, 83]. Thus, an immune stimulus caused more dendritic cells to migrate faster from the skin to the lymph nodes following its application at daytime, as compared to night-time. The number of T-cells in lymph nodes was higher during the day, and T-cell movement from blood to lymph nodes was controlled by the circadian expression of the homing molecule Intercellular

Adhesion Molecule 1 (ICAM-1) [82]. Mathematical modeling revealed that the presence of dendritic cells and T-cells in the lymph node at daytime, during the rest span of mice, increased the probability of immune interactions between antigen-presenting dendritic cells and antigen-recognizing T-cells [78]. Circadian rhythms also moderated the proliferation of T-cells in the lymph nodes at a later stage of an adaptive immune response. One week after an immune stimulus consisting of OVA peptide-loaded dendritic cells, T-cells proliferated more at daytime compared to nighttime, with largest daytime expression of proteins involved in immune regulation [84]. These rhythmic patterns were suppressed in mice lacking clock gene Bmal1 expression in T-cells [85] or in those with constitutive CLOCK gene mutation [84].

The timing when the immune challenge was given also affected the later stages of the adaptative response. The B-cells from mice vaccinated in the daytime produced more antibodies at 14 days and more antigen-specific antibodies at 28 days, compared to mice vaccinated at night [86, 87]. Day-vaccinated mice also had a stronger virus-specific T-cell response at 28 days. This effect was not seen in mice lacking Bmal1 in T-cells [86, 87].

Experimental results combined with mathematical modeling, revealed that the oscillating nature of one stage of the immune response, such as T-cell trafficking, feeds into and is necessary for the next stage such as T-cell proliferation, which is also rhythmic. This mechanism enables the immune response to remain rhythmic for several weeks after an initial challenge [87]. Figure 3 provides an overview of the peak times of relevant cellular, humoral and molecular immune parameters in the circulating blood, the draining lymph nodes and the tumour immune microenvironment in nocturnal rodent models [87–91].

In healthy humans, circadian variations with large amplitudes characterize the circulating counts of most blood cells, including total lymphocytes and their subsets. Total lymphocytes and mononuclear cell counts reach a low point in the morning hours, i.e. between 08:00 and 10:00 then gradually increase with a daily maximum between midnight and 02:00 at night [92]. All subpopulations of interest of T($CD4^+$) and T($CD8^+$) cells including naive, central memory, effector memory, and effector T cells display distinct and significant circadian rhythms in absolute cell counts, except for effector T(CD4) cells. Most lymphocyte subset counts nearly double over the 24-h time scale. Acrophases also clump in the early night span, thus ranging from 01:31 to 02:41 for naive, central memory, and effector memory T(CD4) and T(CD8) cells. In contrast, circulating counts in both effector T(CD8) cells and natural killer cells peak in the morning or early afternoon hours [83]. Immune cell trafficking and functions are regulated in part by hormonal secretions. For instance, cortisol [93] is rhythmically secreted by the adrenal cortex in the early morning hours with reduced levels at night. Cortisol, increases the expression of CXCR4 to mediate bone marrow homing with a 3-h delay, thereby negatively regulating the counts in naive and memory T-cells [83]. Epinephrine is secreted by the adrenal medulla primarily in the afternoon hours [94]. It recruits natural killer cells and effector T cells from the marginal pool to the circulation by increasing the expression of β-adrenergic receptors and the chemokine receptor CX3CR1 and reducing the expression of adhesion molecules [83]. Adrenergic signaling also regulates the expression of vascular cell adhesion molecule-1 [95] in the bone marrow microenvironment to induce homing of leucocytes to the bone marrow and in muscles late in the activity phase [95]. Hence, trafficking to peripheral sites occurs simultaneously, thereby resulting in anti-phasic abundance in the bloodstream.

Circadian rhythms regulate macrophage-mediated immune responses through time-of-day-dependent regulation of macrophage function [96]. Circadian disruption induced the loss or inversion of daily patterns of M1 (proinflammatory) and M2 (anti-inflammatory) macrophages in the spleen [97]. The immune time-of-day variation has also been shown in the magnitude of the response to vaccination. Thus, preclinical and clinical studies have shown that a weaker adaptive immune response is induced when vaccinations are given in the evening than when given earlier in the day [98–102]. Figure 4 critically summarises a snapshot of pertinent available data on the 24-h rhythms in immune functions in peripheral blood demonstrated in humans using repeated measures. These results support strategies for optimizing therapeutic options through treatment timing of administration in a personalized and precise fashion.

Experimental cancer chronoimmunopharmacotherapy

Improved treatment responses have resulted from circadian-timed delivery of interleukin-2 and interferons in laboratory rodents. In B16 melanoma-bearing mice, maximum antitumor activity was achieved following dosing at ZT4, during the early stage of the diurnal rest span of mice, for recombinant human interferon (IFN)α A/D and at ZT16 for recombinant murine IFN-γ, i.e. shortly before the middle of the nocturnal activity span [103]. The mRNA expression of IFN receptor was rhythmic in tumor cells, and peaked synchronously with the proportion of S-phase cells, thus supporting a link between the cell cycle and immunologic tumor responses potential. Highest interferon receptor expression occurred near the middle of the rest phase of mice [104].

The administration of IFN-α or IFN-γ disrupted circadian gene Per2 mRNA expression, both in the SCN and in peripheral organs, as well as the circadian rhythm in rest-activity and body temperature, following daily dosing at ZT12 but not at ZT0. The study further revealed the ability of IFNs to disrupt both the central circadian pacemaker and peripheral clocks following dosing around the beginning of the activity span (ZT12) [104]. The circadian disruption potential of IFN was further shown through the continuous administration of IFN-α to mice using a subcutaneous implanted pump. Constant rate IFN-α infusion decreased the rhythm amplitude of locomotor activity, body temperature, leukocyte counts, and plasma corticosterone levels and suppressed the oscillation in the expression of clock genes in the liver compared to repeat daily administrations at ZT2 [105]. The chronopharmacology of interleukin-2 (IL-2) was shown in tumor-bearing rats, where constant rate infusions of IL-2 induced a 37.5% mortality rate and a 25% objective tumor response rate, whereas animals receiving a "day cycle" of IL-2 had no mortality and a 100% objective response rate [106]. Clinical Phase 1 and 2 studies have shown good tolerance despite a significant increase in dose intensity with a circadian infusion schedule compared to standard or flat continuous infusion schedules of recombinant alpha-interferon-2b in melanoma or renal cell cancer patients [107, 108]. IL-2 chronotherapy also proved to be safe, moderately toxic and active in metastatic RCC patients [109].

Circadian rhythms in key mechanisms of ICI's efficacy were recently demonstrated in mice. Circadian *Pdcd-1* mRNA expression was found in tumor-associated macrophage (TAMs), whilst PD-1-positive TAMs count also displayed a significant circadian rhythm in B16/BL6 melanoma transplanted into C57BL/6J mice [88]. *Pdcd-1* was further characterized as a clock-controlled gene [88]. In this mouse model, BMS-1, a small molecule inhibitor of PD-1/PD-L1, was significantly more effective following its administration at ZT18, near the middle of the activity span, in comparison with ZT6. Both circadian times, respectively correspond to the rise and decrease in PD-1 expression on TAMs. Although a two-timepoints comparison cannot identify the optimal time of administration of BMS-1, the findings suggest that the circadian expression of PD-1 on TAMs, but not in circulating monocytes, could help select the most appropriate time of day to administer PD-1/PD-L1 inhibitors. Wang et al. [89] further demonstrated that the anti-tumor immune responses were ToD-dependent. Fourteen days after the subcutaneous engraftment of a fixed melanoma cells load, tumor volumes were significantly larger in those mice engrafted ZT21

(in the mid-to-late activity span of mice) as compared to ZT9 (in the mid-to-late rest span). This difference was abrogated in mice lacking both adaptive and innate immune cells. Interestingly, T (CD8) cells, the main target cells of ICIs were significantly more numerous in the tumors at ZT9 compared to ZT21. This difference was abrogated in mice whose Bmal1 expression was specifically suppressed in dendritic cells. Thus both above reports identify a 9-h long time span, ranging from ZT9 to ZT18, within which ICIs might be most effective. Further studies are yet mandatory to identify the optimal circadian times of ICIs, and the circadian biomarkers that will help the personalization of the chronoimmunotherapy schedules.

Chronopharmacokinetics

In humans, ICIs have an elimination half-life that ranges between 14 and 27 days, and a mean clearance of about 0.36 L/day [18, 110, 111]. As a result, the steady state is usually reached after 2–4 months [112]. Additionally, it takes 32.4 weeks to reduce T-cell PD-1 occupancy by 50%, after nivolumab discontinuation [113]. These data reveal that ICIs timing might only moderate their pharmacokinetics over the initial 2–4 months of treatment, i.e. before the reach of the steady state of ICIs blood concentrations. Furthermore, the pharmacokinetics of monoclonal antibodies have peculiar aspects and are influenced by target-mediated drug disposition and drug clearance [114], which vary over time [115, 116]. Preclinical studies have shown that pembrolizumab biodistribution depends on PD-1-mediated uptake into lymphoid organs, which oscillates with a circadian rhythm. Thus, there might be circadian changes in the clearance of monoclonal antibodies from tissues with a crucial impact on T-cell antitumor function [117].

These observations call for careful investigations of ICI chronopharmacokinetics, as potential mechanisms for the dosing time dependencies in ICI efficacy over the initial treatment cycles.

CIRCADIAN BIOMARKERS TOWARDS PERSONALIZED CHRONOIMMUNOTHERAPY

The main physiological host biomarkers that inform CTS function and timing include the 24-h patterns in rest-activity, body temperature, and heart rate, as well as cortisol and melatonin secretions. These rhythms help coordinate peripheral clocks over the 24 h. They can be moderated by sex, age, as well as cancer type and stage [49, 118, 119]. In metastatic cancer patients, such circadian biomarkers either remain similar to those in healthy people, or be shifted, damped, or altogether suppressed. Predictable chronopharmacologic effects require well-coordinated and functional circadian clocks [38, 56]. Between-patient differences have been identified for numerous host circadian biomarkers [57, 120, 121]. Furthermore, robust, altered or suppressed clock gene expressions and circadian clock functionality have been found in the tumors from cancer patients [67, 122]. It can thus be hypothesized that the patients with rhythmic biomarkers could benefit the most from chrono-immunotherapy.

Circadian patterns in rest-activity

In mammals, the circadian rhythm in rest activity and that in body temperature are generated by the SCN [123], thus representing biomarkers of the coordination activity of the central circadian pacemaker [123]. In humans, the dampening of the circadian amplitude in rest-activity rhythm, measured with wrist actimetry for a week, has been associated with a significant increase in both the incidence of cancer and other diseases and all causes of mortality among the 92614 participants in the UK Biobank study [124]. In cancer patients, the most clinically relevant metric of the rest-activity rhythm is the dichotomy index (I < O), a measure of the percentage of in-bed activity counts that are less than the median of out-of-bed counts. Nearly half of 436 patients with metastatic colorectal cancer patients had robust rest-activity rhythm, with an I < O of 97.5% or more, i.e. similar to healthy subjects [125], whilst 25% had damped rhythms, and a further 25% had severe circadian disruption with an I < O below 96% [126]. Low I < O values consistently predicted for both poor PFS and OS in a pooled analysis involving these 436 patients [126], and for OS in an independent meta-analysis of 6 studies in a total of 659 cancer patient [127]. Large between patients differences have been reported for the rest-activity circadian pattern in several studies Involving patients with advanced or metastatic lung or esophagus cancer or melanoma, i.e. populations similar to those where early ToD ICIs improved outcomes. The clinical relevance of rest-activity circadian disruption has also been shown especially in lung cancer patients [128–131]. Walking exercise improved sleep quality, as assessed with the Pittsburgh Sleep Index questionnaire, and the rest-activity dichotomy index I < O, only in the lower I < O group, while no effect of the intervention was found in the good I < O group or in the whole population [132]. The restoration of a near-normal rest-activity rhythm with gefitinib was associated with decreased symptom severity and improved quality of life in a pilot study involving 10 NSCLC patients [133]. In such patients, rest-activity rhythm disturbances have been frequently associated with alterations in cortisol and melatonin secretions, core body temperature, and/or circulating immune cell subset counts [134].

Circadian patterns in cortisol or melatonin secretions

Disrupted circadian patterns have been identified for cortisol secretion in individual patients with advanced or metastatic breast or ovarian cancer [135]. Such disruption was further shown as an independent prognostic factor of survival in metastatic breast cancer patients [136], as well as for advanced or metastatic patients with NSCLC [137] or RCC [138], but not for those with metastatic colorectal cancer [139].

In NSCLC patients, Sephton et al. [137] observed a significant increase in early mortality among the 29 patients with flattened diurnal salivary cortisol slope compared with the 32 patients with steep slopes that suggested a near normal circadian pattern. She found that disrupted circadian cortisol profiles were associated with both low circulating total and cytotoxic T-cell lymphocyte counts and male sex. These data were further supported by the documentation of large between-patient differences in 24-h mean level, peak-trough difference and peak time of plasma cortisol in lung cancer patients [140]. In 161 patients with advanced lung cancer, diurnal salivary cortisol slope was identified as the most critical factor influencing the psychoneurological symptom cluster in multiple linear regression models after adjusting for physical performance status and number of comorbidities [141]. The diurnal slope of salivary cortisol also proved clinically relevant in 202 renal cell cancer patients [138]. Indeed, flatter cortisol slopes were significantly associated with decreased patient survival, a finding similar to that found for this endpoint in lung cancer patients [137].

In contrast, plasma melatonin 24-h patterns appeared quite similar, with a nocturnal peak occurring at 02:00 in each healthy subject, as well as in each early or late-stage lung cancer patient. The relation between the circadian patterns in salivary melatonin and cortisol was further investigated in a case control study involving 40 patients with newly-diagnosed lung cancer and 40 healthy adults [142]. The authors reported that the group of patients had lower salivary melatonin levels and higher salivary cortisol levels with flatter slopes for both hormonal concentrations The multivariate linear regression analysis indicated that the cortisol slope and fatigue score significantly predicted the sleep quality score.

Relations between immune functions and circadian host or tumor biomarkers

Scarce studies have examined the relationship between host circadian biomarkers and immune functions in patients with lung, kidney, bladder, or esophageal cancer or melanoma. Mazzocoli et al. [140] compared circadian rhythms in circulating Natural Killer (NK), T and B lymphocyte subsets in a group of nine NSCLC patients versus a group of 11 controls. They confirmed the well-known circadian rhythms in circulating immune cell counts in healthy controls, with maxima at daytime for T(CD8), NK-cell subset (CD8dim and CD16+), Vδ2TCR, and at night for T(CD3), T(CD4), and B cells (CD20). In a group of nine stage I–IV NSCLC patients, between-patient differences characterized the 24-h patterns in circulating T(CD3), T(CD8), and B cells. The circadian rhythmicity in circulating NK-cell counts and phagocyte activity were also disrupted in five patients with stage I–II malignant melanoma, compared to 12 healthy controls [143]. The most typical alterations were discoordination between the cytotoxicity rhythms of NK-cells and phagocytes.

The downregulation of clock gene expressions in some human cancers has been associated with altered circadian patterns in the tumor microenvironment, with a possible impact on survival or immunotherapy efficacy. In human malignant melanomas, as well as in human RCCs, the mRNA expression of most clock genes was reduced, whilst displaying disrupted circadian patterns indicative of molecular clock dysfunction, compared to corresponding nonmalignant tissue [144, 145]. Those few melanoma patients whose tumor was characterized with high BMAL1 mRNA expression had prolonged survival, possibly as a result of reduced key DNA-repair enzyme expressions, increased mutational/neoantigen load, and strong intratumoral T-cell infiltration and activation [144]. Moreover, the patients with high BMAL1 tumor expression also achieved significant clinical benefits from immune checkpoint inhibitors [144, 146]. In 11 primary RCC compared to matched healthy tissues, the mRNA expression of clock genes PER2, TIMELESS, and TIPIN was downregulated, whilst that of clock-controlled gene SERPINE1 was upregulated in the tumor cells [147]. In contrast, in the microenvironment of these tumors, the mRNA expression of clock genes BMAL1, REV-ERBα, PER1, PER2, and RORA was upregulated, whilst those of CLOCK, CRY1, and CRY2 declined [148].

The overexpression of PER 2/3, CRY2, and RORα (among other clock genes) in RCC tissues was correlated with longer OS in patients with RCC [149–151]. The survival improvement was associated with the extent of tumor infiltration with T(CD4) and T(CD8) lymphocytes [151], thus highlighting the important role of the interactions between the circadian and the immune systems.

NSCLC patients with high expression of TIMELESS or low expression of RORA, PER1, PER2, or CRY2 had a significantly worse survival prognosis [152]. TIMELESS and RORA are also significantly correlated with immune checkpoint and immune infiltration levels in NSCLC [152]. Clock-related genes were reportedly repressed in human esophageal squamous cell carcinoma samples [153, 154]. In spite of these alterations, PER2 oscillations still occurred in some human esophageal cancer cell lines [154] suggesting that the response to therapy might be enhanced by linking chronotherapy to PER2 expression pattern as a circadian biomarker of interest for personalized chronotherapy.

DISCUSSION

Here, we discuss how the circadian system can influence the efficacy of ICIs and how the use of circadian biomarkers can help optimize ICI-based treatments in cancer patients.

17 of 18 reported retrospective studies that have examined the influence of ToD administration of ICIs have concluded that predominantly morning or early afternoon infusions significantly improved PFS and/or OS compared with later ToD infusions. These results are consistent with those reported for different vaccines, whose efficacy was usually enhanced following morning (between 9:00 and 11:00) rather than afternoon between 15:00 and 17:00) administration [98, 100]. However, several questions remain unanswered.

When is the optimal circadian rather than daily time of best efficacy and tolerance of ICIs? Despite the consistent finding that early ToD is more effective compared to late ToD, none of the studies reported thus far, have addressed this question. Two main daily cut-off times have been used to differentiate early vs late ToD groups: 12:00–14:00 and 16:00–16:30. Morning dosing might be more advantageous compared to afternoon dosing [32]. Only one-third of the 24-h span has been explored in the 18 studies, thus, questioning whether optimal ICI timing could be located between 18:00 and 9:00. The number of initial ICI infusions to be administered in the morning toward improved efficacy needs to be determined. Thus, four studies highlighted the critical relevance of early ToD administration among the initial four courses. The half-life of ICIs ranges from 14 to 21 days. Thus the circulating steady state is usually reached after 2–4 months [112]. About 8 months are needed to decrease T-cell PD-1 occupancy by 50% after ICIs [113]. The proportion of late ToD courses associated with worse survival also needs to be specified, as it currently ranges from >20 to 67%.

Besides the circadian control of immune functions, other possible explanations have been hypothesized to account for the observed consistency in ICI timing effects.Thus, the ToD of ICI administration could represent a surrogate of socioeconomic status or performance status. Also, imbalances in the total number of ICI administrations between groups of patients treated early or late in the day could moderate treatment efficacy [17]. However, improved efficacy of immunotherapy in randomized trials has been consistently associated with more treatment courses, due to PFS prolongation. Yet, in order to eliminate potential hidden biases, randomized clinical trials are needed. Prospective comparisons should be made between different times of ICI administration to fully appraise the dosing time-related differences in ICI efficacy, which currently appear of the size of a very active new drug.

A possibly overlooked aspect of these studies is the potential impact of sleep, tightly linked to CTS function, on ICI efficacy and optimal ToD administration. Indeed, sleep and immunity are known to be bidirectionally linked, with relevant impact in disease response and health promotion [155]. For instance, in breast cancer patients, aberrant circadian cortisol rhythm was found to be associated with both sleep disruption and suppressed activity of NK cells, ultimately concurring to poorer overall survival [136, 156]. Modern wearable biosensors allow for unintrusive assessment of circadian and sleep cycles, whose alterations have also been associated with immune disruption in night workers [157, 158]. Thus, digital solutions are allegedly going to be paramount in optimization of ICI ToD administration.

Given both the impact of circadian function on outcomes on cytotoxic chemotherapy [62], and the potential for non-pharmacological modulation of ICI efficacy [159, 160], it stands to reason to harness circadian-based behavioural factors to maximize benefits from ICIs.

The available literature suggests that about one-third of the patients with advanced or metastatic cancer display circadian disruption, based upon the suppression or severe dampening of circadian rhythms in rest-activity, cortisol or melatonin secretions or body temperature. These rhythms are critical biomarkers of the circadian coordination system. However, macrophages and other immune cells are endowed with an autonomous molecular clock thus can display rhythmic function and proliferation in vitro, in the absence of endogenous rhythmic signals [161]. Preclinical studies have highlighted the ability of immunologic rhythms to persist despite the complete suppression of the rest-activity and body temperature rhythms [162], whilst altered yet present circadian

rhythms in circulating lymphocytes were identified both in tumor-bearing mice with suprachiasmatic nucleus ablation [48], and in cancer patients with suppressed rest-activity or cortisol rhythms [139]. These results highlight the need for specific studies aiming to determine the relations between circadian coordination biomarkers and immunologic rhythms in patients with various malignancies whose response to immunotherapy vary according to dosing time.

Sex-specific differences in the efficacy or tolerability of cancer chronochemotherapy have been reported, as a result of differences in circadian rhythms between male and female patients. An individual patient-level meta-analysis involving data from 345 females and 497 males with metastatic colorectal cancer revealed that males lived significantly longer on chronomodulated oxaliplatin-5-fluouracil-leucovorin compared to conventional chemotherapy. The opposite was observed for women [76]. Experimental and clinical data support an about 6-h delay in the optimal timing of oxaliplatin, 5-fluorouracil and irinotecan, both in mice and in cancer patients [163]. Indeed, many circadian rhythms in hormonal secretions, as well as gene expressions in several tissues display larger amplitudes in females as compared to males, thus highlighting the relevance of sex for circadian drug responses [163, 164]. Regarding chronoimmunotherapy, four of the 18 studies cited here found a trend toward a larger reduction of the relative risk of an earlier death through Early ToD dosing of ICIs in women, as compared to men [11, 12, 21, 29]. Such finding is consistent with larger amplitude rhythms reported in women compared to men [165], although discrepant results have been published on these topics. Also, no ICI timing effects were reported for sex in the 14 other studies. Large meta-analyses have examined whether sex was influential on immunotherapy efficacy irrespective of timing, with two positive and two negative conclusions [166–169]. Thus, prospective studies are needed to establish possible sex-related differences in immunotherapy efficacy, and to link them to circadian and immunologic biomarkers.

Indeed, the main driving mechanisms for ICI chronoefficacy relate to the robust circadian control of immune function, trafficking and ICI responses, that have been shown in experimental models so far. Similar data are expected in cancer patients in order to account for the dosing time-dependent efficacy of ICIs as well as between-patient variations.

The impact of the time of administration for efficacy and tolerability of chemotherapy followed a similar circadian pattern in preclinical models and in cancer patients, resulting in the same timing corresponding to best efficacy and best tolerability [38, 77]. Regarding immunotherapy, an association between the occurrence of adverse events and improved prognosis has been suggested [170, 171]. Consistently with these observations, Karaboué et al. [11] found significantly more skin reactions in patients who received most nivolumab infusions in the morning as compared to afternoon administration. However, morning treatment was both most effective, and associated with significantly less fatigue. Other reports regarding possible time-dependencies in ICIs adverse events are inconclusive [12, 21]. Thus, despite time-dependencies in ICIs-related toxicities deserve careful investigations.

Randomized trials and concomitant translational studies are mandatory for establishing dosing-time dependences of ICI efficacy and tolerability as well as for identifying circadian biomarkers toward the personalization of cancer chronoimmunotherapy. Indeed, it will be important to take into account patients' robustness and timing or disruption of host circadian rhythms, and sleep disorders, as well as tumor molecular clock functions and genetic mutations.

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AUTHOR CONTRIBUTIONS

Abdoulaye Karaboué (AK) and Francis Lévi (FL) searched the literature and wrote the first draft. AK and Pasquale Innominato (PI) plotted the figures. René Adam and Boris Duchemann critically reviewed the initial manuscript. All co-authors were involved in the reviewing of the final manuscript.

COMPETING INTERESTS

A Karaboué, P Innominato, NWreglesworth, B Duchemann, R Adam, and F Lévi declared no conflict of interest.

ADDITIONAL INFORMATION

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4.3 PERSISTANCE DE LA CHRONOEFFICACITE DE L'IMMUNOTHERAPIE ASSOCIEE A LA CHIMIOTHERAPIE DANS LES CANCERS BRONCHIQUES

Article n°4 *(soumis pour publication)*

Survie globale en fonction de l'heure d'administration de l'immunothérapie chez 713 patients recevant une immunochimiothérapie en première ligne de traitement pour cancer du poumon non-à-petites cellules avancé : une étude bicentrique bicontinentale.

Huang Z, ***Karaboué A****, Zeng L, Lecoeuvre A, Zhang L, Li XM, Qin H, Danino G, Yang F, Malin MS, Deng L, Rigal M, Liu H, Chen X, Xu Q, Grimaldi L, Collon T, Wang J, Adam R, Yang N, Duchemann B, Zhang Y, Lévi F.*

Selon une méta-analyse, l'administration précoce dans la journée d'ICIs a permis de presque doubler la survie globale (OS). On ne sait pas si l'effet de l'heure d'administration des ICIs sur la survie des patients persiste en cas de traitement combinant immunothérapie et chimiothérapie, devenu le traitement de référence des patients atteints de cancer bronchique non-à-petites cellules (CBNPC) avancé ou métastatique.

Méthodes : Cette étude rétrospective porte sur les données anonymisées de 713 patients recevant une immunochimiothérapie standard en première ligne pour un CBNPC de stade IIIC ou IV à Paris, France (Cohorte 1), ou à Hunan, Chine

(Cohorte 2) entre 01/2018 et 10/2023. La médiane des horaires des quatre perfusions initiales d'ICIs a été calculée pour chaque patient. La survie globale a été comparée entre les patients dont l'horaire médian d'administration de l'ICI avait lieu « avant » ou « après » les horaires seuils testés, espacés de 30 min entre 10h30 à 13h00. La survie globale a été estimée à l'aide de la méthode Kaplan-Meier et comparée entre différents groupes de traitement à l'aide du test de Log-rank. L'association entre le groupe horaire de traitement et la survie globale a été évaluée à l'aide du modèle de Cox en tenant compte des principales caractéristiques des patients.

Résultats : La médiane de la survie globale était de 33,0 mois [27,5 - 41,0] chez les patients dont l'heure médiane de traitement se situait avant 11 h 30 (N = 345) et de 19,5 mois [18,0 - 22,5] chez ceux dont l'heure médiane de triatment se situait après 11 h 30 (N = 368) ($p < 0,0001$). Dans l'analyse multivariée, l'administration majoritaire des traitements à ou avant 11h30 était associée à une prolongation significative de la survie globale, avec un HR ajusté de 0,47 [0,37-0,60]. Des différences importantes et significatives des effets de l'heure de traitement ont également été trouvées pour la survie sans progression et les taux de réponse, en faveur d'administration des ICIs avant 11h30.

Conclusion : Dans cette étude bicontinentale, l'administration d'ICIs le matin a

significativement amélioré la survie globale, par rapport à l'administration d'immunochimiothérapie l'après-midi chez les patients atteints de CBNPC. Des essais randomisés sont nécessaires pour confirmer ce résultat important et fournir des recommandations pour la pratique quotidienne.

Title

Overall survival according to time-of-day of treatment administration in 713 patients receiving immunochemotherapy as first line treatment protocol for advanced non-small cell lung cancer: a bicentric bicontinental study.

Authors

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Abstract

Background: Circadian rhythms moderate immune cells trafficking and function. As a result, responses to both vaccines and immune checkpoint inhibitors are improved following dosing near the onset of the activity phase of the endogenous rest-activity cycle in mice. Consistently, a near doubling of overall survival (OS) has resulted from early time-of-day administration (ToDA) of immune checkpoint inhibitors (ICIs) as single agents in patients with metastatic "immunotherapy sensitive" cancer, according to a meta-analysis. It is unknown whether ToDA moderates OS in patients treated with both ICI and chemotherapy for advanced non-small cell cancer (NSCLC), i.e. the current therapeutic mainstay in this disease.

Methods: Patients receiving first line immunochemotherapy for stage IIIC or IV NSCLC in oncology units in Paris, France (Cohort 1), or Hunan, China (Cohort 2) between 01/2018 and 10/2023 were retrospectively included. Primary outcome was OS. The median ToDA of the initial four ICI infusions was computed for each patient. OS was compared between patients, whose median ToDA occurred "Before" or "After" cut-off times ranging from 10:30 to 13:00, with 30-minutes increments. OS was estimated using Kaplan-Meier and compared using log-rank. The association between ToDA and OS was evaluated using the Cox model controlling for main patient characteristics.

Findings: 713 patients with a median age of 62 years were selected, including 84% males. ICIs mostly consisted of pembrolizumab (51% of the patients). They were combined with pemetrexed-carboplatin/cisplatin (49%) or paclitaxel-carboplatin (51%). Median OS (months) was 33.0 [95% CL, 27.5 - 41.0] in the patients, with a median ToDA before 11:30 (N=345 patients) and 19.5 [18.0 - 22.5] in those, whose median ToDA was after 11:30 (N = 368) ($p<0.0001$). In the multivariable analysis, a median ToDA before 11:30 was associated with prolonged OS with an adjusted HR of 0.47 [0.37-0.60]. Large and significant differences in ToDA effects were also found for OS in each individual cohort, as well as for progression-free survival (PFS) and response rates (RR) both in each individual cohort and in the pooled data.

Conclusion: In this bi-continental study, morning ICI administration consistently improved OS, PFS and RR, as compared to afternoon dosing in patients receiving the standard first line immunochemotherapy protocol for NSCLC. Randomized trials can now be designed to confirm this important finding and provide recommendations for daily practice.

Key words

Circadian rhythms, Chronotherapy, Immune checkpoint inhibitors, Time od Day of administration, Lung cancer, Immuno-chemotherapy, Chronopharmacology, Circadian Medicine.

Introduction

Puzzling differences in overall survival (OS) and progression-free survival (PFS) have been reported as a function of time-of-day of administration (ToDA) of immune checkpoint inhibitors (ICIs) in numerous retrospective studies now involving over two thousand patients [1-5]. A meta-analysis of 13 reported studies has recently shown that both OS and PFS were nearly doubled in the patients receiving ICIs mostly in the morning or in the early afternoon hours, as compared to later dosing times [3]. Strikingly ToDA effects were remarkably consistent in patients suffering from one of seven different tumor types, including non-small cell lung cancer, malignant melanoma, or cancer of the kidney, bladder, oesophagus, or stomach, or hepatocarcinoma [3, 5, 6]. Experimental studies have revealed a tight control of both immune cells trafficking and Programmed Cell Death protein 1 (PD-1) expression by the circadian timing system [7-9]. Thus, strong circadian waves characterize tumor infiltration by T(CD8), dendritic cells and M2 macrophages, i.e. the main cells involved in ICIs activities against cancer cells [7, 8]. Moreover PD-1, the main target protein of ICIs, is also rhythmically expressed in immune cells along the 24 hours [7, 9, 10]. Large amplitude circadian rhythms also characterize circulation, function and proliferation of immune cells in humans [10-12]. Here we determine the possible relevance of the daily timing of the initial four immunochemotherapy infusions for OS and PFS in 713 patients with advancer non-small cell lung cancer, following the establishment of immunochemotherapy as the mainstay of first-line treatment for non-small cell lung cancer [13, 14]. We first determine the Hazard ratios (HR) of an earlier death or an earlier progression as a function of median per patient ToDA of a PD-1 inhibitor in combination with chemotherapy separately in a French and a Chinese cohort. Due to the remarkable consistencies of the results in each cohort, we pool all data and report patient outcomes according to anti-PD-1 infusion daily timing.

Patients and Methods

Study Objective, Design and Ethics

This study aimed at the first identification of the clinical relevance of the ToDA of the initial four courses of the combination of an anti-PD-1 ICI and chemotherapy as first-line treatment for advanced or metastatic non-small cell lung cancer (NSCLC).

We conducted a large international retrospective study based on pooled deidentified individual patient data from a French and a Chinese cohort, i.e. Cohort 1 and 2 respectively. Cohort 1 involved patients with metastatic NSCLC from Le-Raincy Montfermeil Hospital, Montfermeil, and Avicenne University Hospital, Bobigny, France. Cohort 2 involved patients with locally advanced or metastatic NSCLC from Hunan Hospital, Changsha.

All study procedures were performed following the ethical standards of the institutional and national research committees and the 2013 revised Declaration of Helsinki. Cohort 1 study was approved by Internal Review Boards of Le Raincy-Monfermeil and Avicenne hospitals (IRB 200224 and CLEA-2023-n°337, respectively). Cohort 2 study was approved by the Ethics Committee of Hunan Cancer Hospital (approval number: 2022YYQ-IIT-015.). The study followed the STROBE guidelines [15].

Patient Selection

For both cohorts, data (**Table S1**) were collected from consecutive patients with (i) histologically- or cytologically-documented NSCLC; (ii) advanced or metastatic disease; (iii) World Health Organization performance status of 0 1, or 2; (iv) No tumor EGFR mutation, ROS1 translocation, or ALK gene abnormality; (v) administration of at least one course of standard first-line protocol combining an anti-PD-1 ICI and pemetrexed-carboplatin (or cisplatin), or paclitaxel-carboplatin. The patients with brain metastases not requiring glucocorticoid therapy were included. Those receiving glucocorticoid daily doses >10 mg/day for any medical condition were not included. Those patients with missing information regarding ICIs infusion timing over the initial four treatment courses were discarded from analyses.

Treatment procedures

For each course, pembrolizumab or another anti-PD-1 ICI, i.e. sintilimab, toripalimab, tirelizumab or camrelizumab, were administered at a fixed dose of 200 mg as a 30-minute infusion just before the delivery of either standard chemotherapy protocol every 3 weeks for 4 courses. Subsequently, patients could receive pembrolizumab or another ICI as a single agent or combined with pemetrexed q 3 weeks until disease progression, severe toxicity or medical or patient decision to stop therapy [13, 14]. For both cohorts, follow-up visits, and laboratory tests were performed before each treatment course, whilst imaging studies were performed after 2nd and 4th course, then every 2-4 courses until protocol withdrawal or completion

Immunochemotherapy Treatment and Timing

ICIs-chemotherapy timing slots were randomly allocated for each course by the day-hospital coordinator on a logistical basis and accurately documented. Subsequent ICI infusion time-slots remained grossly similar thereafter for each patient for logistics reasons. Treatments were administered until disease progression, occurrence of unacceptable toxicities, or intercurrent death.

Assessments

De-identified electronic medical records were reviewed at each site to collect *a priori* selected patient and tumor baseline characteristics, including WHO performance status, ICI type, daily time of ICI administration onset for each of the initial four infusions (date and clock hour), and number of courses given. Anti-tumor responses were assessed, using Computed Tomography scans, Magnetic resonance Imaging, and/or PET scan, after every two to three courses. Responses were categorized according to iRECIST [16] criteria as complete (CR), partial (PR), stable (SD) or progressive disease (PD). Blood test results and adverse events were not collected for this study. Data were independently reviewed by two clinicians, alongside the lead oncologist for each patient, to ensure quality, consistency, and plausibility.

Outcomes

The primary endpoint was OS defined as the time from the first ICI administration to all-cause death or to the last follow-up visit for patients last seen alive. Secondary endpoints were PFS and best response rate (RR). PFS was defined as the time from the first ICI administration to death or disease progression (whichever came first) or to the last follow-up visit for patients last seen alive and without disease progression. Best RR was defined as the percentage of patients whose best tumor response was categorized as a PR or a CR.

Statistical Considerations

Categorical variables were reported as frequencies and percentages and continuous variables as mean and standard deviation (SD) or median and interquartile range (IQR). Normality assumption was assessed graphically. The median follow-up was estimated using the reverse Kaplan–Meier method. For each patient, median ToDA was defined based on the administration start time of the initial 4 immunochemotherapy courses. The intra-patient coefficient of variation was assessed by the ratio of the standard deviation to the mean of all four infusion times for each individual patient. The median between-patients coefficient of variation of the ICI infusion times and its interquartile and range were then computed for each study group.

Different timing cut-offs were studied from 10:30 to 13:00 with 30-minute increments. For each timing cut-off, the population was classified as either "Before" or "After", according to their median ToDA. Characteristics of patients in the "Before" and "After" groups were compared using Student's t-test (or non-parametric Wilcoxon test) for quantitative variables, chi-square test (or Fisher's exact test) for non-ordered categorical variables and Cochran-Armitage test for ordered categorical variables (performance status and PDL-1 expression). OS and PFS were estimated in each group using Kaplan-Meier methods and compared using log-rank test. The associations between ToDA cut-off and OS or PFS were evaluated using the Cox model controlling for country, sex, age, performance status, stage, PDL-1 expression, number of metastastic sites, type of immunotherapy and chemotherapy [17]. Unadjusted Cox models were also performed within different subgroups to explore the consistency of the effect of ToDA cut-off on OS and PFS. The association between ToDA cut-off and RR was assessed using logistic regression model adjusting for the same factors. Missing values were described and assumed to be at random. Whenever a categorical variable had a significant amount of missing data, a new category 'Missing' was created to handle with this situation. The main analysis was performed on pooled deidentified individual data from both cohorts. Sensitivity analyses were also performed for exploratory purposes in each individual cohort. The same cut-off times were studied following the same methods as described above. OS and PFS were estimated in each "Before" and "After" group and compared using log-rank test. The association between cut-off ToDA and OS or PFS were assessed using unadjusted Cox model.

The p-value was set a priori at 0.05 for all analyses and 95% confidence intervals (95% CI) of the estimates were calculated. All tests were two-sided. The analyses were performed using SPSS® v26.0 (Chicago, IL, USA) and R® v4.3.0 [18].

Results

Patient Characteristics and Study Flow

Due to missing ToD of administration of ICI infusions, 165 of 176 patients met selection criteria in Cohort 1 (93.8%), and 548 of 598 patients (91.6%) did so in Cohort 2 (**Figure 1**). Included patients started first-line immuno-chemotherapy between February 5th, 2019 and October 9th, 2023 for Cohort 1, and from January 1st, 2018 to June 30th, 2022, for Cohort 2. Among the 713 patients, 83.9 % were males; median age was 62 [IQR, 56 - 68] years old; 93.5% of the patients had a WHO performance status of 0 or 1; and 87.8% of them had Stage IV disease (**Table 1**). The patients had a median of two metastatic sites, including involvement of bone (33.5% of the patients), brain (17.7%), and liver (11.6%). PD-L1 was expressed in the tumors of 71.1% of those 540 patients with such determination. The majority of patients received pembrolizumab as an ICI (50.5%). Immunotherapy was combined with paclitaxel-carboplatin or pemetrexed-carboplatin for 51.3% and 48.2% of the patients, whilst 3 (0.4%) patients received pemetrexed-cisplatin. The median ToDA of the per patient median ToDA of the initial 4 courses was located at 11:36 [10:49 – 12:48]. The median within patient coefficient of variation of ICI infusion start ToDA was 9.9% [IQR, 5.2 – 18.1]. Overall, the patients received a median number of 9 [IQR, 5 to 18] courses of immunotherapy with or without chemotherapy throughout the protocol treatment (**Table 1**). Median per-patient ICI infusion ToDs was 10:39 [9:57 – 11:30] for Cohort 1 and 11:51 for Cohort 2, with both distribution patterns being skewed to the left (**Figure S1**).The median follow-up was 24.5 months [*95% CL,* 23.2 - 25.6]. The OS and PFS curves of the whole cohort revealed a median OS and a median PFS of 35.5 months [22.5 – 29.7] and 9.0 months [8.3 – 9.9], respectively (**Figure S2**).

Determination of cut-off ToDA for differentiating overall survival and progression-free survival

The HR functions displayed similar trends for both OS (**Figure 2)** and PFS **(Figure 3**). Unadjusted HRs of an earlier death and an earlier progression were statistically significant for cut-off ToD of 11:30 for the pooled cohort, with respective HRs of 0.59 [95% CL, 0.47 – 0.73] and 0.64 [0.54 – 0.76]. Using this cut-off ToDA, HRs of an earlier death were remarkably similar in Cohort 1, i.e. 0.47 [0.29 – 0.77] and in Cohort 2, i.e. 0.50 [0.38 – 0.65] (**Figure S3**). The HRs of an earlier progression were amazingly comparable in both cohorts, i.e. 0.56 [0.37 – 0.84] and 0.57 [0.46 – 0.70] (**Figure S4**). Statistically significant HRs were identified however for cut-off timepoints staggered by 30-minutes, ranging from 11:30 to 13:00, yet with overlapping 95% CL. in the pooled cohort and in each individual Cohort as well (**Figures 2 and 3, Figures S3 and S4**).

Efficacy of immunochemotherapy delivered "Before" of "After" clock hour 11:30

204 There were 345 patients in the "Before 11:30" group and 368 in the "After 11:30" group (**Table S2**). Compared to the "Before
205 11:30" group, the "After 11:30" group included more patients with non-squamous cell NSCLC (p <0.001), fewer metastatic sites (p
206 = 0.005), higher tumor PD-L1 expression (p = 0.057).
207 Using such patient population dichotomization, large and statistically significant differences characterized both OS (**Figure 4**) and
208 PFS curves (**Figure 5**). Median survival times were 33.0 months [27.5 – 41.0] in the "Before 11:30" group, compared to 19.5 months
209 [18.0 – 22.5] in the "After 11:30" group (p<0.0001). The respective median PFS times were 11.8 months [9.9 – 14.0] and 7.2 months
210 [6.6 – 8.5] (p <0.0001). Both OS and PFS differed significantly in each individual cohort using 11:30 as a cut-off time (**Figures S5**
211 **and S6**).
212 RR were also largest in the "Before 11:30" group in Cohort 1 (63.4% vs 45.9%, p = 0.014), Cohort 2 (60.8% vs 53.1%, p = 0.036)
213 and both pooled (61.7% vs 52.4%, p = 0.002) (**Figure S7**). As a result of the quicker disease progression following treatment after
214 11:30, the patients in this group received fewer treatment courses and less loco-regional radiotherapy compared to those treated
215 before 11:30 (p<0.001 for each) (**Table S2**).
216
217 **Multivariable analyses of pooled cohort data and relevance of ToDA in patient subsets**
218 Multivariable analyses revealed statistically significant effects of ToDA for OS and PFS, with corresponding adjusted HR ratios
219 shown for tested ToDA staggered by 30 minutes from 10:30 to 13:00 (**Figures 2 and 3**). Using 11:30 as cut-off ToDA, the adjusted
220 HR of an earlier death was 0.47 [0.37 – 0.60]. Performance status, PD-L1 expression and chemotherapy protocol were jointly
221 associated prognostic indicators, with improved prognosis in the patients with a PS of 0 (HR, 0.30 [0.18 – 0.48]), or tumor PD-L1
222 expression ≥ 50% (HR, 0.54 [0.34 - 0.76]) or those receiving paclitaxel-carboplatin chemotherapy (HR, 0.77 [0.61 – 0.98]) (**Figure**
223 **S8**).
224 The adjusted HR of an earlier progression was 0.54 [0.45 – 0.65], with performance status, tumor PD-L1 expression, and
225 chemotherapy protocol being joint prognostic indicators (**Figure S9).** Sub-groups analyses confirmed that the administration of the
226 majority of the initial 4 immunochemotherapy courses before 11:30 improved both OS and PFS irrespective of patient or tumor
227 characteristics, except for PD-L1 whose tumor expression below 1% might lessen the role of clock hour 11:30 as a relevant cut-off
228 ToDA (**Figure 6**). Logistic regression also confirmed the superiority of ICI infusions "Before 11:30" for RR, with unadjusted OR
229 of 1.5 [1.1-2.0] and adjusted OR of 1.6 [1.1-2.2].
230
231 **Discussion**
232 ***Relevance of ToDA of immunochemotherapy for all efficacy outcomes***
233 Here we show that the ToDA of ICI infusion critically determined the outcomes of 713 patients receiving first-line
234 immunochemotherapy for NSCLC. Indeed, the group of patients receiving the initial 3 or 4 ICI infusions before 11:30 had
235 significantly longer OS and PFS, and significantly larger RR, compared to those getting them after 11:30. Remarkably, the median
236 survival was nearly one year longer in the 'morning' group, as compared to the 'afternoon' group in this large pooled cohort.

237 Moreover, the 'morning' group was significantly superior to the 'afternoon' one for OS, PFS and RR for each cohort. Using 11:30
238 as cut-off time, the unadjusted HRs of an earlier death were 0.47 [95% CL, 0.29 – 0.77] for Cohort 1, and 0.57 [0.46 – 0.70] for
239 Cohort 2, and 0.59 [0.47 – 0.73] for the pooled Cohort, whose adjusted HR was 0.47 [0.37 – 0.60].

240 ***Consistent timing effects between immunochemotherapy and immunotherapy-only***

241 The similarities of these figures highlighted the consistency in the ICI timing responses of NSCLC patients from both continents.
242 The current immunochemotherapy timing effects are also consistent with our recent meta-analysis of 13 retrospective studies, where
243 early ToDA of single agent ICIs nearly doubled both OS and PFS in 1667 patients, with lung, kidney, bladder or oesophagus cancer
244 or melanoma [3]. Similar results have been reported for single-agent pembrolizumab in a large cohort of 361 cancer patients,
245 including 80% with NSCLC, with 11:30 being identified as a relevant cut-off ToDA [4]. Survival improvements through
246 predominantly morning ICI infusions have also been recently reported for patients with stomach cancer [6] and those with
247 hepatocarcinoma [5].

248 ***Possible confounders and study limitations***

249 The main limitation of this study is its retrospective design. Its strengths however are multifold: it involves 713 patients from two
250 cohorts in two different continents. Both cohorts had similar main patient characteristics, and received treatment protocols
251 corresponding to the current standard of care. This study is the first to reveal a critical role of the daily timing of immuno-
252 chemotherapy for OS in the pooled cohort, and in each individual cohort. The near doubling of OS through ICIs dosing before 11:30
253 is based on the timing of the initial 4 treatment courses. The results are consistent with those published for mono-immunotherapy.
254 Another limitation however could result from the selection of patients fit for a specific timing schedule. Thus, statistically significant
255 imbalances were found some of which could favor the "After 11:30" group, and others the "Before 11:30" group. Yet, the magnitude
256 of the timing effect was largest rather than damped following adjustment for the main prognostic factors. Indeed, the collection and
257 analyses of deidentified data from two continents support the statement that the large benefit derived for all three efficacy endpoints
258 through immunochemotherapy dosing before 11:30 is not specific of a care system, genetics or population lifestyle.

259 ***Mechanisms of dosing time dependencies in immuno-chemotherapy efficacy***

260 In mammals, a Circadian Timing System (CTS) rhythmically regulates cellular metabolism, proliferation, and death, as well as drug
261 responses along the 24-h time scale [11]. The CTS involves a network of molecular circadian clocks in each cell, that are coordinated
262 by the supra-chiasmatic nuclei, a hypothalamic pacemaker. This pacemaker also adjusts the timing of the clocks to environmental
263 cycles through the generation of physiological rhythms including those in rest-activity, body temperature and glucocorticoids
264 secretion [11, 19]. The molecular clocks are made of 15 specific genes, including Bmal1, Clock, Rev-erb's, Per's, and Cry's, and
265 have been found in all the cells that participate in immune functions and trafficking [7-10]. As a result, dosing time dependencies
266 characterized efficacy and/or tolerability of interferons [20, 21], interleukin-2 [22], and recently for anti-PD-1 agents in mice [7].
267 Circadian rhythms controlled both tumor infiltration with T(CD8), macrophages and dendritic cells and PD-1 and Pccd1 expressions
268 in immune cells [7, 8]. In humans, the circulating counts in T-lymphocyte subsets nearly doubled between circa 10:00 in the morning
269 (trough) to circa 02:00 at night (peak) [11]. The findings suggest that concurrent tissue depletion from immune cells is operating in

the afternoon hours, when ICIs were found to be less effective in patients. Such chronopharmacodynamic mechanisms may override as yet unexplored ICIs chronopharmacokinetics. The average half-lives of ICIs of about 2 weeks support the hypothesis that ICI chronopharmacokinetics could be influential over the initial 4 courses, i.e. before the reach of plateaued plasma concentrations, a timeframe consistent with the setting of this study.

The CTS also regulates cell cycle phases and apoptosis, hence resulting in established circadian rhythms in the toxicities and/or efficacy of 50 anticancer medications of all pharmacology classes in experimental models [23, 24]. A systematic review of 18 randomized trials of chronomodulated chemotherapy concluded that chronomodulated chemotherapy according to circadian rhythms provided consistent benefits for tolerability, with similar or better efficacy, thus confirming our initial trial results [25, 26]. No circadian data exist regarding pemetrexed chronopharmacology in preclinical models or in patients. In contrast, taxanes appear best tolerated and/or most effective following their administration in the late rest span of mice [27, 28]. with an apparent correlation between clock gene expression Bmal1 and increased tumor cell susceptibility to paclitaxel [29]. Platinum complexes displayed less toxicity and increased efficacy if their delivery occurred near the middle of the activity span of mice, rats or cancer patients [24], although sex-related differences moderated the time of least toxicity [11]. Taken together the results call for further mechanistic explorations and optimization of chrono-immuno-chemotherapy.

Implications for clinical research and oncology practice

For the sake of healthcare logistics convenience, the majority of treatments is administered to patients regardless of their daily times. Circadian rhythms however have long been shown as important determinants of toxicity and efficacy outcomes for hundreds of medications including anticancer therapies [11, 23, 24]. ICIs have largely improved the survival of patients with many different cancer types over the past two decades [30]. Yet none of the pivotal clinical trials for ICIs approval apparently investigated the possible relevance of ToDA, a question that did not seem of concern to the regulatory authorities. Evidence is accumulating however for the need to carefully complement the determinations of optimal dose by that of optimal time for cancer therapies throughout the development of new drugs, especially those which target immune-cancer cell interactions. Randomized trials on their way (e.g. NCT05549037) compare different daily time slots for treatment administration to prospectively measure the extent of improvement in efficacy due to optimal treatment timing selection. They also aim at the identification of possible between-patient differences in optimal timing for efficacy and tolerability, and their mechanisms along the 24-hour time scale.

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Contributions of authors.

Z Huang conceived the study, collected data from Cohort 2, contributed to the analysis and interpretation of the data, manuscript writing, and figures and tables; A Karaboué conceived the study, collected data from Cohort1, contributed to the analysis and interpretation of the data, to manuscript writing, and to figures and tables, L Zeng contributed to the analysis and interpretation of the data, and to manuscript review, and edition; A Lecoeuvre designed and performed the statistical analyses, wrote the statistical methods section, contributed to the preparation of figures and tables, and critically read and improved the manuscript. L Zhang, H Qin, F Yang, and L Deng collected data from Cohort 2; XM Li contributed to all collaborative aspects in the study, and critically

read and improved the MS. G Danino , MS Malin, and M Rigal collected data from Cohort1. H Liu, X Chen, and Q Xu collected data from Cohort 2, and reviewed and edited the manuscript. L Grimaldi designed the statistical analysis plan of each cohort and both pooled, discussed results, and critically read and improved the MS. T Collon contributed to Cohort 1 study design, patient assessments and reviewed the manuscript. J Wang, N Yang and R Adam discussed results and interpretation, and critically improved the MS. B Duchemann conceived the study, was in charge of deidentified data collection in Cohort 1, contributed to all study progress and development, contributed to methods, results, interpretation, and manuscript writing. Y Zhang and F Lévi co-directed this study, including conception, organization, data collection, auditing, supervision, project management, funding acquisition, writing and editing current manuscript. All authors approved the current manuscript.

Data sharing

All the deidentified participant data will be made available 12 months after the publication of this manuscript, to the scientists who will address a request to francis.levi@universite-paris-saclay.fr .

Legends to Figures

Figure 1: Consort diagram: study Flow and endpoints in each cohort, and in both pooled.

Figure 2: Hazard Ratios of an earlier death as a function of cut-off ToDA of immunochemotherapy. *Left column,* tested ToDAs located 30-min apart, i.e. from 10:30 (bottom row) to 13:00 (upper row); *Middle column*, unadjusted and adjusted HRs and their respective 95% CL for each tested ToDA; *Right figure panel,* illustrative display of the corresponding HRs and their respective 95% Confidence Limits.

Note: ToDA at clock hour 11:30 appears as the first timepoint that consistently differentiates the survival of the patients receiving the majority of ICI infusions Before vs After 11:30. Overlapping 95% CI indicate that using later ToDA as cut-off points may not be more discriminant compared with 11:30.

Figure 3: Hazard Ratios of an earlier progression as a function of cut-off ToDA of immunochemotherapy. *Left column,* tested ToDAs located 30-min apart, i.e. from 10:30 (bottom row) to 13:00 (upper row); *Middle column*, unadjusted and adjusted HRs and their respective 95% CL for each tested ToDA; *Right figure panel,* illustrative display of the corresponding HRs and their respective 95% Confidence Limits.

Note: ToDA at clock hour 11:30 appears as the first timepoint that consistently differentiates the progression-free survival of the patients receiving the majority of ICI infusions Before vs After 11:30. Overlapping 95% CI indicate that using later ToDA as cut-off points may not be more discriminant compared with 11:30.

Figure 4: Overall survival curves as a function of ICI ToDA.

A majority of the 4 initial immunochemotherapy courses started before 11:30 in 368 patients, and after 11:30 in 345 patients**.** Both curves differed significantly ($p<0.0001$), with median survival times of 33.0 [27.5 – 41.0] in the "Before 11:30" group, compared to 19.5 [18.0 – 22.5] in the "After 11:30" group.

Figure 5: Progression-free survival curves as a function of ICI ToDA.

A majority of the 4 initial immunochemotherapy courses started before 11:30 in 368 patients, and after 11:30 in 345 patients**.** Both curves differed significantly ($p<0.0001$), with median progression-free survival times of 11.8 [9.9 – 14.0] in the "Before 11:30" group and 7.2 [6.6 – 8.5] in the "After 11:30" group.

Figure 6: Forrest plots of TodA effects in patient subgroups.

HRs of an earlier death (*Left panel*) or an earlier progression (*Right panel)* using clock hour 11:30 as ToDA in selected patient subgroups (*Right column*).

408 **Table 1: Main patient characteristics in both cohorts and overall**

Characteristics	Cohort 1 (N=165)	Cohort 2 (N=548)	Overall (N=713)
Sex (male)	120 (72.7%)	478 (87.2%)	598 (83.9%)
Age			
- Median [IQR]	63.8 [56.2 - 70.0]	61.0 [55.8 - 67.0]	62.0 [56.0 - 68.0]
- (range)	(37.5 - 85.1)	(40.0 - 79.0)	(37.5 - 85.1)
Smoking (yes)	158 (95.8%)	440 (80.3%)	598 (83.9%)
WHO PS			
- 0	43 (26.4%)	87 (15.9%)	130 (18.3%)
- 1	89 (54.6%)	446 (81.4%)	535 (75.2%)
- 2	31 (19.0%)	15 (2.7%)	46 (6.5%)
- Missing	2	0	2
Histological type			
- Adenocarcinoma	111 (67.3%)	284 (51.8%)	395 (55.4%)
- Squamous cell carcinoma	25 (15.2%)	254 (46.4%)	279 (39.1%)
- Poorly differentiated carcinoma	29 (17.6%)	10 (1.8%)	39 (5.5%)
Stage			
- IIIC	0 (0.0%)	87 (15.9%)	87 (12.2%)
- IV	165 (100.0%)	461 (84.1%)	626 (87.8%)
Main sites or metastases			
- Brain	55 (33.7%)	71 (13.0%)	126 (17.7%)
- Liver	35 (21.2%)	48 (8.8%)	83 (11.6%)
- Bone	76 (46.1%)	163 (29.7%)	239 (33.5%)
Number of metastatic sites			
- Median [IQR]	3 [2 - 4]	1 [1 - 2]	2 [1 - 2]
- (range)	(1 – 11)	(0 – 5)	(0 – 11)
PDL1			
- <1%	59 (37.6%)	97 (25.3%)	156 (28.9%)
- 1%-49%	56 (35.7%)	149 (38.9%)	205 (38.0%)
- ≥ 50%	42 (26.8%)	137 (35.8%)	179 (33.1%)
- Missing	8	165	173
Chemotherapy			
- Pemetrexed + Carboplatin	122 (73.9%)	222 (40.5%)	344 (48.2%)
- Paclitaxel + Carboplatin	40 (24.2%)	326 (59.5%)	366 (51.3%)
- Pemetrexed + Cisplatin	3 (1.8%)	0 (0.0%)	3 (0.4%)
Immune Checkpoint Inhibitor (ICI)			
- Pembrolizumab	165 (100.0%)	195 (35.6%)	360 (50.5%)
- Sintilimab	0 (0.0%)	202 (36.9%)	202 (28.3%)
- Toripalimab	0 (0.0%)	43 (7.8%)	43 (6.0%)
- Tirelizumab	0 (0.0%)	55 (10.0%)	55 (7.7%)
- Camrelizumab	0 (0.0%)	53 (9.7%)	53 (7.4%)
Median ToD of initial 4 ICI infusions per patient (clock hours, hh:mm)			
- Median [IQR]	10:39 [9:56 - 11:30]	11:51 [11:12 - 13:37]	11:36 [10:49 - 12:48]
- (range)	(8:53 - 16:59)	(9:16 – 18:11)	(8:53 – 18:11)
Within-patient coefficient of variation of ICI infusion ToD (%)			
Median [IQR]	6.4 [3.5 – 11.1]	11.2 [5.9 – 19.8]	9.9 [5.2 – 18.1]
(range)	(0 – 33)	(0 – 35)	(0 – 35)
Number of ICI+chemotherapy courses per patient			
- Median [IQR]	4 [3 – 4]	4 [4 – 6]	4 [4 - 5]
- (range)	(1 – 6)	(1 – 26)	(1 – 26)
Total number of ICI infusions (+/- chemotherapy) per patient			
- Median [IQR]	7 [3 – 14]	13.5 [6 – 19]	9 [5 – 18]
- (range)	(1 – 49)	(1 – 40)	(1 – 49)
N of patients with local radiotherapy post ICI-chemotherapy (%)	47 (28.5%)	27 (4.9%)	74 (10.4%)

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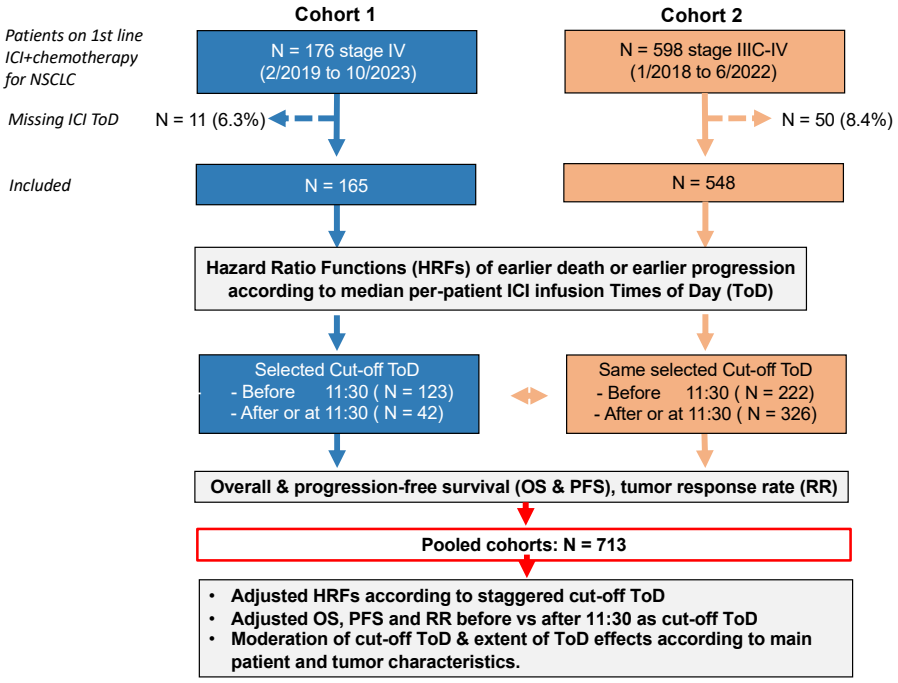
Cohort 1
Cohort 2
Patients on 1st line ICI+chemotherapy for NSCLC
N = 176 stage IV (2/2019 to 10/2023)
N = 598 stage IIIC-IV (1/2018 to 6/2022)
Missing ICI ToD
N = 11 (6.3%)
N = 50 (8.4%)
Included
N = 165
N = 548
Hazard Ratio Functions (HRFs) of earlier death or earlier progression according to median per-patient ICI infusion Times of Day (ToD)
Selected Cut-off ToD
- Before 11:30 (N = 123)
- After or at 11:30 (N = 42)
Same selected Cut-off ToD
- Before 11:30 (N = 222)
- After or at 11:30 (N = 326)
Overall & progression-free survival (OS & PFS), tumor response rate (RR)
Pooled cohorts: N = 713
• Adjusted HRFs according to staggered cut-off ToD
• Adjusted OS, PFS and RR before vs after 11:30 as cut-off ToD
• Moderation of cut-off ToD & extent of ToD effects according to main patient and tumor characteristics.

Figure 1

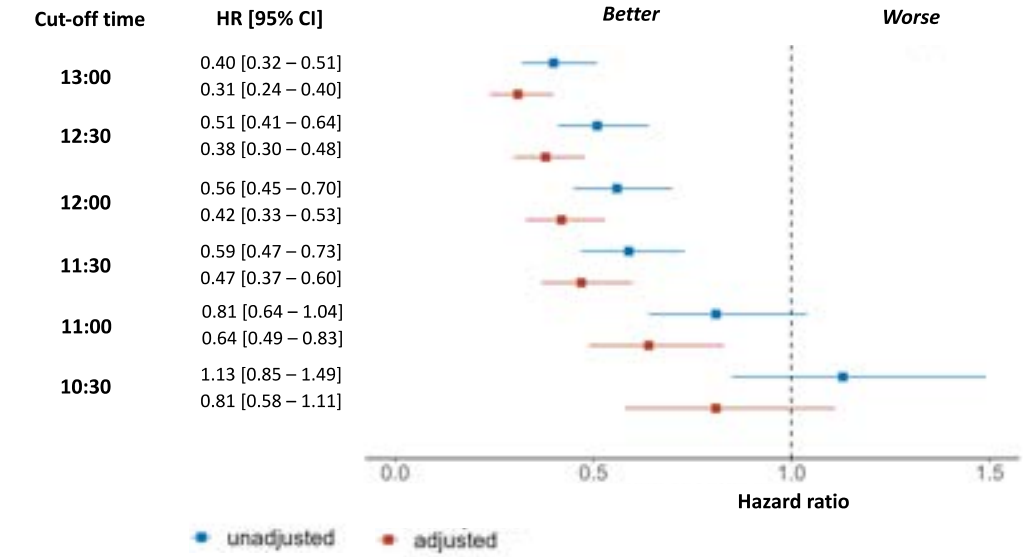

Figure 2

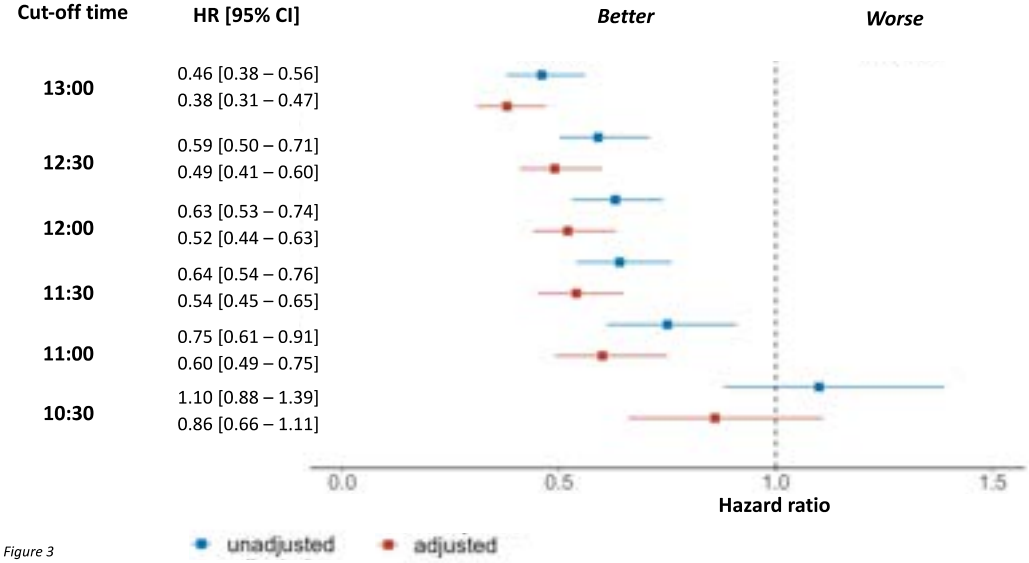

Figure 3

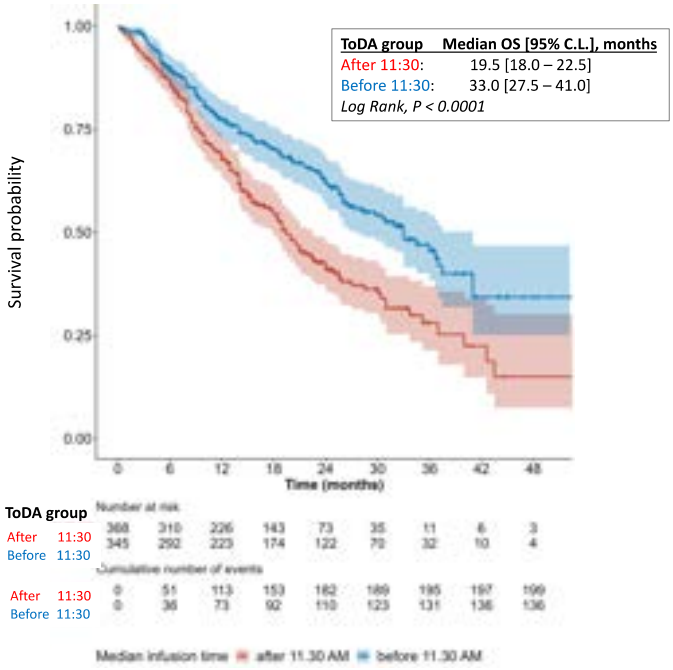

Figure 4

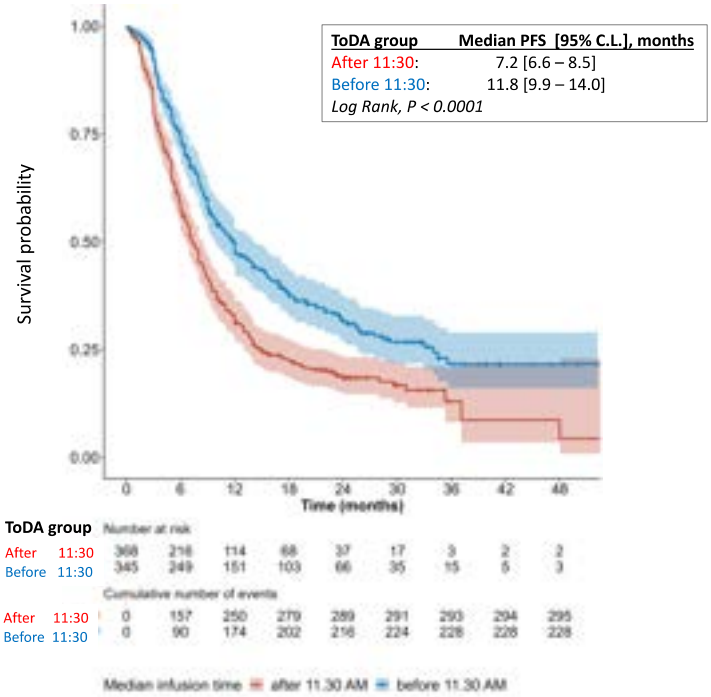

Figure 5

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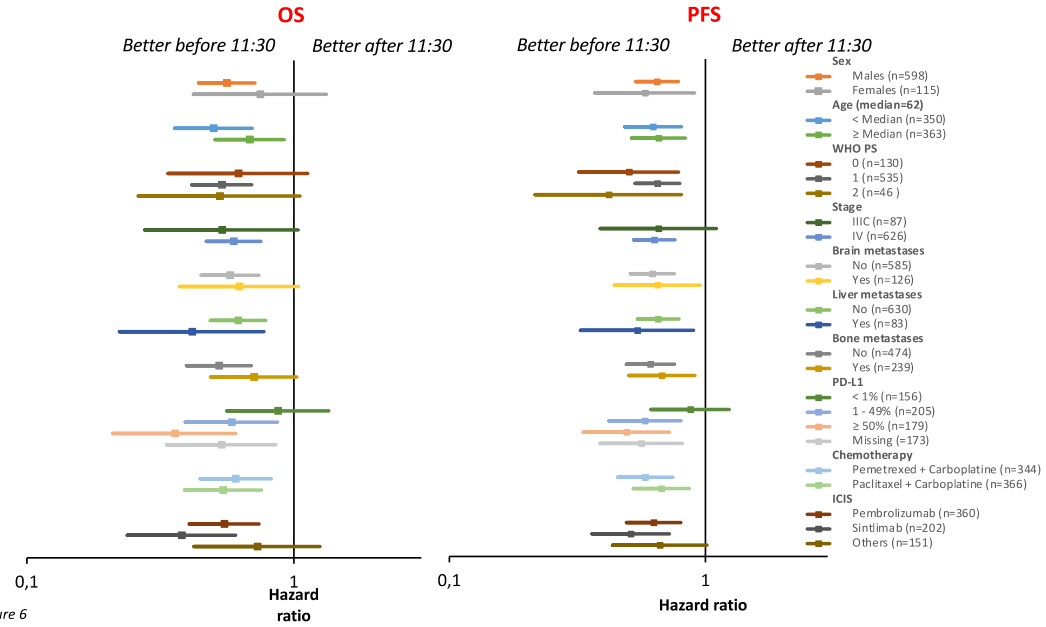

Figure 6

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Overall survival according to time-of-day of administration in 713 patients receiving first line immunochemotherapy for advanced non-small cell lung cancer: a bicentric bicontinental study

Zhe Huang[1,2,*], Abdoulaye Karaboué[3,4,*], Liang Zeng[1], Adrien Lecoeuvre[5], et al.

Supplementary Tables and Figures

Table S1 : List of data collected for each patient in each cohort

Description of data collected in each patient clinical file
Sex: Male/Female
Date of birth
Smoking status: Yes/No
Initial WHO PS[1]: 0/1/2
Disease stage: III / IV
Histologic type: adenocarcinoma, squamous cell carcinoma, other non-small cell carcinoma
Brain metastases Yes/No
Liver metastases: Yes/No
Bone metastases: Yes/No
Number of metastatic sites
PD-L1[2] expression
Chemotherapy regimen combined with the ICI[3]: pemetrexed-carboplatin or cisplatin / paclitaxel-carboplatin
ICI type: Pembrolizumab / Sintilimab / Toripalimab / Tirelizumab / Camrelizumab
Date of cycle 1
Local time of ICI onset at cycle 1
Date of cycle 2
Local time of ICI onset at cycle 2
Date of cycle 3
Local time of ICI onset at cycle 3
Date of cycle 4
Local time of ICI onset at cycle 4
Number of cycles of combined chemotherapy plus ICI
Number of cycles of ICI alone after combined chemotherapy plus ICI
Date of last ICI infusion
Radiotherapy during ICI treatment: Yes/No
Best response: CR[4] / PR[5] / SD[6] / PD[7]
Disease progressed at study cut-off date: yes / No
Date of progression, if yes
Date of last known to be alive or death
Survival status at study cut-off date: dead / alive

[1] WHO PS = WHO performance status; [2] PD-L1 = Programmed-death ligand 1; [3] ICI = Immune checkpoint inhibitor; [4] CR = Complete response; [5] PR = Partial response; [6] SD = Stable disease; [7] PD = Progressive disease.

Table S2: Main patient characteristics in the pooled cohort dichotomized as a function of the administration of the majo of the initial four immunochemotherapy infusions starting before or after 11:30.

	Characteristics	Before 11:30 (N=345)	After 11:30 (N=368)	p-value
	Sex (male)	283 (82.0%)	315 (85.6%)	0.195
	Age			
-	Median [IQR]	61.4 [56.0, 68.0]	62.0 [55.8, 67.1]	0.780
-	(range)	(40.0 - 83.7)	(37.5 - 85.1)	
	Smoking (yes)	298 (86.4%)	300 (81.5%)	0.078
	WHO PS			
-	0	66 (19.2%)	64 (17.4%)	
-	1	249 (72.6%)	286 (77.7%)	0.143
-	2	28 (8.2%)	18 (4.9%)	
-	Missing	2	0	
	Histologic tumor type			
-	Adenocarcinoma	194 (56.2%)	201 (54.6%)	< 0.001
-	Squamous cell carcinoma	119 (34.5%)	160 (43.5%)	
-	Poorly differentiated carcinoma	32 (9.3%)	7 (1.9%)	
	Tumor stage			
-	IIIC	39 (11.3%)	48 (13.0%)	0.494
-	IV	306 (88.7%)	320 (87.0%)	
	Metastases in			
-	Liver	35 (10.1%)	48 (13.0%)	0.228
-	Bone	123 (35.7%)	116 (31.5%)	0.243
-	Brain	66 (19.2%)	60 (16.3%)	0.322
	Number of metastatic sites			
-	Median [IQR]	2 [1 - 3]	2 [1 - 2]	*0.005*
-	(range)	(0 - 11)	(0 - 5)	
	PD-L1 expression			
-	<1%	89 (33.3%)	67 (24.5%)	
-	1%-49%	95 (35.6%)	110 (40.3%)	0.057
-	≥ 50%	83 (31.1%)	96 (35.2%)	
-	Missing	78	95	
	Chemotherapy			
-	Pemetrexed + Carboplatin/Cisplatin	188 (54.5%)	159 (43.2%)	*0.003*
-	Paclitaxel + Carboplatin	157 (45.5%)	209 (56.8%)	
	ICIS			
-	Pembrolizumab	218 (63.2%)	142 (38.6%)	
-	Sintilimab	80 (23.2%)	122 (33.2%)	*< 0.001*
-	Other	47 (13.6%)	104 (28.3%)	
	Median ToD[f] of initial 4 ICI infusions per patient (clock hours, hh:mm)			
-	Median [IQR]	10 :47 [10 :16 – 11 :15]	12 :55 [12 :17 – 15 :42]	< 0.001
-	(range)	(8 :53 – 11 :29)	(11 :30 – 18 :11)	
	Within-patient coefficient of variation of ICI infusion ToD (%)			
-	Median [IQR]	7.5 [4.5 – 14.1]	14.1 [6.6 – 19.8]	< 0.001
-	(range)	(0 – 35)	(0 – 28)	
	Number of ICI+chemotherapy courses per patient			
-	Median [IQR]	4 [4 - 6]	4 [4 - 4]	0.005
-	(range)	(1 – 26)	(1 – 26)	
	Total number of ICI infusions (+/- chemotherapy) per patient			
-	Median [IQR]	14 [6 - 21]	7 [4 - 17]	< 0.001
-	(range)	(1 – 49)	(1 – 40)	
	N of patients with local radiotherapy post ICI-chemotherapy (%)	54 (15.7%)	20 (5.4%)	< 0.001

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Figure S1: Distribution of per-patient median ToDA of ICI infusion onsets, for the initial 4 courses of immunochemothe

Left, Cohort1; *Right*, Cohort 2.

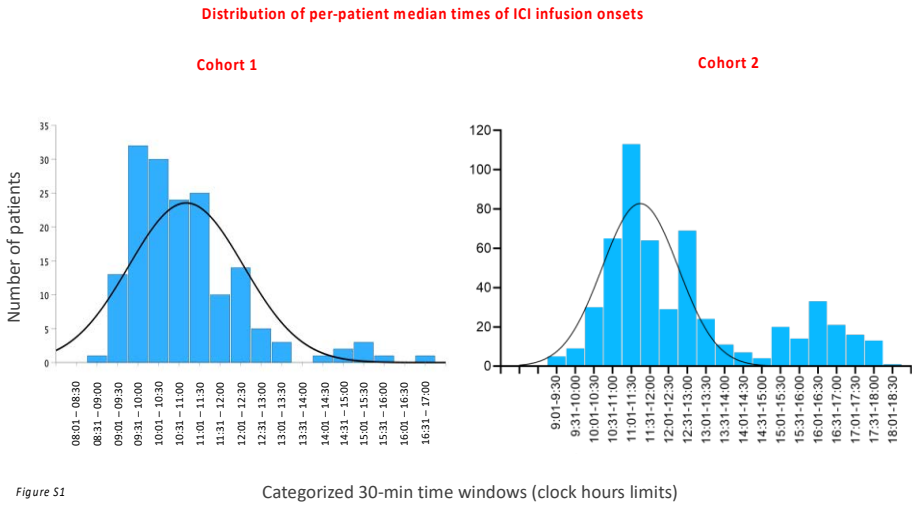

Figure S2: Overall efficacy of the immunochemotherapy protocols in the pooled cohorts.

(a) Overall survival; (b) Progression-free survival.

Note: median values [95% CL] are 35.5 months [22.5 – 29.7] and 9.0 months [8.3 – 10.0], respectively.

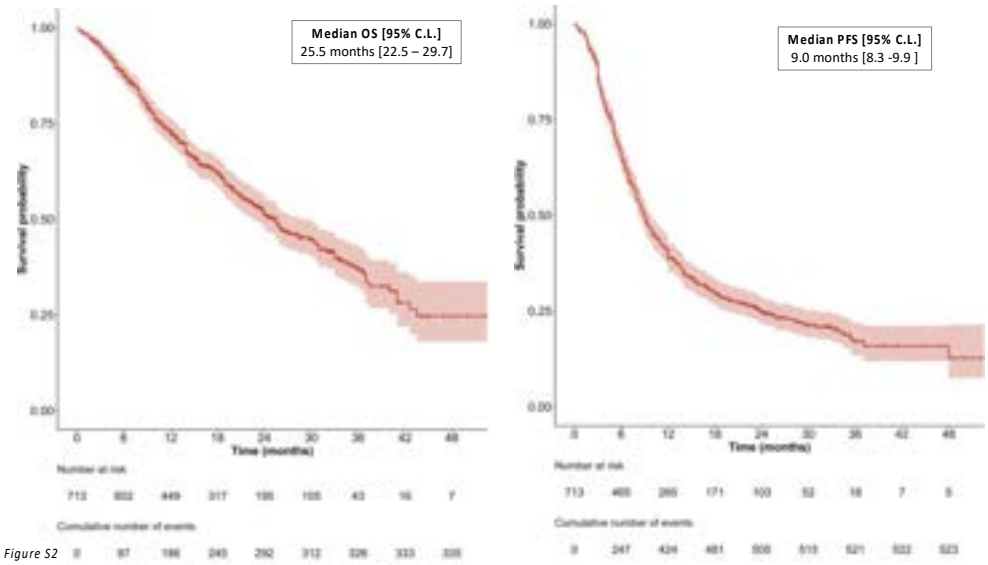

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Figure S3: Hazard Ratios of an earlier death as a function of cut-off ToDA of immunochemotherapy in Cohort 1 (upper panel, S3 a) and Cohort 2 (lower panel, S3b). *Left columns,* tested ToDAs located 30-min apart, i.e. from 10:30 (bottom row) to 13:00 (upper row); *Middle columns*, Unadjusted HRs and their respective 95% CL for each tested ToDA. *Right figure displays* corresponding unadjusted HRs with their respective 95% Confidence Limits.

Note: ToDA at clock hour 11:30 appears as the first timepoint that consistently differentiates the survival of the patients receiving the majority of ICI infusions Before vs After 11:30. Overlapping 95% CI indicate that using later ToDA as cut-off points may not be more discriminating compared to 11:30.

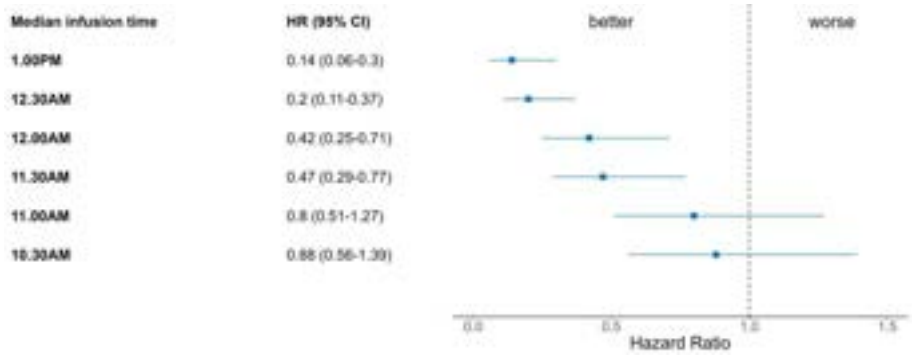

Figure S3a

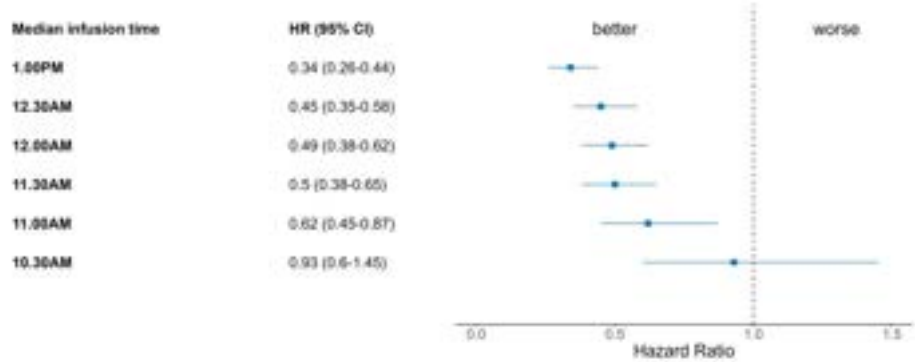

Figure S3b

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Figure S4: Hazard Ratios of an earlier progression as a function of cut-off ToDA of immunochemotherapy in Cohort 1 (upper panel, S4a) and Cohort 2 (lower panel, S4b). *Left columns,* tested ToDAs located 30-min apart, i.e. from 10:30 (bottom row) to 13:00 (upper row); *Middle columns*, unadjusted HRs and their respective 95% CL for each tested ToDA; *Right figure* display: corresponding unadjusted HRs with their respective 95% Confidence Limits.

Note: ToDA at clock hour 11:30 appears as the first timepoint that consistently differentiates the progression-free survival of the patients receiving the majority of ICI infusions Before vs After 11:30. Overlapping 95% CI indicate that using later ToDA as cut-off points may not be more discriminating compared to 11:30.

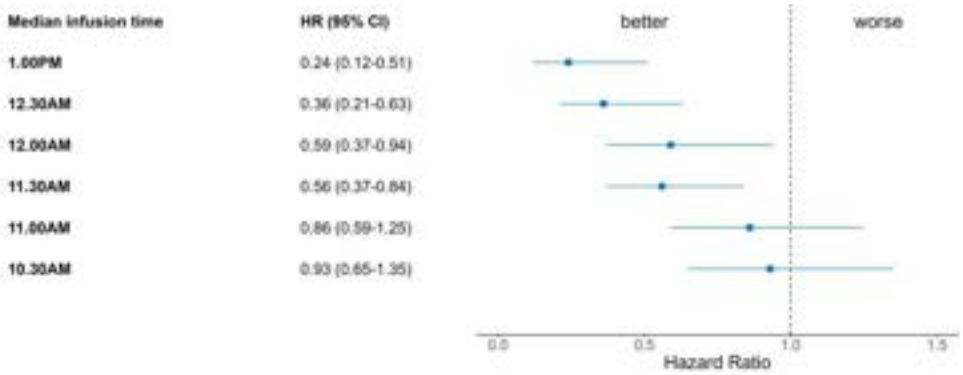

Figure S4a

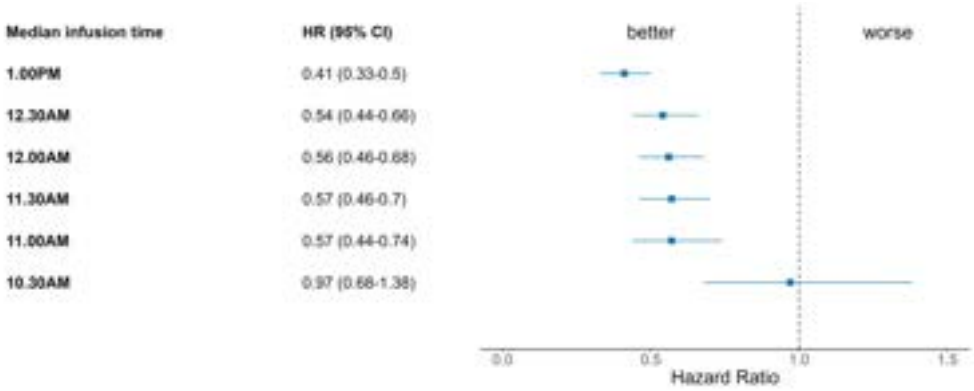

Figure S4b

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Figure S5: Overall survival curves as a function of ICI ToDA in Cohort 1 (Left panel, a) and Cohort 2 (Right panel, b). For Cohort1, a majority of the 4 initial immunochemotherapy courses started before 11:30 in 123 patients, and after 11:30 in 42 patients**.** Both curves differed significantly (p<0.0021), with median survival times of 23.7 months [17.6– NR] in the "Before 11:30" group, compared to 9.3 [3.4 – NR] in the "After 11:30" group. For Cohort 2, a majority of the 4 initial immunochemotherapy courses started before 11:30 in 222 patients, and after 11:30 in 326 patients**.** Both curves differed significantly (p<0.0001), with median survival times of 36.0 months [32.0 – NR] in the "Before 11:30" group, compared to 20.0 [18.5 – 24.0] in the "After 11:30" group.

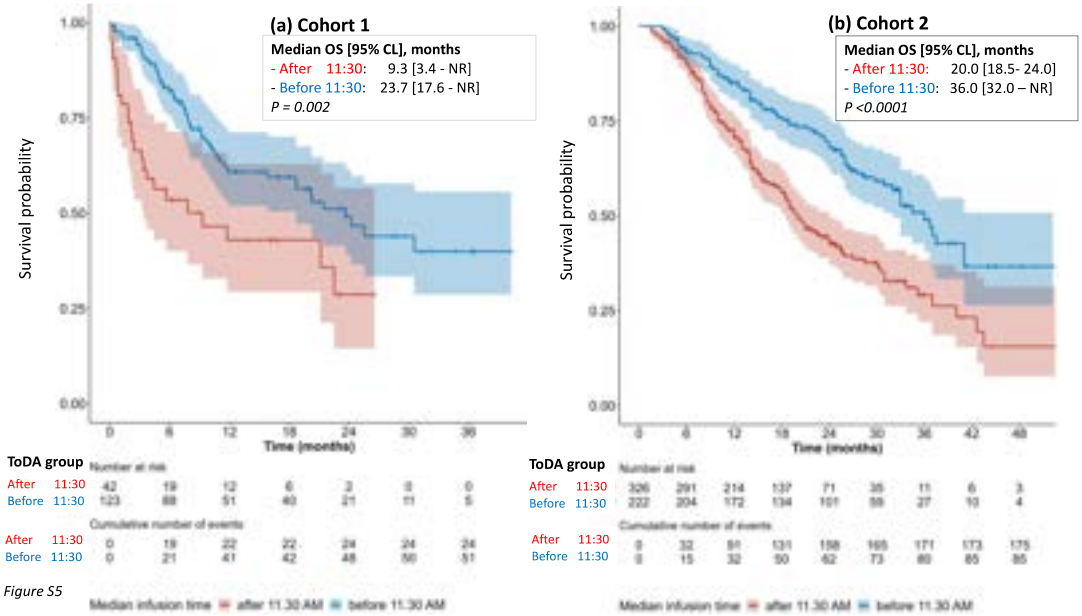

Figure S5

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Figure S6: Progression-free survival curves as a function of ICI ToDA in Cohort 1 (left panel, a), and Cohort 2 (right panel, b). For Cohort1, a majority of the 4 initial immunochemotherapy courses started before 11:30 in 123 patients, and after 11:30 in 42 patients**.** Both curves differed significantly (p = 0.005), with median PFS of 8.0 months [6.3 – 11.8] in the "Before 11:30" group, compared to 3.5 [2.1 – 8.7] in the "After 11:30" group. For Cohort2, a majority of the 4 initial immunochemotherapy courses started before 11:30 in 222 patients, and after 11:30 in 326 patients**.** Both curves differed significantly (p<0.0001), with median survival times of 14.0 months [11.4 – 18.0] in the "Before 11:30" group, compared to 7.8 months [7.0 – 8.8] in the "After 11:30" group.

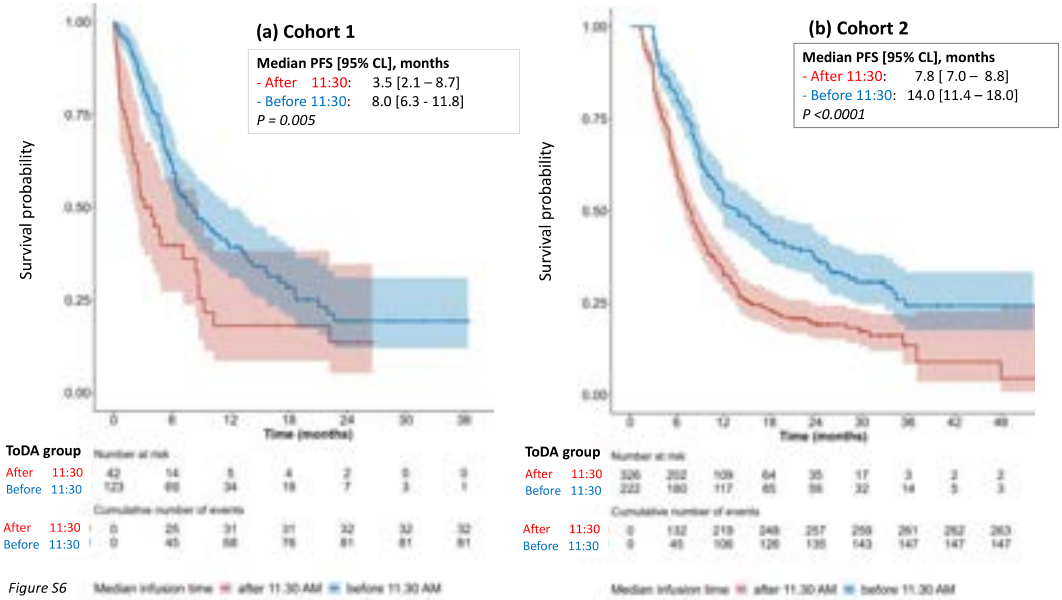

Figure S6

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Figure S7: Best response rates in the "Before 11:30" and the "After 11:30" groups in each cohort and in both pooled. *Note statistically significant larger response rates in the "Before 11:30" groups compared to the "After 11:30" groups for each cohort.*

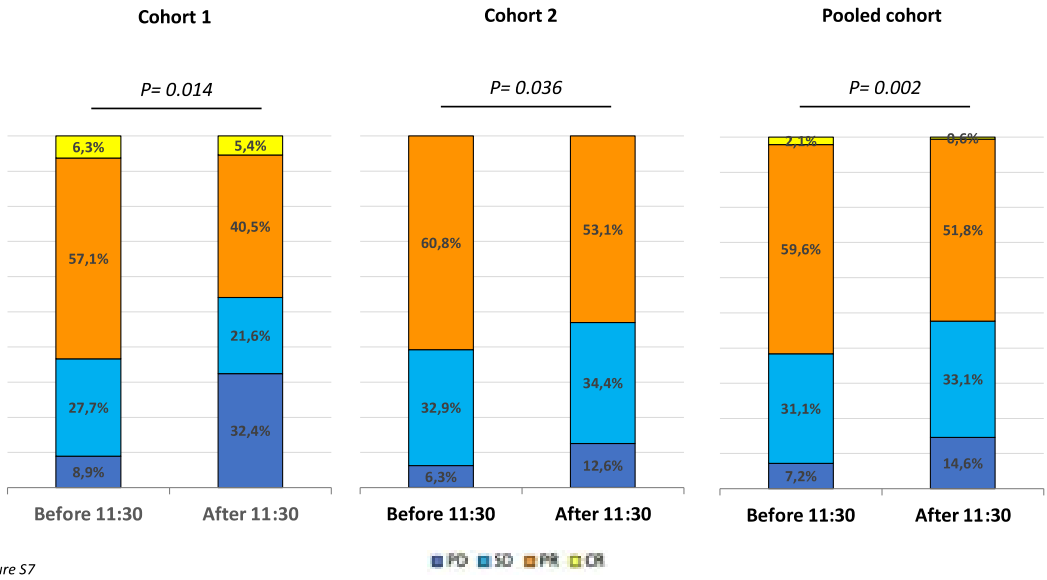

Figure S7

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Figure S8: Multivariable analysis of OS using 11:30 as cut-off ToDA.

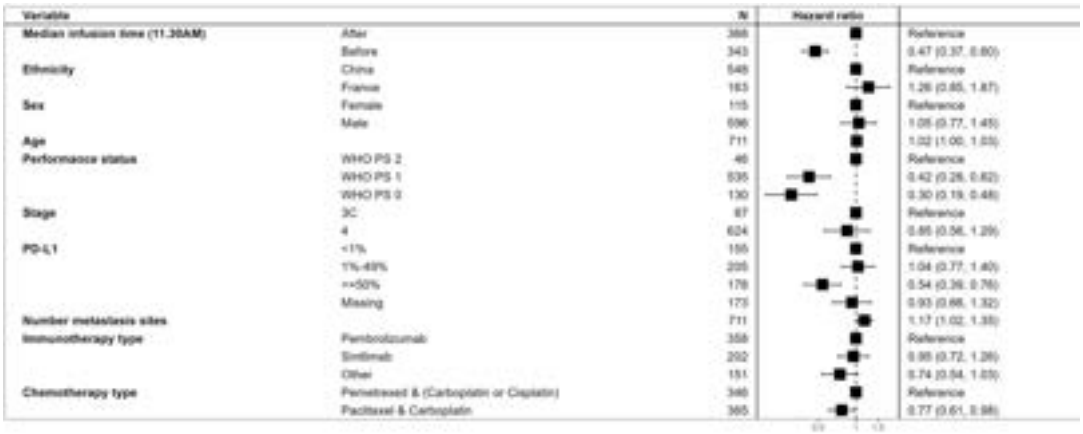

Figure S8

Figure S9: Multivariable analysis of PFS using 11:30 as cut-off ToDA

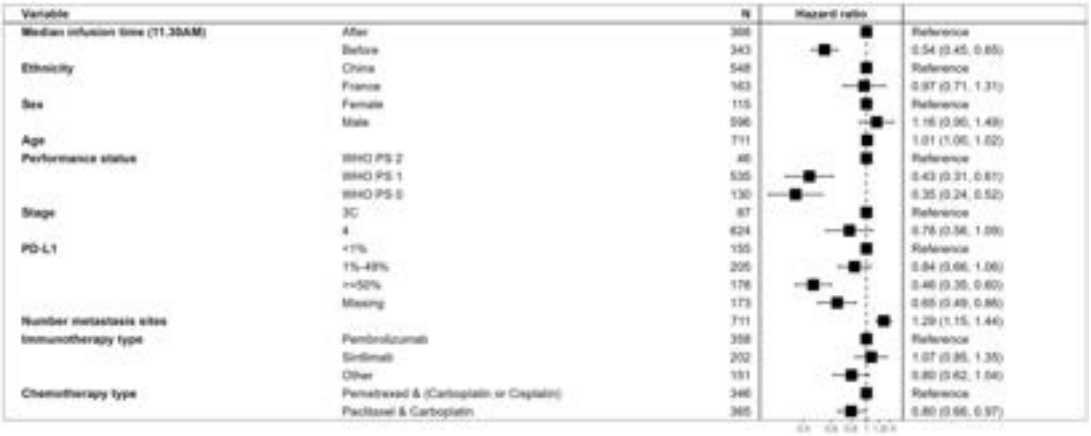

Figure S9

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4.4 AUTRES PRODUCTIONS SCIENTIFIQUES EN RAPPORT AVEC LA CHRONOTHERAPIE DES CANCERS

4.4.1 Variations circadiennes de l'excrétion urinaire de nucléosides modifiés et leurs modifications sous chimiothérapie chez des patients atteints de cancer colorectal métastatique.

Article n°5, *Sci Rep . Décembre 2021*

S. Dulong, Q. Huang, P. F. Innominato, ***A. Karaboué****, M. Bouchahda, A. Pruvost, F. Théodoro, L. A. Agrofoglio, R. Adam, B. Finkenstädt & F. Lévi*

Les niveaux urinaires de nucléosides modifiés reflètent le renouvellement des acides nucléiques et peuvent servir de biomarqueurs non invasifs pour surveiller la dynamique circadienne des tumeurs et les réponses au traitement chez les patients atteints de cancer colorectal métastatique. Chez 39 patients, l'excrétion urinaire médiane pendant la nuit de la pseudouridine déterminée par LC-HRMS était environ dix fois plus importante que celle de la 1-méthylguanosine, de la 1-méthyladénosine ou de la 4-acétylcytidine, et environ 100 fois plus importante que celle de l'adénosine et de la cytidine. L' augmentation de l'excrétion d'un ou plusieurs nucléosides après administration de chimiothérapie a précédé l'augmentation de l'antigène carcinoembryonnaire plasmatique de 1 à 2 mois et a été associée à une diminution apparente de la survie. Dix volumes urinaires fractionnés ont été recueillis pendant deux jours chez 29 patients. La valeur

médiane de l'excrétion urinaire de nucléosides ajustée au rythme variait de 64,3 pour la pseudouridine à 0,61 μg/g de créatinine pour la cytidine. Les enregistrements du rythme d'activité-repos ont révélé que les amplitudes reltives à la moyenne des 24 heures des excrétions de six nucléosides étaient significativement associées à la durée du repos. Ceci confirme l'existence d'un lien étroit entre le métabolisme des nucléosides et le rythme d'activité-repos. De plus, l'amplitude du rythme de la 1-méthylguanosine était corrélée à l'indice de dichotomie du rythme circadien d'activité-repos, un facteur pronostique indépendant et significatif de la survie des patients dans deux méta-analyses. En conclusion, la dynamique de l'excrétion urinaire des nucléosides modifiés semble utile pour caractériser le contrôle circadien de la prolifération cellulaire et détecter précocement l'activité des traitements chez les patients atteints de cancer colorectal.

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Circadian and chemotherapy-related changes in urinary modified nucleosides excretion in patients with metastatic colorectal cancer

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Urinary levels of modified nucleosides reflect nucleic acids turnover and can serve as non-invasive biomarkers for monitoring tumour circadian dynamics, and treatment responses in patients with metastatic colorectal cancer. In 39 patients, median overnight urinary excretion of LC-HRMS determinations of pseudouridine, was ~ tenfold as large as those of 1-methylguanosine, 1-methyladenosine, or 4-acetylcytidine, and ~ 100-fold as large as those of adenosine and cytidine. An increase in any nucleoside excretion after chemotherapy anticipated plasma carcinoembryonic antigen progression 1–2 months later and was associated with poor survival. Ten fractionated urines were collected over 2-days in 29 patients. The median value of the rhythm-adjusted mean of urinary nucleoside excretion varied from 64.3 for pseudouridine down to 0.61 for cytidine. The rhythm amplitudes relative to the 24-h mean of 6 nucleoside excretions were associated with rest duration, supporting a tight link between nucleosides turnover and the rest-activity rhythm. Moreover, the amplitude of the 1-methylguanosine rhythm was correlated with the rest-activity dichotomy index, a significant predictor of survival outcome in prior studies. In conclusion, urinary excretion dynamics of modified nucleosides appeared useful for the characterization of the circadian control of cellular proliferation and for tracking early responses to treatments in colorectal cancer patients.

Colorectal cancer ranks as the second cause of cancer-related deaths, which makes it critical to detect and treat this malignancy as early as possible in order to enhance survival and cures. Elevated circulating levels in carcinoembryonic antigen (CEA) and carbohydrate antigen CA19.9 have proven their clinical usefulness for assessing treatment responses and detecting early relapse[1]. More recently, circulating tumor cells, cell-free DNA and RNA or exosomes, have been proposed as blood biomarkers for monitoring colorectal malignancies[2]. The several nucleosides that result from DNA or RNA breakdown undergo post-transcriptional chemical modifications yielding ultrafilterable modified nucleosides that cannot be re-utilized or be degraded further, and are excreted as such into the urines. These modified nucleosides belong to the family of the ribosyl-nucleosides, twenty of which have been proposed as tumor markers for cancers of the colon or liver[3,4], breast[5–7] or lung[8]. More specifically, the isomerisation of uridine, a nucleic acid base that composes RNA, results in pseudouridine, which is incorpored into tRNA and released in the systemic circulation then ultrafiltrated into the urine

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following nucleic acids breakdown, primarily including tRNA[9]. The desamination of adenosine yields inosine. The methylation of common bases that compose DNA or RNA lead to the formation of 1-methyladenosine, 1-methylinosine, 1-methylguanosine, and N2-N2-dimethylguanosine. Modified DNA base 4-acetylcytidine results from the desacetylation of cytidine. All these biochemical reactions occur at post-transcriptionnal level, and have been reported as being enhanced in cancer, as a result of increased cellular metabolism and accelerated RNA turnover in tumor cells[10,11].

The current study aimed to determine whether these modified nucleosides could serve as non-invasive and quantifiable markers of dynamic changes in tumors, along the 24-h and/or after chemotherapy. Indeed, 24-h changes have been documented for several circulating tumour biomarkers, including carcinoembryonic antigen (CEA) and CA125[12–14], as well as for urinary excretion patterns of polyamines or myelomatous immunoglobulin[15]. While rhythms were scarcely detectable in groups of patients, strikingly rhythmic patterns were demonstrated in individual patients[12,14]. The identification of such patient-specific circadian control of cancer processes could in turn help optimize treatment timing and enhance efficacy.

The circadian timing system (CTS) is a hierarchically coordinated network of molecular clocks that regulates mammalian physiology, as well as cellular metabolism, proliferation, and survival along the 24-h[16,17]. The disruption of the rhythms that are generated by the CTS has been associated to disease processes and impaired treatment effects (for review see[18–20]). Circadian rhythms are generated at single-cell level by molecular clocks, consisting of interwoven transcriptional translational feedback loops involving some 15 known specific "*clock*" genes including Bmal1, Clock, Period 1 and 2, Cryptochrome 1 and 2 and Rev-erbα[21,22]. The molecular clocks are coordinated at the whole organism level by the suprachiasmatic nuclei, a hypothalamic pacemaker, which also helps circadian rhythms adjust to light–dark and other environmental 24-h cycles through the rhythmic control of rest-activity, body temperature, feeding, as well as cortisol and melatonin secretions and autonomic nervous sytem activities[1]. Both glucocorticoids and body temperature rhythms reset molecular clocks and cellular circadian rhythms both in vitro and in vivo[23–27].

The evaluation of CTS robustness or disruption in healthy or sick humans during their daily routine has mostly involved the non-invasive recording of the rest-activity rhythm, using a wrist-worn accelerometer. In cancer patients, the relative amount of activity in bed that was below the median activity out of bed, the so called dichotomy index I < O, has been identified as an independent predictor of progression-free survival and overall survival among 436 patients with metastatic colorectal cancer[28,29].

Here we determined the urinary excretions of up to 8 nucleosides in patients with metastatic colorectal cancer recruited in two studies. We assessed inter- and intra-patient changes in these potential biomarkers along the 24 h (St1), or before, during and after chronochemotherapy (St2). We linked the 24-h variations in nucleosides excretion with the rest-activity circadian rhythm and characterized the potential clinical significance of their dynamics.

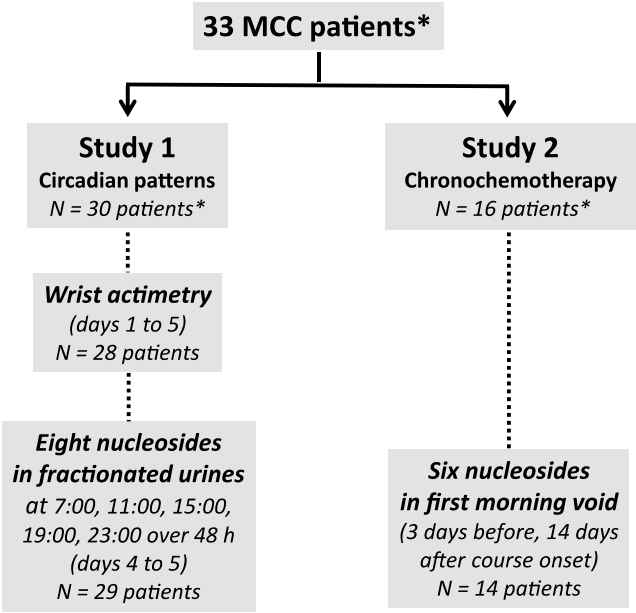
Results

Patients characteristics. Both observational studies (St) were conducted in a total of 33 patients with metastatic colorectal cancer. Thirteen patients participated to both studies at various stages of their disease (Fig. 1, Table 1). St1 recruited 30 patients, the majority of whom had a WHO performance status of 0 (N = 23, 76.7%), primary colon cancer (N = 66.7%), and liver metastases (N = 20, 86.7%). Thirteen patients had two or more metastatic sites (43%). All but one patient had received prior chemotherapy, with 2 prior protocol lines being administered for 93.3% of the patients. Sixteen patients (53.8%) had undergone prior surgery of metastases. Five-days wrist watch monitoring was complete for 28 out of 30 patients (93.3%). There were two technical failures, with no data collection. Two hundred and ninety-three urine samples were collected. The number of samples was insufficient for a single patient. Overall, a total of 226 nucleoside excretion data points were available for analysis in 29 patients, corresponding to a protocol compliance rate of 95.9%. St2 involved 16 patients who received a chronochemotherapy protocol. The majority of the patiens had colon cancer (N = 11, 69%) with liver metastases (N = 12, 75%). 94% of them had prior resection of the primary tumor (N = 15), and 63% of them also had prior metastases surgery (N = 10). The majority of the patients (N = 14, 88%) had received more than 2 lines of chemotherapy. A total of 239 urinary samples were analysed. Overall, nucleosides excretions were determined in 465 urinary samples in both studies.

Overnight nucleosides excretions in the absence of treatment. Within each St, the early morning levels of urinary nucleoside excretion, corresponding to overnight elimination into the urine, varied by up to nearly 100-fold according to nucleoside type in the absence of any treatment for the past 2 weeks (Fig. 2). The excretion of pseudouridine was nearly tenfold as large as those of 1-methylguanosine, 1-methyladenosine and N2-N2-dimethylguanosine, and 20–30-fold as large as those of 4-acetylcytidine and 1-methylinosine. The nucleosides with lowest excretions were adenosine and cytidine. Interpatient variability was large with coefficients of variation ranging from 84.2% for adenosine in St 1 to 21.8% for 4-acetylcytidine in St 2, with a median value of 46.5% [IQR, 33.9 to 60.9%] across nucleosides and studies. Two-sample *t*-test revealed that the overnight excretion of pseudourine was higher in patients with co-morbidities ($p < 0.001$), that of 1-methyladenosine was higher in female as compared to male patients ($p = 0.039$), and that of adenosine was higher in those patients with primary colon rather than rectum cancer ($p = 0.015$).

Twenty-four hour changes in urinary nucleosides excretions. In St 1, detectable urinary levels were found in all the patients for eight nucleosides, but not for 8-hydroxy-2′-deoxyguanosine.

Average 24-h excretions (mesors). The median level of urinary nucleoside excretion mesors varied by up to 110-fold according to nucleoside type among the 29 patients (Fig. 3.1). Thus, the excretion of pseudouridine



* 13 patients participated in both studies

Figure 1. Consort diagram of both studies in patients with metastatic colorectal cancer (MCC). Thirteen patients participated in both studies.

(median, 64.3 µg/g of creatinine) was nearly tenfold as large as those of 1-methylguanosine, 1-methyladenosine and N2-N2-dimethylguanosine, and 20–30-fold as large as those of 4-acetylcytidine and 1-methylinosine. The nucleosides with lowest excretions were adenosine and cytidine (medians of 0.69 and 0.61 µg/g of creatinine respectively). Large inter-patient variations were demonstrated for the urinary excretion of the modified nucleosides, with coefficients of variation ranging from 25.0% (pseudouridine) to 42.3% (1-methylguanosine).

The mesors of nucleosides excretion were correlated among patients, except for cytidine. More specifically, two clearly distinct groups of inter-correlated nucleosides were identified (Fig. 3.2). A first one, involving pseudouridine, 1-methyladenosine, 1-methylinosine, and adenosine, and a second one involving 1-methylguanosine, N2-N2-dimethylguanosine and 4-acetylcytidine.

Twenty-four-hour patterns. Using multiple-component cosinor analysis, the number of nucleoside excretion rhythms per patient ranged from null to 7, with a median of 3. Raw nucleosides measurements and cosinor model fitting are shown as examples for three patients (A, B and C) (Fig. 4). Compared with the relatively flat fitting curves for patient B and C, patient A displayed a large circadian variability in nucleosides excretion and consistent day-to-day patterns. As a result, patient A had rhythmic excretions for 7 nucleosides, with significantly reproducible 24-h patterns (plotted with solid lines), while patient B had 3 excretion rhythms and patient C a single one.

Overall, the urinary excretion of each nucleoside displayed a 24-h rhythm for 4-acetylcytidine in 6 of 29 patients (23.1%), and for pseudouridine in 11 of 29 patients (37.9%). A 12-h rhythm was also found 7 of 29 patients (24.1%) for pseudouridine and for both 1-methylguanosine and 1methyladenosine in 5 of 29 patients (17.2%). Thus a 24-h and/or a 12-h rhythm characterized pseudouridine urinary excretion for 14 of 29 patients (48.3%) of the patients (highest rate) as compared to 4-acetylcytidine for 10 of 29 patients (34.6%) of the patients (lowest rate).

Statistically significant correlations were found for the urinary excretion rhythm amplitudes as % of mesor for 1-methylinosine (Spearman's correlation with p-value = 0.001), 1-methylguanosine ($p < 0.001$), 1-methyladenosine ($p = 0.004$), N2-N2-dimethylguanosine ($p < 0.001$), cytidine ($p < 0.001$) and 4-acetylcytidine ($p = 0.017$). Circadian mesor, amplitude and acrophase estimates for the eight nucleosides excretions are summarised in Table 2.

	St1	St2
Number of patients	30	16
Sex		
Male	18	8
Female	12	8
Age, years		
Median	66.2	67.5
Range	23–74	44–81
Performance status (WHO)		
0	23	12
1	7	4
Primary site		
Colon	20	11
Rectum	10	5
Metastatic site		
Liver	22	12
Lung	10	8
Others	18	10
Number of metastatic sites		
1	17	6
2	6	3
3	7	7
Comorbidities		
Yes	14	10
No	16	6
Prior primary tumor surgery		
Yes	27	15
No	3	1
Prior metastatic surgery		
Yes	16	10
No	14	6
Number of prior chemotherapy lines		
0	1	0
1	1	2
2–3	22	11
More than 3	6	3
Prior chemotherapy modality		
Chronotherapy	23	12
Conventional	7	4
Plasma CEA (mg/L)		
< 5	6	3
6–100	17	10
> 100	4	2
Unknown	3	1

Table 1. Main characteristics of the patients in both studies (St).

Circadian patterns in rest-activity. The 24-h pattern in rest-activity of the 28 patients in St 1 ranged from robust to weak as indicated by the results from Hidden Markov modelling, dichotomy index I < O, autocorrelation coefficient r24, circadian period estimates and 24-h cosinor estimates of amplitude and acrophase (Table S1). Interestingly, the median I < O value was 97.9%, in good agreement with prior studies on larger sample sizes, where this value was 97.5%[28]. Interpatient differences in rest-activity patterns were obvious (Fig. 5). The timeseries of wrist-watch activity measurements, the corresponding displays resulting from HMM, and the temporal distribution of High, Intermediate and Low Activities over the 24 h are shown in Fig. 5, for the same three patients as those taken as illustrative examples for nucleosides rhythmic excretions (Fig. 4). Patient B had a regular and robust rest-activity rhythm, patient C had a severely disturbed pattern and patient A displayed an intermediate profile.

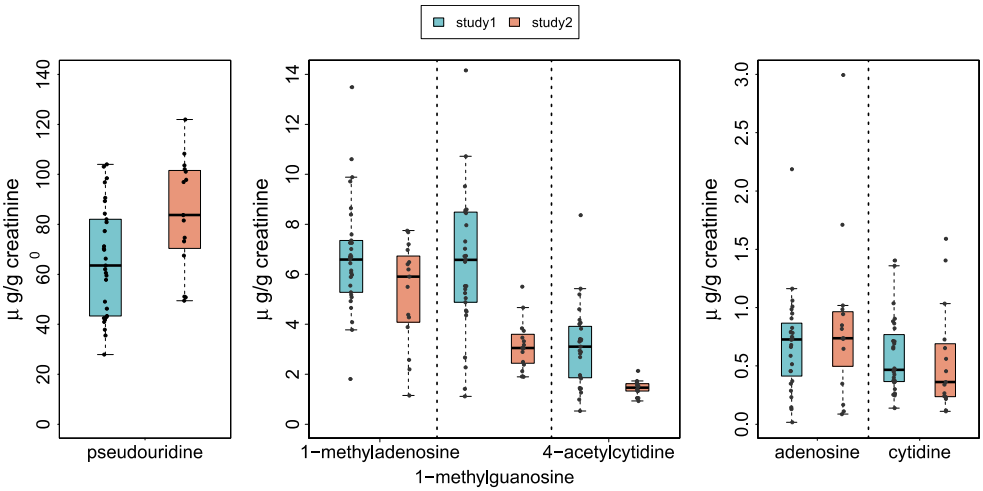


Figure 2. Distribution of early morning urinary excretion values of the six modified nucleosides at baseline in both studies. Nucleosides excretions are ranked from highest (pseudouridine) to lowest (cytidine). Median values, interquartile and extreme values are shown as boxplots for each nucleoside in each study. Slight differences in median values and/or range of pseudouridine, 1-methylguanosine and 4-acetylcytidine excretions between patients in St 1 and patients in St 2.

The dichotomy index I < O, the Rhythm Index, the transition probability of remaining in IA state (p1-1), and the autocorrelation coefficient at 24-h (r24) were strongly correlated with each other, as the Spearman rank coefficients ρ ranged from 0.49 ($p = 0.009$) between I < O and the Rhythm Index, up to 0.80 ($p < 0.001$) between I < O and p1-1. The median level of the HA state was also correlated with both I < O ($\rho = 0.40$ with $p = 0.033$) and r24 ($\rho = 0.58$ with $p = 0.001$). The overall daily rest duration was negatively correlated with the Rhythm Index ($\rho = -0.49$ with $p = 0.007$) (Fig. S1).

Multivariate regression analysis revealed that a good performance status (PS = 0) was significantly associated with high values in I < O ($p = 0.007$), p1-1 ($p < 0.001$), and Rhythm Index ($p = 0.007$). Female sex was associated with high values in p1-1 ($p = 0.024$) and median activity at HA state ($p = 0.011$), and possibly with high values in both I < O and r24 ($0.05 \leq p < 0.1$).

Relations of nucleosides excretion changes with patient characteristics and rest-activity pattern. Multivariate regression analysis showed that the mesor of 1-methylguanosine excretion was higher in patients with a performance status of 1 as compared to those whose PS was 0 ($p = 0.026$). The mesor of pseudouridine and that of adenosine were lower in the patients who had prior liver metastases surgery ($p = 0.049$ and $p = 0.007$, respectively). The circadian amplitudes of adenosine and N4-acetylcytidine were higher in female as compared to male patients ($p = 0.011$ and $p = 0.01$, respectively).

Spearman's rank correlation analysis revealed striking relations between the relative circadian amplitude of the 24-h patterns of urinary nucleosides excretions and rest-activity indices (Fig. 6). For example, the larger the rest duration, the larger the relative circadian amplitude of pseudouridine ($p = 0.015$), adenosine ($p = 0.049$), 4-acetylcytidine ($p = 0.008$) and cytidine ($p = 0.002$). Moreover, the more robust the rest activity rhythm, as indicated with a higher I < O, the higher the relative circadian amplitude of 1-methylguanosine ($p = 0.0182$).

Changes after chronochemotherapy, and relations with outcomes. No urinary excretion data were available for one patient in St2. Chronochemotherapy administration was followed with increasing or decreasing trends in overnight urinary nucleosides excretions in six patients or no change in the other nine patients (see four illustrative examples (one with positive, one with negative, two with no trend for cytidine in Fig. S2). The data thus highlighted patient-specific treatment responses and dynamics, which were recapitulated by the changes in 1-methylguanosine, acetylcytidine, adenosine and cytidine. Having an increasing or a decreasing trend (slope of regression line with p-value ≤ 0.05) in these urinary nucleosides after chemotherapy was associated with a > 50% delayed increase in plasma CEA (Fisher's exact test p-value = 0.0275) and a poor survival (two-sample t-test, p-value = 0.0054) (Table 3). In contrast, no change ($p > 0.05$) in nucleosides was associated with no change in plasma CEA, no toxicity > Grade 1, and a far better survival in this limited sample of metastatic colorectal cancer patients.

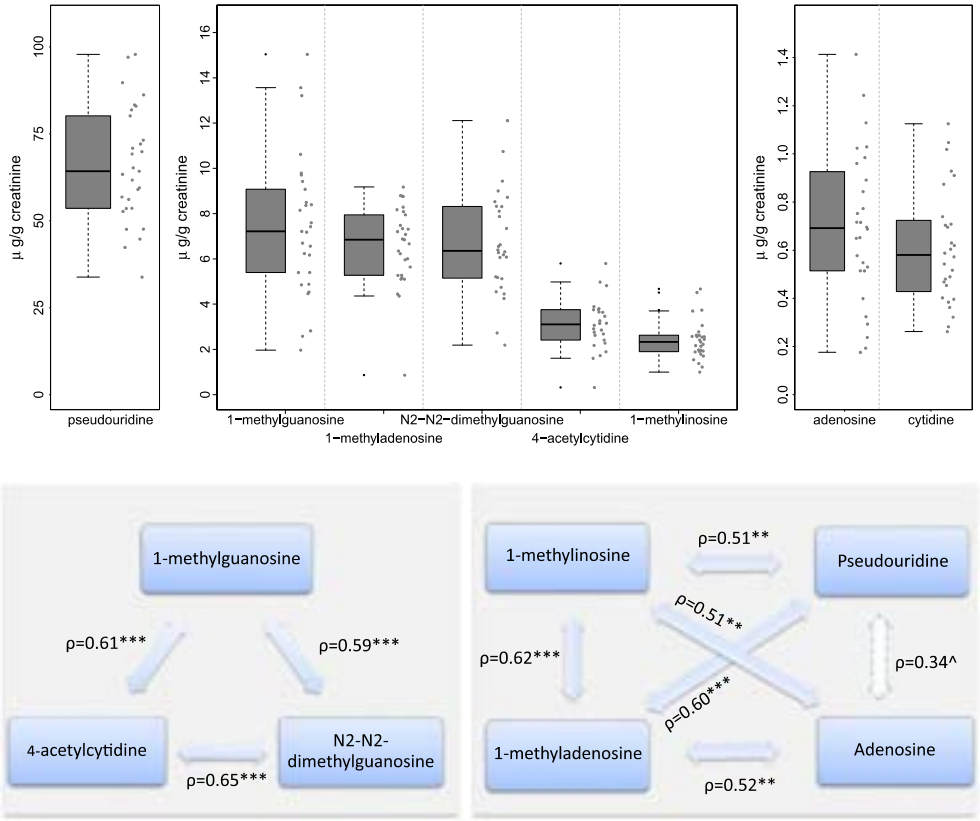


Figure 3. Distribution of urinary excretion mesor values for the 8 modified nucleosides and their intercorrelations in St 1. (1) Mesor estimates with the boxplots (median, interquartile and extreme values) for each nucleoside, ranked from highest to lowest median excretion values. (2) Diagram of intercorrelations. Spearman's rank correlation coefficients ρ with stars indicating p-value levels, i.e. 0 '***' 0.001 '**' 0.01 '*' 0.05 '^' 0.1. Insignificant correlation in bootstrap uncertainty check, i.e. pseudouridine and adenosine, is marked with dashed line.

Discussion

In patients with previously treated metastatic colorectal cancer, the average overnight urinary excretions of six modified nucleosides varied by over 100-fold according to the nucleoside considered, being highest for pseudouridine, intermediate for 1-methyladenosine, 1-methylguanosine and 4-acetylcytidine, and lowest for cytidine and adenosine. Large differences were found between patients before liver surgery or chronochemotherapy, as well as along the 24-h time scale, that partly related to sex, co-morbidities, and primary colon or rectum site.

Consistently with our findings, large between- and within-patients variations were reported for pseudouridine, 1-methyladenosine and 1-methylguanosine in patients with breast, lung, or gastric cancer. In these patients however cytidine and adenosine urinary excretions were larger, and 4-acetylcytidine was only occasionally determined[10,11,30]. In metastatic colorectal cancer patients, we found striking similarities between the urinary excretions of all the nucleosides in both studies. As compared to results in other cancer studies mentioned in our manuscript, we found similar levels for pseudouridine (~ 100 μg/g of creatinine), N2-N2-dimethylguanosine (~ 6 μg/g of creatinine) and 1-methylguanosine (between 0.43 and 9.3 μg/g of creatinine, pending on the studies , i.e. 6 or 3 μg/g of creatinine respectively). In contrast, large between-studies variabilities stood out for the other urinary nucleosides excretion that possible related to differences in determination techniques, cancer types, and/or stage or grade. Thus, mean urinary excretions ranged from 6.5 μg/g of creatinine (our studies 1 and 2) up to 42 μg/g of creatinine for 1-methyladenosine[11], and from 2 μg/g of creatinine (our studies) up to 36 μg/g of creatinine for 1-methyl1inosine[11]. Moreover, our mean adenosine and cytosine excretion levels were 20 times and 6 times as low as those reported for earlier stage colorectal cancer patients[30,31].

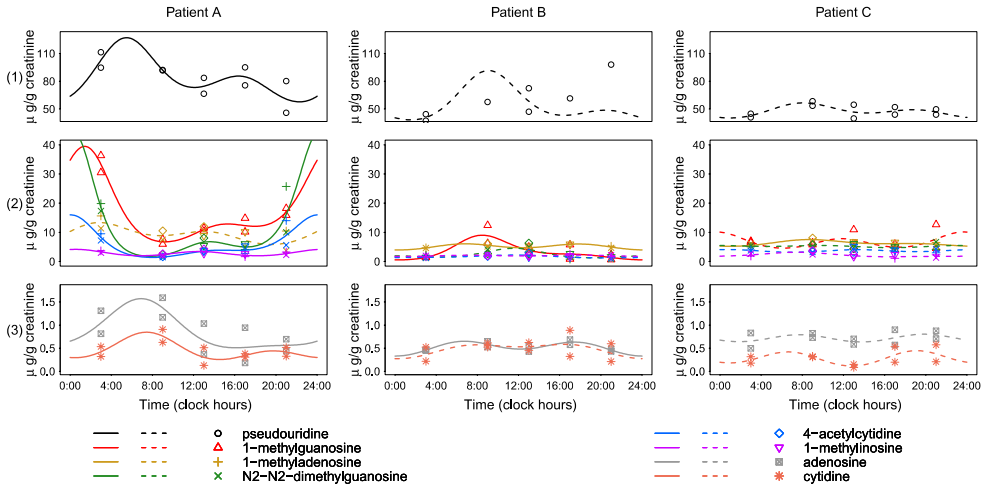


Figure 4. Examples illustrating interpatient differences in 24-h excretion patterns of urinary nucleosides in St 1. Urinary nucleoside excretion measurements (dots) and multiple-component cosinor model fitting (lines) for example patients A, B and C. Nucleosides identified with significant circadian rhythmic pattern are plotted in solid line, while non-significantly rhythmic ones are shown in dashed line. Nucleoside patterns from each patient are plotted in three panels with different ordinate scales.

	1-methylinosine	pseudouridine	1-methylguanosine	1-methyladenosine	N2-N2-dimethylguanosine	adenosine	cytidine	4-acetylcytidine
Number of patients	29	29	29	29	28	28	28	26
Number of patients with significant rhythmicity pattern	7	11	10	10	9	9	10	6
Mesor (μg/g creatinine)	2.41 (0.99–4.67)	66.1 (33.8–97.9)	7.44 (1.97–15.04)	6.57 (0.86–9.18)	6.70 (2.19–12.11)	0.69 (0.18–1.41)	0.61 (0.26–1.13)	3.09 (0.31–5.80)
	2.32 [1.90, 2.63]	64.3 [53.6, 80.2]	7.22 [1.97, 9.08]	6.84 [5.27, 7.95]	6.35 [5.16, 8.31]	0.69 [0.51, 0.91]	0.58 [0.44, 0.72]	3.11 [2.46, 3.73]
Amplitude (μg/g creatinine)	0.98 (0.21–2.95)	26.9 (5.74–60.7)	5.57 (1.02–16.36)	1.94 (0.66–4.69)	5.63 (0.38–21.48)	0.39 (0.08–1.24)	0.36 (0.13–0.81)	2.28 (0.29–7.30)
	0.71 [0.46, 1.31]	26.7 [16.3, 34.7]	5.24 [3.21, 7.28]	1.76 [0.66, 4.69]	4.74 [2.75, 6.55]	0.24 [0.16, 0.53]	0.28 [0.20, 0.52]	1.49 [0.85, 3.18]
Relative amplitude (% mesor)	0.40 (0.09–1.26)	0.42 (0.06–0.99)	0.80 (0.17–2.07)	0.31 (0.15–0.76)	0.79 (0.07–2.59)	0.69 (0.12–3.11)	0.59 (0.24–1.07)	0.73 (0.12–2.51)
	0.33 [0.23, 0.48]	0.41 0.25 0.54]	0.72 [0.59, 1.00]	0.28 [0.20, 0.38]	0.71 [0.49, 1.01]	0.41 [0.31, 0.68]	0.58 [0.43, 0.72]	0.66 [0.32, 0.95]
Acrophase (clock hours and min.)	08:26 (00:06–23:18)	13:06 (01:30–23:42)	13:00 (00:06–23:42)	10:08 (00:01–23:36)	11:13 (00:00–23:30)	10:44 (00:24–23:36)	11:39 (00:06–23:48)	13:30 (00:06–23:54)
	06:42 [02:36, 12:24]	12:54 [09:06, 19:00]	12:00 [7:42, 19:18]	9:48 [04:18, 14:42]	10:24 [05:88, 19:01]	10:27 [05:42, 12:48]	12:27 [05:20, 17:50]	13:06 [06:45, 20:48]

Table 2. Results from cosinor analyses of nucleosides urinary excretion in St 1. For each parameter, i.e. Mesor, Amplitude, Relative amplitude and Acrophase, mean values and (ranges) are reported in upper rows, and median values and [1st, 3rd] quartiles are given in lower rows, using imputed rhythm parameter values from each individual patient.

In our study, chronochemotherapy resulted in patient-specific responses in the overnight urinary excretions of the six modified nucleosides. Increasing, stable, or decreasing trends were captured in the urinary excretions of 1-methylguanosine, 1-methyladenosine, adenosine and cytidine. Consistent increases in urinary nucleosides excretions were related to disease progression on chronochemotherapy (St2). These observations suggest that these four urinary nucleosides levels could reflect cellular proliferation and serve as early markers of efficacy of chemotherapy.

The 24-h mesors of urinary excretion levels of the modified nucleosides in St1 ranked similarly as the overnight excretions that were determined in St2. Eight-hydroxy-2-deoxyguanosine, the ninth modified nucleoside selected for St1, was not detected in 68% of the diluted urine samples. A circadian rhythm in the urinary excretion of this oxidized form of guanosine had been previously reported both in healthy adults and in patients with

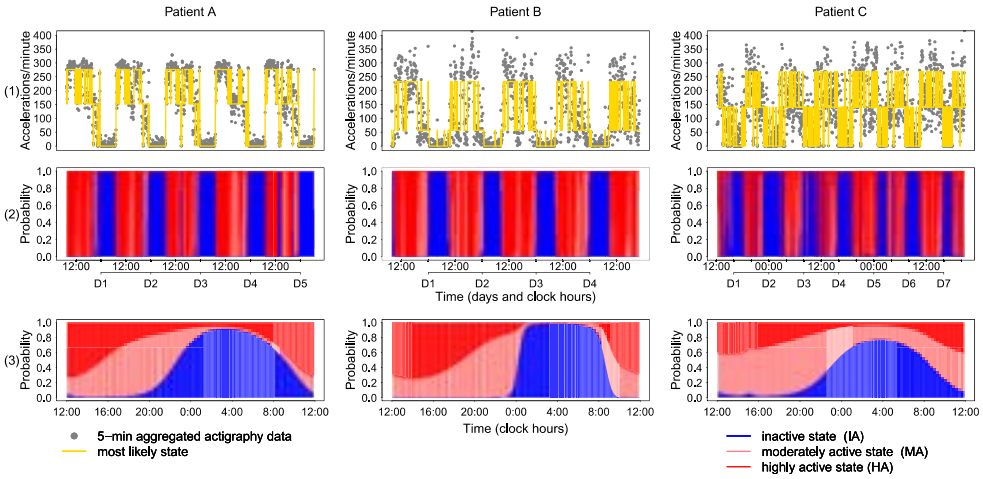


Figure 5. Interpatient differences in 24-h patterns in rest-activity. Profiles of the same three patients in St 1 that were selected for Fig. 4. (1) Plotted time series of wrist watch activity measurements. Time series of activity in dots, and corresponding HMM-decoded most likely states connected with a yellow line (lowest level for IA—inactive, middle level for MA—moderately active, and highest level for HA—highly active). (2) Corresponding most likely states, i.e. IA, MA or HA, with frequent state transitions between MA and HA during daytime. Cumulative state probability plots for IA (blue), MA (light red) and HA (dark red) states. (3) Temporal distribution of the three state probabilities over the 24 h, providing a fingerprint of the individual's typical daily rest-activity behaviour. Patients A and B had solid blue areas (IA) at night while patient C had many interruptions. This was translated into low values for p1-1 and I < O. A sharp incline/decline of the blue IA profile is indicative of regular times for retiring in the evening and awakening in the morning. The prolonged IA period of Patient B started around 0:00–1:00 a.m. and ended around 8:00–9:00 a.m. every day with no noticeable interruptions to the IA state in-between, hence this patient has the highest Rhythm Index among the three patients shown. Values of rest-activity indices: patient A: I < O: 99.7%, Rhythm Index = 67.3%, p1-1 = 0.969, r24 = 0.654; patient B: I < O: 98.2%, Rhythm Index = 91.2%, p1-1 = 0.972, r24 = 0.392; patient C: I < O: 90.6%, Rhythm Index = 54.6%, p1-1 = 0.910, r24 = 0.247.

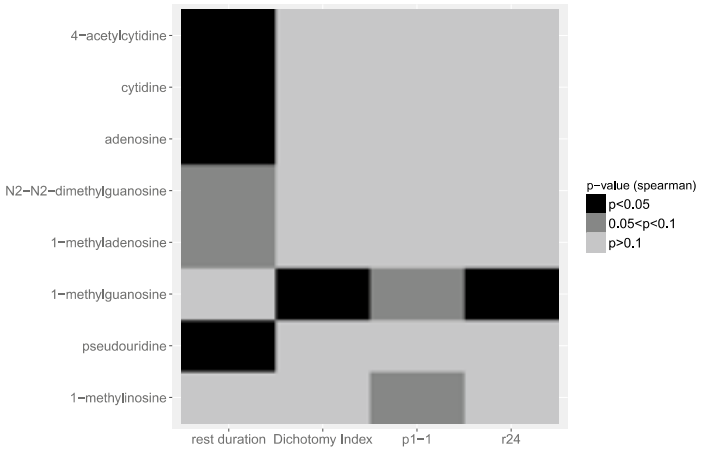


Figure 6. Grid plots of intercorrelations between selected rest-activity indices and urinary nucleoside excretion relative amplitudes in St 1. Black boxes correspond to statistically significant positive (+) or negative (−) Spearman's correlations ($p \leq 0.05$). Dark grey boxes correspond to trends ($0.05 < p \leq 0.1$), and light grey boxes correspond non significant correlations ($p > 0.1$).

Trend in nucleosides excretion	CEA			
	Decrease	No change	Increase	Sum
No trend	1	8	0	9
Trend	0	2	3	5
Sum	1	10	3	14

Table 3. Relations between chemotherapy-related changes in urinary nucleosides and those in plasma carcinoembryonic antigen (CEA) for the patients in St 2. Fourteen patients with both nucleosides and CEA determinations are considered . Changes in plasma CEA are categorized according to whether this tumor marker increased or decreased by more than 50% (trend) or remained stable for at least 1 month and up to 2 months after a course of chronochemotherapy. Changes in nucleosides are classified as trend, in case of a positive or a negative slope of regression line (p-value ≤ 0.05), respectively or as no change in case of no slope (p-value > 0.05). The interaction between nucleoside excretion trend and CEA change was evaluated by Fisher chart, p-value = 0.0275.

multiple sclerosis, but not in diabetic patients[32,33]. Moreover, elevated levels of 8-hydroxy-2-deoxyguanosine were reported in cancer patients[34,35]. Here, owing to the dilution of the samples before injection, this modified nucleoside could only be determined in 32% of the urine samples.

Robust correlations were found between the mesor values of pseudouridine, 1-methyladenosine, 1-methylinosine, and adenosine, on the one hand, and between those of 1-methylguanosine, N2-N2-dimethylguanosine and 4-acetylcytidine on the other hand. Cytidine excretion appeared as independent from that of the other nucleosides.

Circadian and/or 12-h rhythms characterized the urinary excretion of pseudouridine for 48.3% of the patients (highest rate) and that of 4-acetylcytidine for 34.6% of the patients (lowest rate). Individual patients displayed marked rhythmic excretion patterns for up to seven nucleosides. Interestingly, the relative circadian amplitudes of nucleosides excretions were positively associated with the duration of rest for pseudouridine, adenosine, 4-acetylcytidine and cytidine, and with the dichotomy index I < O for 1-methylguanosine (Spearman correlation, $p < 0.05$). These results suggested that the rhythmic excretion patterns of modified nucleosides could reflect a host-mediated circadian control of cancer progression. Indeed, progression-free survival and overall survival were significantly improved in metastatic colorectal cancer patients whose I < O exceeded 97.5%[14]. This finding has been confirmed in other oncological clinical settings[36,37].

A main limitation of our work involves the limited sample size in each St. Healthy controls would also be needed for establishing the physiological relations between host circadian biomarkers and the rhythmic turnover of nucleic acids in healthy tissues, as reflected in the urinary excretions of the modified nucleosides. A circadian rhythm was demonstrated in healthy adults for 8-hydroxy-2′-deoxyguanosine, with high values in the afternoon[33], and for both pseudouridine and N2-N2-dimethylguanosine, with highest excretions occurring between 06:00 and 12:00[38]. The percent variations between average maximum value at acrophase and average minimum value at bathyphase (double amplitudes) were respectively 36%, 25.6%, and 12.7% of the mean for 8-hydroxy-2′-deoxyguanosine, pseudouridine and N2-N2-dimethylguanosine. In a circadian and sleep metabolomic study, sleep deprivation was associated with selective increase or decrease in 16 urinary metabolites[39]. This finding further supported the link between sleep duration and modified nucleosides excretion reported here.

Purine and pyrimidine synthesis and catabolism are rhythmically controlled by the circadian clock in mouse liver[40]. Clock gene Bmal1 silencing ablated these metabolic rhythms. Such Bmal1 silencing could result from host circadian disruption, as shown in mice on chronic jet lag[41]. Here, host circadian disruption was determined using 5-day time series of rest-activity monitoring. The median value of the dichotomy index I < O in our patient population was 97.9%, as compared to 97.5% for our prior cohorts of metastatic colorectal cancer patients, where this parameter was shown as an independent prognostic indicator of progression-free and overall survival[29]. I < O was here correlated with the circadian amplitude of 1-methylguanosine, and it was significantly inter-correlated with other rest-activity parameters that helped assess circadian timing system function in individual patients. The duration of rest over the 24 h was the host parameter, that displayed the strongest links with the rhythmic excretions of modified nucleosides, suggesting that the nucleic acids turnover pattern over the 24 h could be predominantly linked to the rest span. However, such correlations were not significant in the additional bootstrap uncertainty check, with 90th quantiles of Spearman correlation p-value > 0.1, a finding supporting the need for confirmation within a larger sample size. Interestingly, however, short sleep duration has been found to be associated with higher mortality in a large cohort of colorectal cancer survivors[42].

In conclusion, we identified and ranked the main modified nucleosides that are excreted into the urines of patients with colorectal cancer metastases. Large interpatient variations were identified, that could reflect tumour or host nucleic acids turnover. Intrapatient changes in urinary nucleosides excretions over the 24-h occurred in nearly 1/3 to half of the cancer patients, consistently with the robustness of the circadian rhythm in rest-activity in these patients. The clinical relevance of urinary nucleosides excretions as biomarkers was further supported by the association of early changes in modified nucleosides on chemotherapy with disease progression and poor survival.

Methods

Study design. The patients had a histological proof of metastatic colorectal cancer, a performance status of 0 or 1 according to the classification of the World Health Organisation and adequate biology. They had received one, two or three prior chemotherapy protocols and provided a signed informed consent for their participation. Two studies (St) were implemented at the Department of Oncology in Paul Brousse University Hospital (Villejuif, France). The rest activity rhythm was monitored using a wrist-worn piezoelectric accelerometer watch (Mini-Motionlogger Actigraph, Ambulatory Monitoring, Ardsley, New-York, USA) for 5 days in St1 and for 17 days in St2 (data not reported here). The user-defined time interval for the count of wrist accelerations was 1 min. Each patient also completed a sleep and feeding diary questionnaire during the days of recording.

Both studies were sponsored by the National Institute for Health and Medical Research of France (INSERM), approved by the Ethics Committee of Kremlin Bicetre hospital (France) and registered with clinical trial numbers NCT01693835 and NCT01693861, respectively. They were conducted according to the "Ethics and methods for biological rhythm research on animals and human beings" and complied with the in force declaration of Helsinki principles[43].

St1 investigated circadian changes in the urinary excretion of nine nucleosides according to patients' characteristics and rest-activity rhythms. The wrist actigraph was worn continuously for 5 days, including 3 days of baseline, and 2 days during which urinary samples were self-collected (Fig. S3). The patients collected urine samples of 5–20 mL into pre-labelled sterile tubes, at 07:00 or upon awakening, then at 11:00, 15:00, 19:00 and 23:00 or just prior to retiring for two consecutive days, resulting in a total of 10 samples per patient.

St2 investigated changes in the urinary excretions of six nucleosides according to patients' characteristics before, during and after a course of chrono-chemotherapy[44] (Fig. S3). Early morning voids were collected daily for 3 days before and 14 days after the onset of a treatment course. Adverse events were rated after the chemotherapy course that was administered while on study, according to Common Terminology Criteria for Adverse Events (CTCAE) v4.0. Plasma CEA and CA19.9 were determined before and up to 2.5 months following inclusion. The survival of each patient was computed from the date of inclusion to the time of death or the last time known to be alive. These data were updated in March 2019.

Urinary collections and nucleosides determinations. Nine modified nucleosides, namely pseudouridine, 1-methylguanosine, N2-N2-dimethylguanosine, 1-methyladenosine, 4-acetylcytidine, 1-methylinosine, adenosine, cytidine, and 8-hydroxy-2′-deoxyguanosine were determined with mass spectrometry. The quantification of urinary nucleosides was determined using the Q-Exactive, composed of the Orbitrap mass analyzer combined with a quadruple mass filter as front-end. This system was chosen because of its high sensitivity and the linearity performance of the High Resolution Mass Spectrometry (HRMS) based quantification[45]. Three of the nine nucleosides to be monitored were selected to be used as labeled internal standards for the entire quantification process (adenosine [ribose-13C5], 8-hydroxy-2′-deoxyguanosine (15N5) and 1-methyl-adenosine-D3). Due to the high concentration of most nucleosides in urine, the technique of "dilute and shoot" was chosen[46]. A dilution of the urine sample in water by a factor of 5 was applied as a pre-treatment overcoming the major matrix effects encountered in LC/MS bioanalysis techniques. Then, a 10 µL aliquot of the diluted urine was injected into the analytical system. The ultra-high pressure chromatographic (UHPLC) method was developed using a reverse phase column. The separation was performed on a column Acquity UPLC BEH Shield 1.7 µm 2.1 × 100 mm (Waters, France). Mobile phases were distributed in a gradient mode (Dionex Ultimate 3000 system). Detection by high-resolution mass spectrometry in full scan mode was accomplished using a Q-Exactive (Orbitrap technology, ThermoFisherScientifics) after ionization in positive mode. The total analysis runtime was 7 min. This method was successfully validated according to the European recommendations[47].

Statistical analysis. *Hidden Markov Model (HMM) for quantifying of circadian rhythmicity in telemetric activity data.* A recently developed harmonic Hidden Markov Model (HMM) approach[48] was applied to compute numerical quantifiers associated with sleep quality and circadian rhythm in actigraphy data. The HMM is a well-known statistical model which assumes that the observed time series data are a realization of a Markov process with unobserved states. In this study, the HMM approach was applied to threshold the actigraphy measurement into three states in a probabilistic way, namely inactive/rest state (IA), moderately active (MA) state and highly active (HA) state. The following circadian rhythm associated parameters derived from HMM[34] were p1-1 (the transition probability of staying in IA), which describes the probability of non-interrupted inactive/rest; median activity value at HA state; rest duration (integral of IA profile over the 24 h, i.e. dark grey area *a* in Fig. S4; center of rest time (gravity center of IA, i.e. *c* in Fig. S4); Rhythm Index, which assumes a value of 100% for a subject with regular daily bedtimes, an IA state with a transition probability of one (case in light grey, Fig. S4), and in contrast, a value of 0% in the absence of a circadian regulation of the rest duration (case in black, Fig. S4).

We also computed the autocorrelation at 24-h (r24), a measure of the regularity of the 24-h rest-activity pattern from day to day, as well as the dichotomy index (I < O), defined as the percentage of activity in-bed (I) that was less than the median activity out-of-bed (O)[28,49]. Furthermore, we also computed for each patient the dominant period using the spectrum-resampling (SR) algorithm of Costa et al.[50], as well as the acrophase and amplitude using a cosinor model.

Cosinor analysis for quantifying of circadian rhythmicity in urinary nucleosides excretion. A multiple-component cosinor model[51] was applied to describe the circadian pattern of nucleosides excretion for St 1. More specifically the model contained two cosine functions with respective periods $T_{12} = 12$-h and $T_{24} = 24$-h:

$$y(t) = M + A_{12}\cos\left(\frac{2\pi t}{T_{12}} + \theta_{12}\right) + A_{24}\cos\left(\frac{2\pi t}{T_{24}} + \theta_{24}\right) + e(t), \quad (1)$$

where $y(t)$ is the urinary nucleosides excretion at time t; M is the mesor (mean level); A_{12}, A_{24} and θ_{12}, θ_{24} are the corresponding amplitude and acrophase for each cosine term; $e(t)$ is the error.

The five parameters, i.e., $M, A_{12}, A_{24}, \theta_{12}, \theta_{24}$ in the non-linear model (1), were estimated using the *R* package *nlme*[52] To avoid over-fitting, individuals with less than seven timed nucleosides excretion samples were excluded from the cosinor analysis. We report the overall circadian amplitude A, i.e. half difference of the highest and lowest values of curve fitting $\widehat{y}(t)$, relative amplitude (% of mesor) and the overall acrophase θ, i.e. acrophase of $\widehat{y}(t)$. Nucleosides excretions were identified to have significant rhythmic pattern at 24-h if either amplitude (A_{12} or A_{24}) was significantly different from zero (t-test with p-value ≤ 0.1). Note that the liberal 0.1 p-value threshold was selected due to limited sample size, i.e. six parameters in model (1) need to be estimated by up to 10 data samples. In order to improve upon the model fit and the assumption of Gaussianity for the residuals, the log transformation was applied to the raw nucleosides excretion before the cosinor analysis. The mesor and amplitude results were transformed back to the original scale for reporting.

Correlation between circadian parameter estimates. The Spearman rank correlation was computed between any pair of parameters related to rest-activity and urinary nucleosides excretion patterns. Since large uncertainties could be expected for all circadian parameter estimates related to nucleosides excretion, correlations related with nucleosides circadian parameters were further verified by bootstrap uncertainty estimates[53]. One thousand bootstrap trials were generated via sampling the residuals in Eq. (1) with replacement and 1000 bootstrap nucleosides paramters were computed. The correlations were identified to be significant if the 90% quantiles of bootstrap Spearman correlation p-values ≤ 0.1.

Regression analysis of parameter estimates versus patients' characteristics. Multivariate regression analysis was applied to estimate the relation between main patient characteristics and response variables. The latter included the estimated parameters characterizing the rest-activity cycle, namely the dichotomy index I < O, the estimated probability of staying in the rest state p1-1, the rest duration, the Rhythm Index RI, the median value of the HA state and the autocorrelation r24; and those in the nucleosides urinary excretion, such as the mesor estimates, the relative amplitude (% of mesor) and the number of nucleosides with significant rhythmicity pattern at 24-h. Stepwise model selection based on Akaike's information criterion[54], as implemented in the *R* function *stepAIC,* was applied to obtain an appropriate model for each response variable.

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Author contributions

S.D., Q.H., P.F.I., A.K., M.B., A.P., F.T., L.A.A., R.A., B.F., and F.L. contributed to the work as follows : Conceptualization and design of the clinical study (S.D., LF, P.F.I., R.A.); patients recruitment (F.L., R.A., P.F.I., M.B.); study conduct (S.D., A.K.); nucleosides quantification (A.P., F.T.); data collection and organisation (S.D., A.K., Q.H.), mathematical and statistical analyses (Q.H., B.F., F.L.); first draft manuscript writing (S.D., Q.H., F.L.); review and editing (all); funding acquisition (S.D.,F.L., A.P., L.A.A.); project administration (S.D., F.L., KA).

Competing interests

The authors declare no competing interests.

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4.4.2 L'avenir de la chronothérapeutique de précision contre le cancer.

***Article n°6**, Lancet Oncol, Juin 2022*

*Pasquale F Innominato, **Abdoulaye Karaboué**, Mohammed Bouchahda, Georg A Bjarnason, Francis A Lévi*

Nous avons lu avec grand intérêt la récente révue systématique sur la chimiothérapie chronomodulée réalisée par Markella Printezi et ses collègues (140). Une révision critique et actualisée des données disponibles s'imposait, étant donné que la précédente a été publiée il y a près de 30 ans (86). Bien que seul un petit nombre d'essais cliniques contrôlés randomisés impliquant une chimiothérapie chronomodulée ou fondée sur le rythme circadien aient été réalisés dans l'intervalle entre ces deux revues systématiques, deux évolutions majeures doivent être soulignées.

Tout d'abord, la compréhension des mécanismes moléculaires et physiologiques qui sous-tendent les différences de toxicité et d'efficacité des médicaments anticancéreux en fonction du moment où ils sont administrés a considérablement progressé (141). La découverte des déterminants génétiques de l'horloge circadienne moléculaire, pour laquelle Hall, Rosbash et Young ont reçu le prix Nobel de physiologie ou de médecine en 2017, a joué un rôle essentiel dans l'approfondissement de ces connaissances. Depuis cette découverte, les preuves

expérimentales et translationnelles se sont rapidement multipliées, apportant une justification moléculaire et physiologique supplémentaire à l'optimisation de l'index thérapeutique des traitements anticancéreux grâce à l'administration précise et personnalisée des traitements fondée sur la prise ne compte des rythmes circadiens. Bien que ces connaissances croissantes ne se tradusient pas encore en évidence clinique au sens large, on peut raisonnablement supposer que des essais cliniques conçus de manière appropriée testeront bientôt la pertinence clinique des approches chronothérapeutiques anticancéreuses sur la base de données expérimentales affinées et solides.

Enfin, de nouveaux agents ciblés ont été identifiés, testés, et couramment utilisés en combinaison avec des médicaments cytotoxiques pour améliorer la thérapeutique des cancers. La découverte et le développement des ICIs, qui ont valu à Allison et Honjo le prix Nobel de médecine ou de physiologie en 2018, a constitué un évènement récent dans le domaine de la thérapie anticancéreuse. En effet, trois mois seulement avant la publication de la revue systématique de Printezi et de ses collègues (140), les premières preuves de l'effet du moment de la perfusion des ICIs sur la survie globale des patients cancéreux ont été publiées (142). Des résultats conformes à ces conclusions ont été rapportés dans un autre contexte clinique au cours des derniers mois (143). Ces résultats remettent en

question notre compréhension actuelle des mécanismes à l'œuvre sur une échelle de 24 heures, compte tenu de ce que l'on sait de la pharmacocinétique des ICIs (142, 143). Néanmoins, les preuves irréfutables d'une efficacité accrue grâce à l'administration matinale des ICIs pourraient facilement être mises en œuvre dans la pratique à un coût faible ou nul.

Nous félicitons Printezi et ses collègues pour leur investigation exhaustive et leur discussion approfondise de la chronothérapeutique clinique des cancers . Nous espérons que leur revue systématique stimulera la recherche clinique sur les horaires circadiens optimaux d'administration de toutes les classes pharmacologiques d'agents anticancéreux et de la radiothérapie (140). Ces travaux sont déjà en cours et devraient se traduire par des avantages tangibles pour les patients atteints de cancer.

The future of precise cancer chrono-therapeutics

We have read with great interest the recent comprehensive systematic review on chronomodulated chemotherapy by Markella Printezi and colleagues.[1] An updated critical revision of available evidence was overdue, given that the previous one was published nearly 30 years ago.[2] Although only a small number of randomised controlled clinical trials involving chronomodulated or circadian-based chemotherapy have been done in the interval between these two systematic reviews, two major developments need to be highlighted.

First, there have been substantial advances in understanding the molecular and physiological mechanisms that underpin the differences in toxicity and efficacy of anticancer drugs according to the timing of their administration.[3] The discovery of the genetic determinants of the molecular circadian clockwork—for which Hall, Rosbash, and Young were awarded the 2017 Nobel Prize in Physiology or Medicine—has been paramount in furthering this understanding. Since this discovery, experimental and translational evidence has been rapidly increasing, providing further molecular and physiological rationale for optimising the therapeutic index of anticancer treatments through precise and personalised circadian-based treatment delivery. Although this expanding knowledge has not yet been translated into broader clinical evidence, one can reasonably speculate that appropriately designed trials will soon test the clinical relevance of anticancer chronotherapeutic approaches based on robust experimental data.

Second, new targeted agents have been identified, tested, and routinely used alongside cytotoxic drugs to treat cancers. The most recent disruptive change in cancer therapeutics has resulted from the discovery and development of immune checkpoint inhibitors, which led to Allison and Honjo being awarded the 2018 Nobel Prize in Medicine or Physiology. Indeed, only 3 months before the publication of the systematic review by Printezi and colleagues,[1] the first evidence of the effect of the timing of infusion of immune checkpoint inhibitors on overall survival was published.[4] Results consistent with these findings have been reported in another clinical setting within the past few months.[5] These findings challenge our current understanding of the mechanisms at work along the 24-h scale, given what is known about the pharmacokinetics of immune checkpoint inhibitors.[4,5] Nonetheless, the compelling evidence of increased efficacy through morning dosing could easily be implemented in practice at little to no cost.

We commend Printezi and colleagues for their careful review and discussion, which we hope will stimulate more clinical research on the optimal timing of all pharmacological classes of anticancer agents and radiotherapy.[1] This work is already in progress and should result in tangible benefits for patients with cancer.

We declare no competing interests.

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4.4.3 Le rythme activité-repos comme biomarqueur circadien clinique dans le cancer.

Article n°7, *Lancet Healthy Longev, juillet 2023*

Pasquale F Innominato, Nicholas I Wreglesworth, ***Abdoulaye Karaboué****, David Spiegel, Francis A Lévi*

Dans leur article, Hongliang Feng et ses collègues (128) ont analysé les données de séries temporelles d'accéléromètrie et ont montré une forte association entre une faible amplitude du rythme circadien d'activité-repos et une augmentation de l'incidence des maladies et de la mortalité. Les résultats rapportés par les auteurs confirment le lien déjà reconnu entre la perturbation du rythme circadien et le risque de cancer (144). Nous saluons leur approche analytique approfondie d'un ensemble de données aussi vaste.

Cependant, nous encourageons les auteurs à étayer davantage leur analyse en incluant l'indice de dichotomie actigraphique (I<O) dans leur liste de paramètres. L'I<O est une mesure qui intègre la régulation circadienne du sommeil et de l'activité en considérant l'activité physique et le repos sur 24 heures différemment de l'amplitude relative. I<O représente le pourcentage de minutes pendant la période de repos d'un individu où son niveau d'activité est inférieur à son activité médiane pendant la période d'éveil (82). Sur 30 paramètres d'actigraphie au

poignet testés pour leur pertinence clinique, I<O s'est classé comme le meilleur prédicteur des résultats cliniques, surpassant systématiquement les autres paramètres dans neuf études prospectives sur le cancer réalisées par notre groupe. L'I<O a permis de prédire de manière indépendante la survie globale dans une méta-analyse portant sur 436 patients atteints d'un cancer colorectal métastatique (82), et ce succès a été confirmé par une méta-analyse indépendante de six études portant sur 659 patients atteints d'un cancer (129). L'I<O était également le marqueur comportemental le plus discriminant entre 226 patients atteints de cancer et 182 individus témoins en bonne santé, surpassant à la fois les paramètres d'activité physique et du sommeil (130). L'association significative de l'I<O avec la qualité de vie liée à la santé et la prédiction réussie de l'hospitalisation d'urgence soulignent encore la pertinence clinique de cette métrique pour les patients atteints de cancer. L'ensemble des données de la Biobank UK offre une occasion unique d'examiner si l'I<O peut également aider à prédire l'incidence des principales causes de morbidité et de mortalité, y compris le cancer.

L'inclusion de I<O dans la liste des paramètres dérivés de l'accéléromètre utilisés dans l'analyse de l'ensemble des données de la Biobank UK réalisée par Feng et ses collègues pourrait améliorer la valeur prédictive des altérations du rythme

circadien pour l'incidence des maladies et la mortalité, en complétant les résultats importants déjà présentés dans l'article. Les auteurs fournissent des preuves irréfutables que la dysrégulation circadienne a un effet omniprésent sur la santé humaine, en particulier en augmentant la mortalité due au cancer. Ces résultats, qui modifient la pratique, appellent à la conception de méthodes diagnostiques optimales pour évaluer le rythme d'activité-repos et proposer des interventions ciblant les déterminants circadiens responsables de la qualité de vie des patients atteints de cancer ou d'autres maladies graves, ainsi que de la tolérance et de l'efficacité de leurs traitements.

Rest–activity rhythm as a clinical biomarker in cancer

In their Article, Hongliang Feng and colleagues[1] analysed accelerometer time series data and showed a strong association between a low amplitude of rest–activity rhythm and increased disease incidence and mortality. The authors' findings support the previously acknowledged link between circadian disruption and cancer risk.[2] We commend their thorough analytical approach in such a large dataset.

However, we would encourage the authors to further substantiate their analysis by including the actigraphic dichotomy index (I<0) in their list of parameters. I<0 is a metric that integrates the circadian regulation of sleep and activity by considering physical activity and rest over 24 h differently from relative amplitude. I<0 represents the percentage of minutes during an individual's rest span in which their activity level is lower than their median activity during the bout of wakefulness.[3] Of 30 wrist actigraphy parameters tested for clinical relevance, I<0 ranked as the best predictor of clinical outcomes, consistently outperforming other parameters in nine prospective cancer studies from our group. I<0 independently predicted overall survival in an individual patient-level meta-analysis involving 436 patients with metastatic colorectal cancer,[3] with confirmation of success reported in an independent meta-analysis of six studies involving 659 patients with cancer.[4] I<0 was also the most discriminatory behavioural marker between 226 patients with cancer and 182 healthy control individuals, outperforming both physical activity and sleep parameters.[5] The significant association of I<0 with health-related quality of life and the successful prediction of emergency hospitalisation further highlights the clinical relevance of this metric for patients with cancer. The UK Biobank dataset provides a unique opportunity to investigate whether I<0 can also help to predict the incidence of (and mortality from) the main causes of morbidity and mortality, including cancer.

Including I<0 in the list of accelerometer-derived parameters used in Feng and colleagues' analysis of the UK Biobank dataset could enhance the predictive value of circadian rhythm alterations for disease incidence and mortality, complementing the important results already shown in the Article. The authors provide compelling evidence that circadian dysregulation has a pervasive effect on human health, particularly by increasing cancer mortality. These practice-changing results call for the design of optimal diagnostic methods to assess rest–activity rhythm, and of dedicated interventions that target the actionable circadian determinants of clinical outcomes for patients with cancer and other major diseases.

We declare no competing interests.

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4.5 AUTRES PRODUCTIONS SCIENTIFIQUES

- ***Article n°8*** : Bouchahda M, Saffroy R, **Karaboué A**, Hamelin J, Innominato P, Saliba F, Bosselut N, Lemoine A, Lévi F. Reply to P. Gazzaniga et al. *JCO Precis Oncol. 2021 Nov;5:391-392. doi: 10.1200/PO.20.00465. PMID: 34994602.*

- ***Article n°9*** : Bouchahda M SR, **Karaboué A**, Hamelin J, Innominato P, Saliba F, Lévi F, Bosselut N, Lemoine A. Efficacité d'un anti-EGFR après conversion de mutation RAS. *ONKO+ 2021;13(103):152-9.*

- ***Article n°10***: Innominato PF, Komarzynski S, Dallmann R, Wreglesworth NI, Bouchahda M, **Karaboué A**, Ulusakarya A, Subbe CP, Spiegel D, Lévi FA. Impact of assessment frequency of patient-reported outcomes: an observational study using an eHealth platform in cancer patients. *Support Care Cancer. 2021 Nov;29(11):6167-6170. doi: 10.1007/s00520-021-06262-1. Epub 2021 May 8. PMID: 33963910.*

- ***Article n°11***: Innominato PF, **Karaboué A**, Focan C, Chollet P, Giacchetti S, Bouchahda M, Ulusakarya A, Torsello A, Adam R, Lévi FA, Garufi C. Efficacy and safety of chronomodulated irinotecan, oxaliplatin, 5-fluorouracil and leucovorin combination as first- or second-line treatment against metastatic colorectal cancer: Results from the International EORTC 05011 Trial. *Int J*

Cancer. 2021 May 15;148(10):2512-2521. doi: 10.1002/ijc.33422. Epub 2020 Dec 10. PMID: 33270911; PMCID: PMC8048520.

- ***Article n°12*** : Bouchahda M, Saffroy R, **Karaboué A**, Hamelin J, Innominato P, Saliba F, Lévi F, Bosselut N, Lemoine A. Undetectable *RAS*-Mutant Clones in Plasma: Possible Implication for Anti-EGFR Therapy and Prognosis in Patients With *RAS*-Mutant Metastatic Colorectal Cancer. *JCO Precis Oncol. 2020 Sep 16;4:PO.19.00400. doi: 10.1200/PO.19.00400. PMID: 33015528; PMCID: PMC7529530.*

- ***Article n°13***: Ulusakarya A, **Karaboué A**, Ciacio O, Pittau G, Haydar M, Biondani P, Gumus Y, Chebib A, Almohamad W, Innominato PF. A retrospective study of patient-tailored FOLFIRINOX as a first-line chemotherapy for patients with advanced biliary tract cancer. *BMC Cancer. 2020 Jun 3;20(1):515. doi: 10.1186/s12885-020-07004-y. PMID: 32493242; PMCID: PMC7268699.*

- ***Article n°14*:** Innominato PF, Ballesta A, Huang Q, Focan C, Chollet P, **Karaboué A**, Giacchetti S, Bouchahda M, Adam R, Garufi C, Lévi FA. Sex-dependent least toxic timing of irinotecan combined with chronomodulated chemotherapy for metastatic colorectal cancer: Randomized multicenter

EORTC 05011 trial. *Cancer Med. 2020 Jun;9(12):4148-4159. doi: 10.1002/cam4.3056. Epub 2020 Apr 22. PMID: 32319740; PMCID: PMC7300418.*

5 DISCUSSION GENERALE

Cohérence des études cliniques en faveur d'une administration matinale des ICIs quel que soit le type de cancer et le type d'ICI.

Mes résultats originaux (*article n°1 et article n°4 soumis*) (143), ainsi que la méta-analyse (*article n°2*) (145) et la revue de synthèse (*article n°3*) (146) que j'ai effectuées, m'amènent à conclure que 17 des 18 études rétrospectives rapportées qui ont examiné l'influence de l'horaire d'administration des ICIs indiquent que les perfusions d'ICIs effectuées principalement le matin ou en début d'après-midi améliorent de manière significative la survie sans progression et/ou la survie globale par rapport aux perfusions effectuées plus tard dans la journée. Ceci concerne des patients atteints de huit types différents de cancer au stade métastatique et traités dans 4 continents. En moyenne, l'administration matinale de nivolumab, de pembrolizumab, d'ipilimumab, de durvalumab, d'atezolizumab, de sintilimab, ou de camrelizumab permet de doubler la survie et/ou la survie sans progression des patients cancéreux traités par ces ICIs, administrés seuls, en combinaison, ou en association avec un antiangiogénique ou une chimiothérapie. Six nouvelles études parues en 2024 ont examiné également l'influence de l'administration des ICIs (**Figure 4**). Cinq d'entre elles indiquent que les perfusions d'ICIs effectuées principalement le matin ou en début d'après-midi améliorent de

manière significative la survie sans progression et/ou la survie globale (**Figure 5 & Tableau 1**).

Ces horaires d'administration associés à une meilleure efficacité antitumorale de l'immunothérapie sont cohérents avec ceux rapportés pour plusieurs vaccins, dont les critères d'efficacité étaient généralement améliorés après une administration le matin entre 9:00 et 11:00 plutôt que l'après-midi entre 15:00 et 17:00 (147, 148).

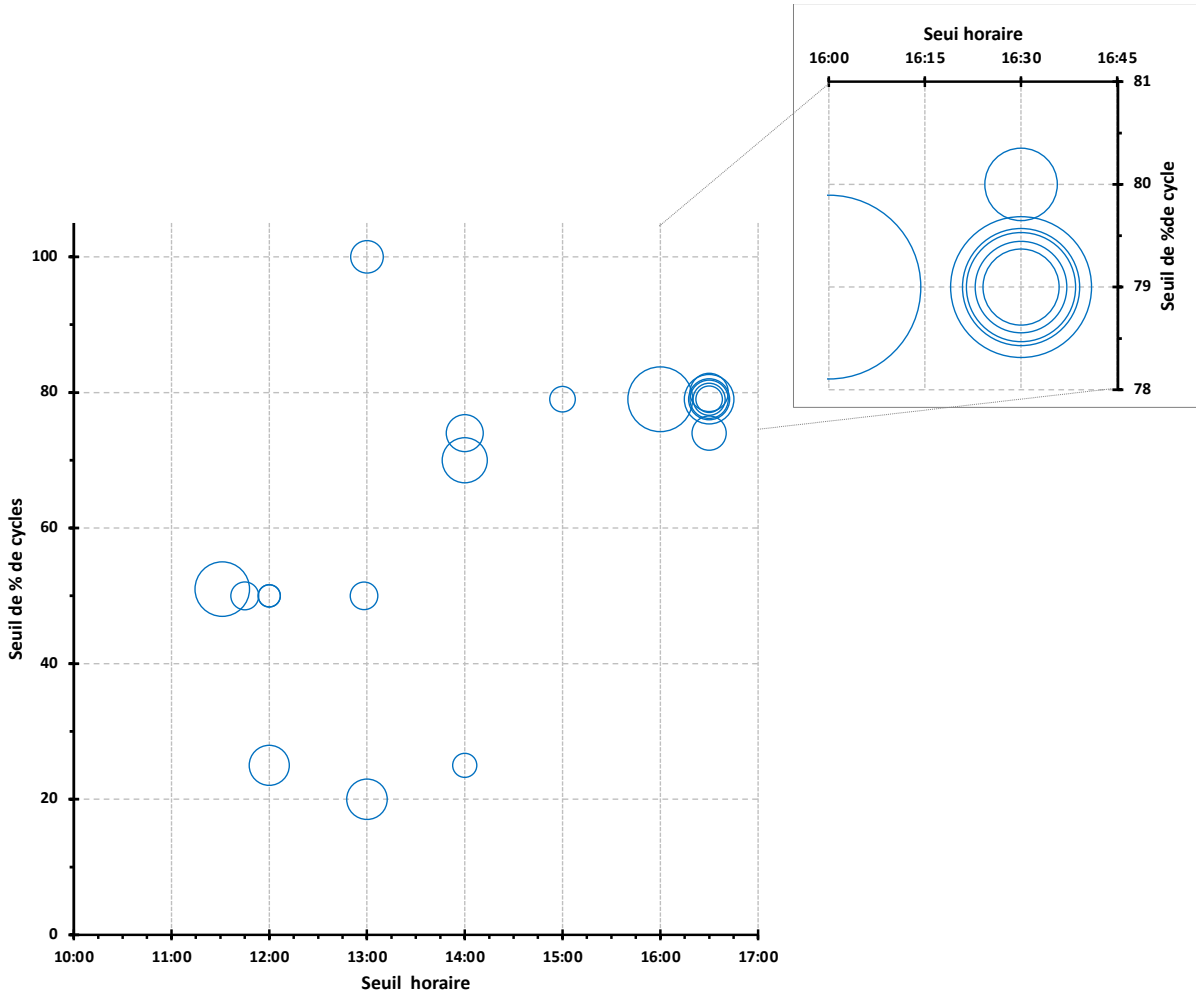
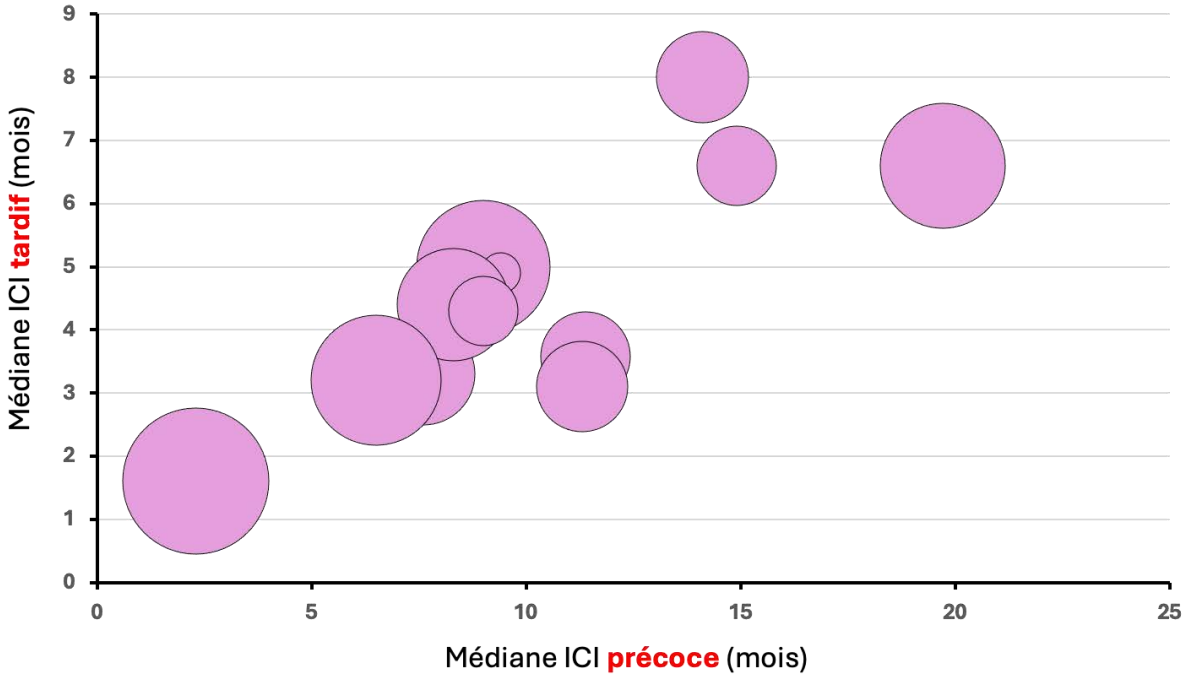


Figure 4: Répartition des seuils horaires, des pourcentages de cycles administrés avant le seuil et des nombres de patients inclus dans les 24 études rapportées qui evaluent l'impact de l'heure d'administration des ICIs sur la survie des patients atteints de cancer.

Chaque cercle repère une étude par l'horaire seuil en abscisse et le pourcentage de cycles administrés avant l'horaire seuil et considéré comme critique pour l'amélioration de l'efficacité. Le diamètre de chaque cercle est proportionnel au nombre de patients inclus dans l'étude.

(a) Survie sans progression



(b) Survie globale

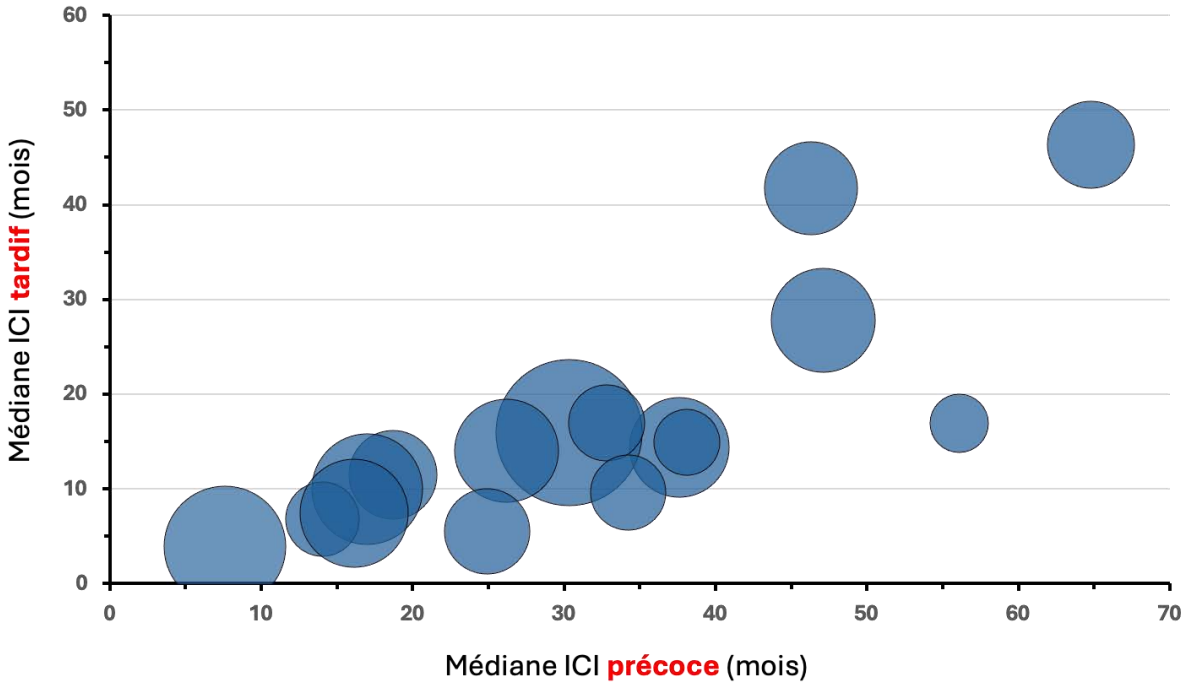


Figure 5 : Répartition des médianes de SSP (a) et SG (b) des patients cancéreux dans les groupes « Avant » (précoce) ou « Après » (tardif) le seuil horaire sélectionné dans les études évaluant l'impact de l'heure d'administration.

Chaque cercle repère une étude par la médiane de survie du groupe « précoce » en abscisse et du groupe « tardif » en ordonnée. Le diamètre de chaque cercle est proportionnel au nombre de patients inclus dans l'étude. Noter les differences d'échelles des ordonnées et des abscisses.

Quel moment optimal pour administrer l'immunothérapie ?

Une tendance en faveur d'une administration matinale plutôt qu'en début d'après-midi est apparue dans la méta-analyse, où les « Hazard Ratio » de progression ou de décès précoces étaient significativement moindres dans les études dont les horaires seuils étaient entre 12h00 et 14h00 plutôt qu'entre 16h00 et16h30 (8). Il en est de même dans 23 des 24 études publiées (**Tableau 1**) Cependant, seul un tiers de la période de 24 heures a été exploré dans toutes ces études, ce qui pose la question de la localisation possiblement nocturne du moment optimal d'administration des ICIs, puisqu'aucune donnée n'existe pour des administrations d'ICIs entre 18h00 et 09h00. Catozzi et al. (149) ont estimé que l'heure optimale d'administration des ICIs se situait à 01h36, après analyse de risque périodique logarithmique selon le modèle de régression périodique de

Cox appliqué à la survie globale de leurs patients PS 0-1(149). Cependant, les études expérimentales montrent que le nombre de cellules immunitaires infiltrant les tumeurs et la réponse immunitaire antitumorale sont maximaux au début de la phase d'activité nocturne des souris (119, 150). Ceci correspondrait à une efficacité optimale des ICIs au début de la journée chez l'Homme, en accord avec toutes les données cliniques précédemment citées. L'intérêt thérapeutique d'une administration nocturne des ICIs fondé sur l'analyse mathématique devrait ainsi faire l'objet d'études cliniques exploratoires.

Tableau 1: *Tableau récapitulatif des 24 études ayant évalué l'impact de l'heure d'administation des ICIs sur la survie sans progression (SSP) et/ou la survie globale (SG) des patients atteints de cancer au stade localement avancé ou métastatique. Classement par date de publication.*

Pays	Auteur *Références*	Tumeur	Traitement	Patients	Seuil	% de cycle: avant//après	Patients: avant vs après	SSP médiane : avant vs après (p)	SG médiane: avant vs après (p)	Cycles analysés
Japon	**Hirata T. et al.** *JTO Clin Res Rep. 2024 Mar 5;5(4):100659*	CBNPC[1]	Durva	82	15:00	//≥20	70 vs 12	NA vs 7,4 (p=0,027)	NA vs 22,4 (p=0,20)	Tous
Japon	**Ishizuka Y. et al.** *JCO 42, 268-268(2024)*	Estomac	Nivo	248	14:00	≥70//	140 vs 108	2,3 vs 1,6 (p<0,01)	7,6 vs 3,9 (p<0,01)	Tous
USA	**Ruiz Torres D.A. et al.** *Oral Oncol. 2024 Apr:151:106761*	Tête et cou	Pembro, Nivo	113	15:00	//≥20	NR	NA[3] (p=0,0001)	NA (p=0,0003)	Tous
France	**Pascale A. et al.** *JCO 42, 457-457(2024)*	CHC	Atezo	131	13:00	//100	90 vs 41	NR	18,7 vs 11,5 (p=0,015)	2 cycles
Chine	**Sun L.Y. et al.** *JCO 42, e14634-e14634(2024)*	Tumeur solide	Cam +CT, Tis +CT	174	16:00	// ≥1 fois	92 vs 82	avant < après (p<0,01)	NR	Tous
Mexique	**Ramirez A.M.A. et al**	CR	Nivo + Ipi	127	16:30	//≥20	84 vs 43	NR	64,8 vs 46,3 (p=0,03)	Tous

	JCO 42, e16537-e16537(2024)									
Portugal	**Goncalves L. et al.** *JCO 42, 9542-9542(2024)*	Melanome	Pembro, Nivo…	168	14:00	//≥75	131 vs 37	NR	37,6 vs 14,4 (p=0,014)	Tous
Chine	**Zheng Y. et al.** *JCO 42, e16186-e16186(2024)*	Voies biliaires	ICI	207	16:30	//≥20	170 vs 37	9 vs 5 (P=0,00183)	17 vs 10 (p=0,00645)	Tous
France	**Catozzi S. et al.** *Eur J Cancer. 2024 Mar:199:113571*	CBNPC	Nivo, Atezo, Durva, or Ave, +/- CT	361	11:37	>50//	136 vs 225	NR	30,3 vs 15,9 (P=0,0024)	Tous
USA	**Patel J.S. et al.** *J Immunother Cancer. 2024 Mar 26;12(3):e008011*	CR	Pembro, Nivo, ou Nivo+Ipi	201	13 :00	≥20//	101 vs 100	NR (p=0,020)	NR (p=0,033)	Tous
France	**Rousseau A. et al.** *Eur J Cancer. 2023 Mar:182:107-114*	CBNPC	Nivo,Pembro ou Atezo	180	16:30	//≥20	115 vs 65	9,4 vs 4,9 (p=0,020)	26,2 vs 14,0 (p=0,090)	Tous
France	**Karaboué A. et al.** *JCO 41, e21055-e21055(2023)*	CBNPC	Pembro +/- CT	97	11:45	≥50//	48 vs 49	14,1 vs 8,0 (p=0,145)	32,8 vs 17,0 (p=0,012)	4 cycles
Canada	**Yeung C. et al.** *Immunotherapy. 2023 Aug;15(11):819-826*	Melanome	Nivo, Pembro, ou Nivo+ipi	121	13 :00	.//.	NR	7,6 vs 3,3 (p=0,009)	24,9 vs 5,5 (p< 0,001)	1er cycle
USA	**Dizman N. et al.** *JCO 41, 678-678(2023)*	CR	Nivo +/- ipi	145	16:30	//≥25	110 vs 35	8,3 vs 4,4 (p=0,32)	46,3 vs 41,7 (p=0,20)	Tous
Espagne	**Fernandez-Mañas L. et al.**	CR	ICI	56	16 :30	//≥20	NR	9 vs 4,3 (p=0,013)	56,1 vs 16,9 (p=0,01)	Tous

	JCO 41, 681-681(2023)					//≥50	NR	9 vs 2 (p=0,007)	36,6 vs 16,9 (p=0,1)	Tous
Portugal	**Gonçalves L. et al.** *Cells. 2023 Aug 15;12(16):2068.*	Mélanome	Nivo, Pembro, ou Nivo+Ipili	73	14 :00	//≥75	48 vs 25	14,9 vs 6,6 (p=0,320)	38,1 vs 14,9 (P<0,01)	Tous
Japon	**Nomura M. et al.** *Esophagus. 2023 Oct;20(4):722-731*	CEE	Nivo	62	13:00	.//.	27 vs 35	NR (p=0,002)	NR (p=0,036)	1er cycle
						≥50//	30 vs 32	NR (p=0,005)	NR (p=0,324)	3 mois
						≥50//	26 vs 36	NR (p=0,303)	NR (p=0,821)	Tous
Royaume Uni	**Cortellini A. et al.** *J Immunother Cancer. 2022;10(Suppl 2):A536-A7*	CBNPC	Pembro	180	16 :30	//≥20	136 vs 44	19,7 vs 6,6 (p=0,056)	47,1 vs 27,8 (p=0,11)	Tous
Brésil	**Barrios CH et al.** *J Clin Oncol 40, 2022 (suppl 16; abstr e21126)*	CBNPC	Nivo, Pembro ou Atezo	508	16 :00	//≥20	465 vs 43	NR (p=0,05)	NR (p=0,5)	Tous
Espagne	**Ortego I et al.** *JCO 40, e16541-e16541(2022)*	Carcinome urothelial	Anti-PD-1 ou anti-PD-L1	92	16:30	//≥20	62 vs 26	11,38 vs 3,58 (P=0,001)	14,04 vs 6,80 (P=0,001)	Tous
Canada	**Rensburg HJJv et al.** *JCO 40, 2589-2589(2022)*	Tête et cou, sein, ovaire, melanome, et autres tumeurs solides	Pembro	106	12 :00	.//.	NR	NR (P=NS)	NR (P=NS)	1er cycle
					15:06	.//.	NR	NR (P=NS)	NR (P=NS)	1er cycle
					12 :00	≥50//	NR	NR (P=NS)	NR (P=NS)	Tous
					15 :11	≥50//	NR	NR (P=NS)	NR (P=NS)	Tous
					16 :30	//≥20	NR	NR (P=NS)	NR (P=NS)	Tous

France	**Karaboué A et al.** *Cancers (Basel). 2022 Feb 11;14(4):896*	CBNPC	Nivo	95	12:54	≥50//	48 vs 47	11,3 vs 3,1 (p<0,001)	34,2 vs 9,6 (P<0,001)	Tous
Espagne	**Vilalta A et al.** *Ann Oncol. 2021;32:S835*	CBNPC	Anti-PD-1 +/- CT	197	12:00	≥25//	104 vs 93	6,5 vs 3,2 (P=0,066)	16,1 vs 7,4 (P=0,003)	4 cycles
USA	**Qian DC et al.** *Lancet Oncol. 2021 Dec;22(12):1777-1786*	Melanoma	Nivo, Pembro, ou Nivo+ipi	299	16 :30	//≥20	225 vs 74	NA	NA vs 57,6 (p=0,038)	Tous

[1]*CBNPC= Cancer bronchique non à petites celulles ; CHC=Carcinome hépatocellulaire ; CR=carcinome renal ; CEE=Carcinome epidermoïde de l'œsophage.*

[2]*Durva=Durvalumab ; Nivo=Nivolumab ; Pembro=pembrolizumab ; Atezo=Atezolizumab ; Tis=Tislelizumab ; Cam=Camrelizumab ; Ipi=Ipilimab ; Ave=Avelumab ; CT=Chimiothérapie.*

[3]*NA=Non atteint ; NR=Non renseigné ; NS=Non statistiquement significatif*

Nombre de cycles et pharmacocinétique

Le nombre de cycles de perfusions matinales d'ICIs nécessaires à l'amélioration de l'efficacité antitumorale nécessite d'être précisé. Cinq études ont mis en évidence l'importance cruciale de l'administration matinale des deux ou quatre premières cures (151-155), alors qu'une étude ne retrouve pas cette relation (156). L'importance des 4 premières cures est cohérente avec un possible rôle de la chronopharmacocinétique des ICIs dans leur chronoefficacité. En effet, la demi-vie des ICIs varie de 10 à 30 jours. Ainsi, l'état d'équilibre dans la circulation sanguine est généralement atteint après 5 demi-vies, soit 2 à 4 mois (157). C'est donc durant les 2 à 4 mois initiaux du traitement qu'une chronopharmacocinétique pourrait être observée et jouer un rôle dans la chronotolérance et/ou la chronoefficacité des ICIs. De plus, la liaison des ICIs aux récepteurs PD-1 des lymphocytes-T est réduite de 50% en environ 8 mois après perfusion d'ICI (158). Cela pourrait expliquer le maintien de la réponse pendant une durée longue lorsque l'ICI anti-PD-1 se lie aux récepteurs PD-1 à un stade circadien où leur expression est maximale. C'est donc durant les 2 à 4 mois initiaux du traitement qu'une chronopharmacocinétique pourrait être observée et jouer un rôle dans la chronotolérance et/ou la chronoefficacité des ICIs (146, 158).

Les résultats des études rétrospectives ne permettent pas de connaître la

proportion de cycles de perfusion d'ICIs associée à une moindre survie en cas d'administration l'après-midi ou en soirée. Selon les études, le pourcentage de cycles débutant après le seuil horaire et associé à une moindre survie varie de 20 à 80% (**Tableau 1**)

Relations entre chronoefficacité et tolerance de l'immunothérapie

L'impact de l'heure d'administration sur l'efficacité et la tolérance de la chimiothérapie suit une variation circadienne similaire dans les modèles précliniques et chez les patients cancéreux, avec pour résultat que le moment de meilleure efficacité correspond au moment de meilleure tolérance (13, 140). En ce qui concerne l'immunothérapie, une association entre la survenue d'effets indésirables et l'amélioration du pronostic a été suggérée (159, 160). Cependant, sur les 24 articles qui ont évalué l'impact de l'heure d'administration des ICIs, seuls deux rapportent les effets secondaires (142, 143). Mon étude (*article n°1*) a montré que la fatigue grade 3-4 était significativement plus fréquente chez les patients traités l'après-midi en comparaison de ceux traité le matin (14,9% vs 6,3%, p=0.024), et ce, bien que le nombre de cycles administrés soit significativement moindre dans le groupe « après-midi ». L'inverse a été observé pour la toxicité

cutanée de grade 2-3 avec 31,9% pour le groupe du matin contre 12,7% dans le groupe de l'après-midi, (p=0.049), possiblement en raison de la moindre exposition du groupe de l'après-midi au traitement par nivolumab. La seconde étude (Qian et al. Lancet 2021) n'a pas rapporté de relation entre chronoefficacité et tolerance de l'immunothérapie, possiblement en raison des multiples traitements possibles des mêmes patients, par anti-PD-1 seuls ou associés à un anti-CTLA-4. Les autres publications sont peu informatives quant à une éventuelle chronotolérance des ICIs, possiblement en raison de leur nature rétrospective.

L'hypothèse du système immunitaire circadien

Notre revue de la litterature (*article n°3*) (146) a décrit et analysé de façon exhaustive les mécanismes potentiels pouvant expliquer l'impact de l'heure d'administration des ICIs sur leur efficacité. Ceux-ci résultent en premier lieu du contrôle circadien du système immunitaire, bien démontré dans plusieurs études expérimentales (15, 37, 41, 43, 119, 150, 161). Wang C et al. ont montré tout récemment que les cellules dendritiques contrôlaient plus efficacement les tumeurs greffées chez des souris en fin de phase de repos diurne (ZT9, 9h après le début de la lumière) plutôt qu'en fin de phase d'activité nocturne (ZT21) (118). La plupart des fonctions du système immunitaire sont contrôlées sur 24 heures (15,

43, 44, 161-164). En effet, chez la Souris, les leucocytes migrent du sang vers les ganglions lymphatiques et d'autres tissus profonds de manière rythmique (35, 165-168) avec pour conséquence, l'existence d'un pic d'infiltration dans les organes lymphatiques à ZT8, qui suit le pic de la numération sanguine de ces cellules à ZT4. Ce rythme circadien endogène de l'infiltration tissulaire par les leucocytes et des lymphocytes, en particulier les lymphocytes T CD8 rendent compte d'une meilleure reconnaissance antigénique en deuxième moitié de la phase de repos diurne (ZT7) en comparaison de la seconde moitiés de la phase d'activité nocturne (ZT21) (169). Outre les caractéristiques circadiennes de ce trafic leucocytaire, le phénotype des cellules immunitaires suit aussi un rythme circadien, attesté notamment par l'expression circadienne de *pdcd1* et de PD-1, rendant aussi compte d'une efficacité optimale de la vaccination et des anti-PD1 en début de la journée (170, 171).

Cependant, bien que le système immunitaire soit fortement rythmé en l'absence de pathologie tumorale, il se pose la question de savoir si les rythmes circadiens régulent aussi la distribution et le phénotype des cellules immunitaires dans les tumeurs cancéreuses ? Wang C et al. (Cell 2024)(119) rapportent en 2024 l'existence de variations circadiennes de l'infiltration tumorale par les lymphocytes-T (CD8+), ainsi que de l'expression de *pdcd1* et de PD-1 dans ces lymphocytes. Ils

montrent aussi que les gènes (notamment *Pdcd1*) associés à l'activation, à la suppression, à la migration et aux fonctions effectrices des cellules T CD8 présentent une oscillation au cours de la journée. Ainsi la différence d'expression de PD-1 au cours des 24 heures est abrogée après suppression sélective du gène horloge *Bmal1* dans les lymphocytes. Ce qui indique que l'expression rythmique de PD-1 dans les cellules T $CD8^+$ est circadienne et intrinsèque à la cellule. Ils ont montré aussi qu'un rythme circadien robuste caractérisait le microenvironnement immunitaire de la tumeur (119, 172). Cette dynamique circadienne du microenvironnement tumoral démontrée chez la Souris rend compte au moins pour partie des différences d'efficacité et peut-être aussi de tolérance liées aux horaires d'administration des ICIs. Elle nécessite d'être explorée en clinique, afin d'en préciser la pertinence pour la chrono-immunothérapie des cancers.

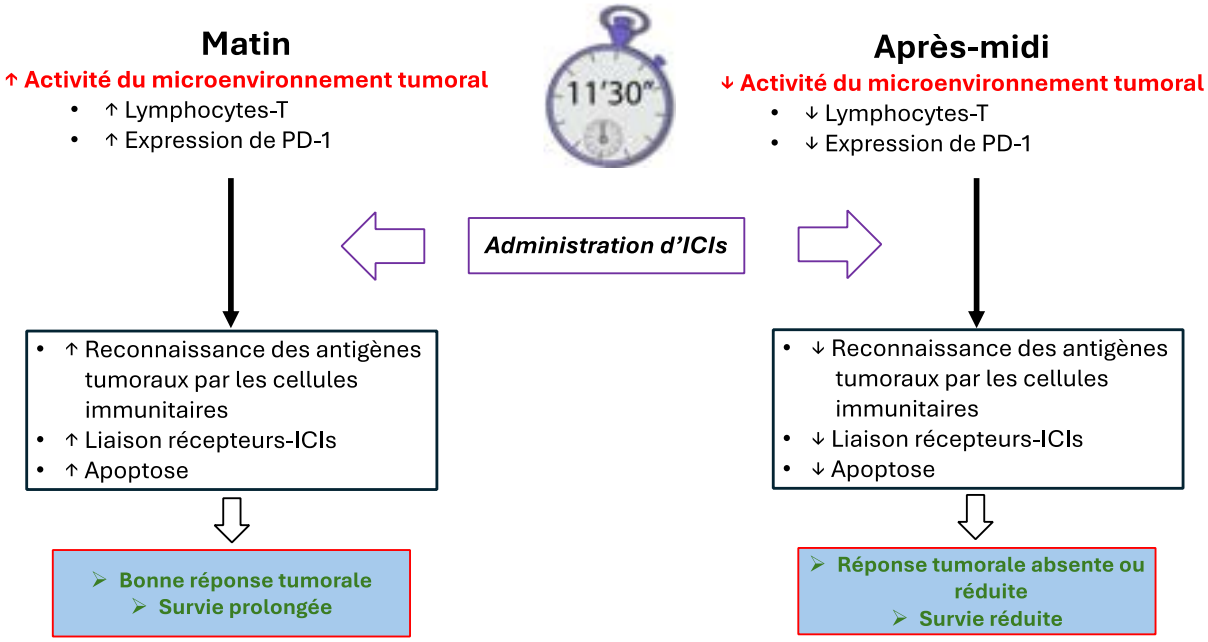


Figure 5: Schéma illustrant les mécamismes qui rendent compte de la meilleure efficacité des ICIs après administration matinale chez les patients cancéreux

Limites et explications alternatives

D'autres explications possibles ont été avancées pour expliquer la cohérence observée de l'effet du moment de l'administration des ICIs sur leur efficacité. Ainsi, l'administration tardive des traitements pourrait refléter un statut socio-économique défavorisé (173), une distance accrue entre le domicile et le centre hospitalier, ou une altération de l'état de performance physique du patient. Alors que le dernier facteur a été éliminé par les analyses multivariées réalisées dans la

plupart des études, ni le statut socio-économique, ni la distance par rapport au centre de traiement ne semblent jouer un rôle essentiel en France, compte tenu de la prise en charge médicale à 100% des patients cancéreux et du bassin de population des hôpitaux concernés, notamment dans les études que j'ai réalisées (article n°1 et Cohorte 1 dans article n°4).

De même, le nombre de perfusions d'ICIs est moindre chez les patients traités après le seuil horaire sélectionné en comparaison de ceux traités avant ce seuil, situé entre 12h00 et 16h30 selon les études. Certains ont considéré que ce déséquilibre de l'intensité du traitement défavorisait l'efficacité des administrations vespérales d'immunothérapie (174).

Cependant, c'est bien l'amélioration de l'efficacité de l'immunothérapie qui justifie la prolongation de la durée des traitements. En effet, une meilleure efficacité des ICIs a toujours été associée à un plus grand nombre de cycles de perfusion dans les essais randomisés (175-177). Cependant, afin d'éliminer les biais potentiels cachés, des essais cliniques randomisés sont nécessaires. Des comparaisons prospectives doivent être effectuées entre les différents moments d'administration des ICIs afin d'évaluer pleinement les différences d'efficacité liées au moment d'administration, équivalentes à celles liées à la découverte d'un nouveau médicament très actif.

Un aspect non exploré dans ces études est l'impact potentiel de la qualité du sommeil sur l'efficacité des ICIs et leur horaire optimal d'administration. En effet, le sommeil est à la fois étroitement lié à la fonction circadienne, et, de façon bidirectionnelle, à l'immunité. Les interactions entre ces trois systèmes dynamiques ont un impact important sur la réponse de l'organisme aux agents pathogènes et au développement de pathologies notamment cancéreuses (178). Par exemple, chez les patientes atteintes d'un cancer du sein, on a constaté que l'altération sévère du rythme circadien de la cortisolémie était associée à la fois à une perturbation du sommeil et à une suppression de l'activité des cellules NK, ce qui se traduisait par une réduction de la survie globale (179, 180). Les biocapteurs portables modernes permettent une évaluation non invasive des cycles circadiens et du sommeil, dont les altérations ont également été associées à une perturbation immunitaire chez les travailleurs de nuit (181, 182). Ainsi, les solutions numériques devraient jouer un rôle primordial dans l'optimisation de l'administration dans la journée des ICIs.

Compte tenu de l'impact de la fonction circadienne sur les résultats de la chimiothérapie cytotoxique (138) et du potentiel d'interventions non pharmacologique pour augmenter l'efficacité des ICIs tels que l'alimentation, l'activité physique, la gestion du stress, la synchronisation circadienne des

perfusions et le renoncement à des co-médicaments inutiles (183, 184), il est logique de chercher à optimiser l'efficacité des ICIs par des interventions ciblant le système circadien.

La littérature suggère qu'environ un tiers des patients atteints d'un cancer avancé ou métastatique présentent une perturbation circadienne, qui consiste en la suppression ou l'amortissement sévère des rythmes circadiens d'activité-repos, de sécrétion de cortisol et/ou de mélatonine et/ou de la température corporelle. Ces rythmes sont des biomarqueurs essentiels du système de coordination circadienne. Cependant, les macrophages et d'autres cellules immunitaires sont aussi dotés d'une horloge moléculaire autonome et peuvent donc présenter une fonction et une prolifération rythmiques *in vitro*, en l'absence de signaux rythmiques endogènes (30). Des études précliniques chez le Rat ont mis en évidence la persistance des rythmes immunologiques malgré la suppression complète (185). De même, l'ablation des noyaux suprachiasmatiques des souris abolit les rythmes circadiens d'activité-repos et de température corporelle, mais laisse persister des rythmes circadiens altérés des lymphocytes circulants, y compris chez les animaux porteurs de tumeur (24). Des observations similaires ont été faites chez des patients cancéreux sans rythme détectable d'activité-repos ou de cortisol (186). Ces résultats soulignent la nécessité d'études spécifiques visant à préciser les

relations entre les biomarqueurs de la coordination circadienne et les rythmes immunologiques chez les patients cancéreux, dont la réponse tumorale à l'immunothérapie varie en fonction de l'heure d'administration.

Des différences d'efficacité ou de tolérance de la chronochimiothérapie anticancéreuse en fonction du sexe ont été signalées, en raison des différences de rythmes circadiens entre les patients masculins et féminins. Une méta-analyse portant sur 345 femmes et 497 hommes atteints d'un cancer colorectal métastatique a révélé que les hommes vivaient significativement plus longtemps sous oxaliplatine-5-fluouracile-leucovorine chronomodulés que sous chimiothérapie conventionnelle. L'inverse a été observé chez les femmes (102). Des données expérimentales et cliniques confirment un retard d'environ 6 heures dans l'horaire optimal d'administration de l'oxaliplatine, du 5-fluorouracile et de l'irinotécan, à la fois chez la Souris et chez les patients atteints de cancer (138). En effet, de nombreux rythmes circadiens des sécrétions hormonales, ainsi que les expressions génétiques dans plusieurs tissus, présentent des amplitudes plus importantes chez les animaux femelles en comparaison des mâles, ce qui souligne l'importance du sexe pour les réponses circadiennes aux médicaments (135, 138). En ce qui concerne la chrono-immunothérapie, quatre des 24 études citées ici ont constaté une tendance à une réduction plus importante du risque relatif de décès

précoce en cas d'administration d'ICIs en début de journée chez les femmes, par rapport aux hommes (142, 143, 149, 187). Cette constatation est cohérente avec les rythmes de plus grande amplitude rapportés chez les femmes par rapport aux hommes (188) bien que des résultats divergents aient été publiés sur ce sujet. En effet, les 20 autres études analysées n'ont pas rapporté de différences d'efficaté selon l'heure de traitement, en fonction du sexe. Quatre méta-analyses portant sur de grands effectifs de patients ont examiné si le sexe avait une influence sur l'efficacité de l'immunothérapie, indépendamment du moment d'administration. Deux ont conclu positivement, et deux négativement (189-192). Des études prospectives sont donc nécessaires pour établir d'éventuelles différences d'efficacité et de tolérance de l'immunothérapie selon le sexe biologique des patients, et pour identifiier les biomarqueurs circadiens et immunologiques pertinents. En effet, les principaux mécanismes de la chrono-efficacité des ICIs sont liés au contrôle circadien robuste de la fonction immunitaire, du trafic, des réponses aux ICIs et du microenvironnement tumoral qui ont tout récemment été démontrés dans les modèles expérimentaux (117, 119, 150). Fortin et al. (172) ont montré que le contrôle circadien de l'immunosuppression tumorale affectait aussi l'efficacité des ICIs en raison d'une production circadienne de cellules myéloïdes suppressives exprimant les récepteurs PD-1. Des études prospectives

translationnelles sont donc indispensables pour identifier précisément ces mécanismes chez l'Homme.

6 CONCLUSION ET PERSPECTIVES

Les objectifs de ma thèse étaient d'identifier le rôle de l'heure d'administration dans la journée des ICIs sur leur efficacité et leur tolérance chez des patients atteints de cancer, préciser les heures d'administration associées à une meilleure survie et leur reproductibilité, identifier les mécanismes circadiens potentiels et préciser l'importance des horaires des premiers cycles dans la chronoefficacité des ICIs et de l'immunochimiothérapie.

Dans trois articles publiés et un article soumis pour publication, je montre que le moment d'administration des ICIs dans la journée constitue un facteur pronostique majeur de la survie globale et de la survie sans progression chez les patients cancéreux, indépendamment des paramètres cliniques, biologiques et moléculaires connus. Ici, je démontre que l'administration matinale des ICIs en monothérapie ou en association avec un protocle standard de chimiothérapie augmente de deux à quatre fois la survie sans progression et la survie globale. Je démontre aussi que l'heure d'administration des quatre premiers cycles est la plus importante et semble fonder la chronoefficacité de l'ensemble du traitement dans sa durée qui peut atteindre plusieurs années. Enfin, j'ai demontré que l'efficacité des ICIs persiste malgré l'association à une chimiothérapie immunomodulatrice.

La revue exhaustive de la littérature a permis de comprendre les principaux mécanismes impliqués dans la chronoefficacité des ICIs. Ainsi, les composantes physiologiques et moléculaires du système circadien régulent les fonctions immunitaires qui contrôlent la cancérogenèse, et notamment la circulation des cellules immunitaires dans l'organisme et leur infiltration du microenvironnement tumoral.

En résumé, mes travaux mettent en évidence une augmentation majeure de l'efficacité des ICIs seuls ou combinés à une chimiothérapie, par une administration matinale en comparaison d'une administration l'après-midi. Le critère d'évaluation est la survie, le plus robuste qui soit. Malgré la surprise et l'incrédulité initiale suscitée par cette observation dans ma première publication chez des patients atteints de cancer pulmonaire, ce résultat n'a fait que se confirmer, maintenant pour 8 cancers différents dans 4 continents, alors que trois études fondamentales en révélaient les mécanismes en cause dans des modèles expérimentaux, mécanismes suspectés en clinique dans la revue exhaustive que j'ai réalisée et publiée. Dans leur globalité, ces résultats constituent à mon sens une avancée majeure dans la prise en charge des patients atteints de cancer, avec un probable impact significatif sur l'amélioration du rapport coût-efficacité de l'immunothérapie. Ces résultats devraient induire des modifications de la

logistique hospitalière et de soins, et de technologie d'administration des traitements afin d'optimiser les horaires d'administration.

Cependant, des essais cliniques randomisés et des études translationnelles concomitantes sont indispensables pour définir précisément les relations entre horaires d'administration, efficacité et tolérance des ICIs, ainsi que pour identifier les biomarqueurs circadiens qui vont permettre la personnalisation de la chrono-immunothérapie des cancers. A ma connaissance trois essais randomisés financés sont en cours ou en préparation en France, aux USA et en Chine. L'essai Immunotime, au plan duquel j'ai contribué, comparera la survie à un an après administration matinale ou l'après-midi d'immunochimiothérapie chez 242 patients atteints de CBNPC dans 33 centres français. Il sera alors important de tenir compte de la robustesse ou de la perturbation du système circadien, et du chronotype des patients, ainsi que de la fonctionnalité des l'horloges moléculaires tumorales. L'objectif est d'obtenir un bénéfice de survie cliniquement significatif par une administration personnalisée des ICIs afin de justifier des recommandations pour la chrono-immunothérapie des cancers auprès des autorités sanitaires.

7 ANNEXES

7.1 ANNEXE I : ETUDE « IMMUNOTIME »

Evaluation de l'effet de l'heure d'administration sur l'activité du pembrolizumab en association avec le carboplatine pemetrexed en première ligne dans le cancer du poumon métastatique non épidermoïde. Essai de phase III randomisé et multicentrique (IMMUNOTIME).

Coordinateur et Investigateur Principal : Boris Duchemann

Coordination Scientifique : ***Abdoulaye Karaboué*** *et Francis Lévi*

8.1.1 Résumé

Introduction : Les rythmes circadiens influencent les horloges moléculaires des lymphocytes T, des macrophages et des cellules dendritiques avec un impact important sur le fonctionnement du système immunitaire (118, 193). On a ainsi observé des réponses aux vaccinations améliorées après une administration matinale (118). Dans des modèles murins, les inhibiteurs de PD-1 (ICI) sont plus efficaces en cas d'administration au début de la phase d'activité (nocturne), avec une infiltration plus marquée de la tumeur par les cellules immunitaires (117). En accord avec ces rythmes circadiens des réponses immunitaires, de nombreuses

études ont observé l'augmentation de la survie sans progression (SSP) et de la survie globale (SG) en cas d'administration matinale d'ICIs. Le pembrolizumab, seul ou associé à une chimiothérapie, est devenu le traitement standard de première intention du cancer du poumon non à petites cellules (CPNPC). Il se lie à la protéine PD-1 sur les lymphocytes T CD8, les macrophages et les cellules dendritiques, et entraîne la destruction des cellules cancéreuses par les cellules immunitaires. Dans une méta-analyse de 13 études comprenant 1663 patients avec divers types de tumeurs nous avons rapporté qu'une administration matinale des ICIs était associée à un Hazard-Ratio (HR) de décès précoce de 0,53 [95% CI, 0,34-0,80] (145).

Notre hypothèse est que l'administration matinale du pembrolizumab en association avec une chimiothérapie à base de carboplatine et de pemetrexed améliorera de manière significative le taux de survie à un an par rapport à l'administration l'après-midi chez les patients atteints d'un cancer du poumon non-épidermoide métastatique

Méthodes : Immunotime est un essai multicentrique, randomisé, contrôlé, et ouvert. Cet essai compare l'impact de l'horaire d'administration du pembrolizumab en association avec la chimiothérapie chez les patients atteints de CPNPC métastatique sur la survie à 1 an (194). Les patients seront randomisés en deux groupes : (i) le groupe du matin dans lequel le début de l'administration aura lieu

entre 8h00 et 12h00 et (ii) le groupe de l'après-midi dans lequel le début de l'administration aura lieu entre 13h00 et 17h00. Le critère principal d'évaluation sera le taux de survie à un an. Sur la base de l'essai Keynote-189 (149) et de notre méta-analyse des données rétrospectives disponibles (145), nous émettons l'hypothèse que les taux de survie à un an des patients recevant une administration le matin ou l'après-midi seront respectivement de 80 % et 64 % (HR = 0,50). En considérant un risque alpha bilatéral de 5%, une puissance de 80%, un HR de 0,50 et un taux de patients perdus de vue de 5%, il sera nécessaire d'obtenir 65 événements, soit 242 patients randomisés, pour montrer une différence significative entre les deux groupes.

Discussion : Cette étude sera le premier essai prospectif randomisé multicentrique qui étudie l'impact sur la survie de l'horaire d'administration du pembrolizumab en association avec une chimiothérapie par carboplatine pemetrexed dans le cancer du poumon. Si Immunotime confirme l'impact positif important de l'administration matinale des ICIs, il provoquera un changement majeur de l'utilisation de ces thérapeutiques dans la prise en charge individuelle de ces patients. Ces résultats pourraient aussi être étendus à d'autres types de cancer. *Immunotime* stimulera aussi la recherche sur la personnalisation des horaires de traitement des patients cancéreux.

L’impact économique des modification de pratiques et l’optimisation du cout efficacité des immunothérapie sera évalué lors d'une étude médico-économique concomitante.

Pour améliorer la faisabilité de notre essai suite aux commentaires des relecteurs, nous avons augmenté la taille de la population étudiée et avons inclus tous les patients métastatiques atteints de carcinome pulmonaire non épidermoide, indépendamment de l'expression tumorale de PD-L1. Ce choix a été conforté par l'amélioration de la survie lors de l'administration matinale de la combinaison d'immuno-chimiothérapie en première ligne de traitement de 713 CBNPC métastatiques (Karabaoué ASCO 23; Lévi ASCO 24). Immunotime implique 33 centres français dont deux CLCC (Gustave Roussy, Institut Curie), les CHU (dont APHP, Lille, Marseille) et de nombreux centres nationaux du groupe coopératif GFPC. La durée prévue du recrutement est de 18 mois avec un suivi de 2 ans. L'association de patients "moncancerdupoumon" a collaboré au protocole en particulier par le biais d'une enquête nationale qui a confirmé l'intérêt des patients et l'acceptabilité de cet essai indiquée dans plus de 400 réponses.

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INSTITUT NATIONAL DU CANCER

APPEL À PROJETS 2024

Programme hospitalier de recherche clinique en Cancérologie
Programme for Hospital Clinical Research in Cancer

PHRC-K 2024

Dossier de candidature / *Full project*

DATE LIMITE DE SOUMISSION DES CANDIDATURES : 12 mars 2024 -18h

Le projet doit être rédigé en anglais
Document à soumettre en ligne (uploader) dans la rubrique "Descriptif du projet"

n° du dossier : Veuillez indiquer le n° de dossier attribué par le portail PROJETS (Menu "Dépôt de projets")	**PHRC-K24-036**
Acronym (15 characters max without any space):	IMMUNOTIME
Titre du projet :	Evaluation de l'effet de l'heure d'administration sur l'activité du pembrolizumab en association avec le carboplatine pemetrexed en première ligne dans le cancer du poumon métastatique non épidermoïde. Essai de phase III randomisé et multicentrique
Project title :	Evaluation of the effect of time of administration on the activity on first line of pembrolizumab in association with carboplatin pemetrexed in metastatic non-squamous lung cancer. A randomized multicenter phase III trial

Research domain **-Organ, tumor location:** **-Others:**	**Oncology** **Lung cancer** **Chronotherapy**
Keywords **-Coordinator domain:** **-Required reviewer's field of expertise:**	**- Oncology, Lung cancer, immunotherapy, Chronotherapy** **- Same as coordinator**

Le projet concerne-t-il une maladie rare ? / Does the project concern a rare disease ?	**Oui ☐ / Non ☒**
-Si oui, préciser son code ORPHA / If yes, please specify the ORPHA number	

-et le nom de la maladie rare / If yes, specify the rare disease name	

1- GENERAL INFORMATION

First name and name of coordinator	**Boris DUCHEMANN**
Specialty	Pneumologist
Service ou département - *Unit or department*	Oncology médicale et thoracique
Name and address of the hospital	Hôpital Avicenne, HUPSSD, Bobigny, France Research unit : CCT Chronothérapie, cancers et transplantation Université Paris Saclay
Phone number	0649078059
E-mail	boris.duchemann@aphp.fr
Physician, dental practitioner / Biologist / Nurse, other healthcare professional:	(scientific coordinator) Francis LEVI Oncologist CCT Chronothérapie, cancers et transplantation Unité de recherche, Université Paris Saclay
First name and name of the methodologist	Lamiae GRIMALDI
Name and address of the hospital	URC (Clinical Research Unit) GH APHP Paris-Saclay Hôpital Bicêtre Pavillon Lasjaunias (Secteur Bleu, Porte 89, 1er étage) 78 rue du Général Leclerc, 94275 Le Kremlin Bicêtre Cedex DRCI-URC project advisor: Pr Lamiae GRIMALDI
Phone number	+33(0)171167728
E-mail	lamiae.grimaldi@aphp.fr

Nom de l'établissement coordonnateur du projet et également gestionnaire des fonds - *sponsor :* **Etablissement de santé (ES) ou Groupement de coopération sanitaire (GCS) ou Maison de santé ou Centre de santé, coordonnateur du projet et également gestionnaire des fonds**	Assistance Publique – Hôpitaux de Paris (AP-HP) and by delegation: Délégation à la Recherche Clinique et à l'Innovation - DRCI (Clinical Research and Innovation Department) Hôpital Saint-Louis 1, avenue Claude Vellefaux
Montant demandé *Funding requested*	1 089 600 euros Si la somme demandée sur le dossier final est différente de celle indiquée sur la lettre d'intention, merci de le préciser

Typologie de la demande *Application typology* ***(barrer les mentions inutiles)***	
☐: **1ère soumission au PHRC cancer** *First submission.* ☒: **re soumission** *re-submission* (soumis en 2023) * case à cocher	
Avis d'un comité de patients : *Patients' committee notice*	Ce dossier a –il été soumis au préalable à un comité de patients : *Has this project be submitted to a patients committee?* **Oui** ☒ **/ Non** ☐ Yes / No Si le projet est financé, cette procédure devra être mise en œuvre.

Avant de rédiger le dossier il est indispensable de prendre connaissance des éléments d'informations ci -après :

PUBLICATION

Toute publication, sous quelque forme que ce soit (notamment articles, abstracts), réalisée dans le cadre du projet devra obligatoirement :

- reprendre l'identifiant unique et scientifique du projet communiqué sur le Portail PROJETS. Ce code permettra de mener des analyses quantitatives et qualitatives des publications issues des financements apportés par l'Institut et la DGOS.

- faire état du soutien apporté par l'Institut et la DGOS à la réalisation du projet. Les citations devront être effectuées comme suit : « Avec le soutien de l'Institut national du cancer et de la DGOS » ou « INCa-DGOS et le code attribué au projet ». Toute autre utilisation devra faire l'objet de l'accord préalable et écrit de l'Institut et, le cas échéant, de la DGOS. Le texte sera communiqué pour information à l'Institut.

L'Institut recommande de privilégier les publications dans des revues ou ouvrages nativement en accès ouvert. A défaut, le Bénéficiaire ainsi que les équipes participant à la réalisation du projet s'engagent à déposer les publications scientifiques issues des projets de recherche financés dans une archive ouverte.

COMMUNICATION

Les communications relatives à la réalisation du projet et, particulièrement les communications en direction de la presse, mentionneront le soutien apporté par l'Institut (ainsi que, le cas échéant, celui du partenaire désigné par l'Institut).

Les citations devront être effectuées comme suit : « Avec le soutien de l'Institut national du cancer et de la DGOS » ou « INCa-DGOS et le code attribué au projet ».

Toute autre utilisation devra faire l'objet de l'accord préalable et écrit de l'Institut et de la DGOS.

2- RÉSUMÉ EN FRANÇAIS ET EN ANGLAIS / *ABSTRACT IN FRENCH AND IN ENGLISH*

Attention, ce résumé est indispensable pour l'expertise de votre projet. Un résumé grand public vous est également demandé sur le portail PROJETS

Résumé: (max. 700 mots=689)

Introduction : Les rythmes circadiens influencent les horloges moléculaires des lymphocytes T, des macrophages et des cellules dendritiques avec un impact important sur le fonctionnement du système immunitaire (1,2). On a ainsi observé des réponses aux vaccinations améliorées après une administration matinale (1). Dans des modèles murins, les inhibiteurs de PD-1 (ICI) sont plus efficaces en cas d'administration au début de la phase d'activité (nocturne), avec une infiltration plus marquée de la tumeur par les cellules immunitaires (3). En accord avec cette sensibilité circadienne des fonctions immunitaires, de nombreuses études ont observé l'augmentation de la survie sans progression (SSP) et de la survie globale (SG) en cas d'administration matinale d'ICIs.
Le pembrolizumab, seul ou associé à une chimiothérapie, est devenu le traitement standard de première intention du cancer du poumon non à petites cellules (CPNPC). Il se lie à la protéine PD-1 sur les lymphocytes T(CD8), les macrophages et les cellules dendritiques, et entraîne la destruction des cellules cancéreuses par les cellules immunitaires. Dans une méta-analyse de 13 études comprenant 1663 patients avec divers types de tumeurs nous avons rapporté qu'une administration matinale des ICIs était associée à un Hazard-Ratio (HR) de décès précoce de 0,53 [95% CI, 0,34-0,80] (4).
Notre hypothèse est que l'administration matinale du pembrolizumab en association avec une chimiothérapie à base de carboplatine et de pemetrexed améliorera de manière significative le taux de survie à un an par rapport à l'administration l'après-midi chez les patients atteints d'un cancer du poumon non-épidermoide métastatique
Méthodes : Immunotime sera un essai multicentrique, randomisé, contrôlé, en ouvert. Cet essai comparera l'impact de l'horaire d'administration du pembrolizumab en association avec la chimiothérapie chez les patients atteints de CPNPC métastatique sur la survie à 1 an(5). Les patients seront randomisés en deux groupes : (i) le groupe du matin dans lequel le début de l'administration aura lieu entre 8h00 et 12h00 et (ii) le groupe de l'après-midi dans lequel le début de l'administration aura lieu entre 13h00 et 17h00. Le critère principal d'évaluation sera le taux de survie à un an. Sur la base de l'essai Keynote-189 (6) et de notre méta-analyse des données rétrospectives disponibles (4), nous émettons l'hypothèse que le taux de survie à un an des patients recevant une administration le matin et l'après-midi sera respectivement de 80 % et 64 % (HR = 0,50). En considérant un risque alpha bilatéral à 5%, une puissance à 80%, un HR à 0,50 et un taux de perdu de vue à 5%, il sera nécessaire d'obtenir 65 événements, soit 242 patients randomisés, pour montrer une différence significative entre les deux groupes.
Discussion : Cette étude sera le premier essai prospectif randomisé multicentrique qui étudie l'impact sur la survie de l'horaire d'administration du pembrolizumab en association avec une chimiothérapie par carboplatine pemetrexed dans le cancer du poumon. Si Immunotime confirme l'impact positif important de l'administration matinale des ICIs, il s'agira d'un changement majeur de l'utilisation de ces thérapeutiques dans la prise en charge individuelle de ces patients. Ces résultats pourraient aussi être étendus à d'autres types de cancer. Il stimulera aussi la recherche sur la personnalisation des horaires de traitement des patients cancéreux.
L'impact économique des modification de pratiques et l'optimisation du cout efficacité des immunothérapie sera évalué lors d'une étude médico-économique concomitante.

Pour améliorer la faisabilité de notre essai suite aux commentaires des relecteurs, nous avons augmenté la taille de la population étudiée et avons inclus tous les patients métastatiques atteints de carcinome pulmonaire non épidermoide, indépendamment de l'expression tumorale de PD-L1. Ce choix a été conforté par l'amélioration de la survie lors de l'administration matinale de la combinaison d'immuno-chimiothérapie en première ligne de traitement de 713 CBNPC métastatiques (Karabaoué ASCO 23;Lévi ASCO 24 soumis). Immunotime implique 33 centres français dont le CLCC (Gustave Roussy, Institut Curie), les CHU (dont APHP, Lille, Marseille) et de nombreux centres nationaux du groupe coopératif GFPC. La durée prévue du recrutement est de 18 mois avec un suivi de 2 ans. L'association de patients "moncancerdupoumon" a collaboré au

protocole en particulier par le biais d'une enquête nationale qui a confirmé l'intérêt des patients et l'acceptabilité de cet essai à travers plus de 400 réponses.

Abstract: (max. 700 words = 678)

Introduction: Pronounced circadian rhythms in immune functions and cell trafficking are generated by cell-autonomous molecular clocks in T lymphocytes, macrophages and dendritic cells (1,2). Human T(CD8) cells-mediated responses to vaccinations are significantly improved following morning administration (1). In mice, the functionality and timing of the molecular clock in dendritic cells determine their tumor infiltration pattern over the 24 hours and drive circadian anti-tumour immune responses (1). In tumor-bearing mice, a PD-1 inhibitor was most effective following dosing in the early nocturnal-activity phase, due to the molecular clock dependency of both tumor infiltration by immune cells and PD-1 expression (3). Consistently with such temporal organization of immune functions, many studies have revealed the prolongation in progression-free survival (PFS) and overall survival (OS) through early vs late Time-of-Day (ToD) of administrations of Immune-Checkpoint-Inhibitors (ICIs).
Pembrolizumab, alone or combined with chemotherapy, has become standard first-line treatment for non-small-cell-lung cancer (NSCLC). It blocks the PD-1 protein on T(CD8) lymphocytes (CD8), macrophages, and dendritic cells, resulting in cancer cells killing by the immune cells.
We have reported that early ToD of ICIs was associated with a Hazard-Ratio (HR) of an earlier death of 0.53 [95% CI, 0.34-0.80] in a meta-analysis of 13 studies from 9 countries in 4 continents comprising 1663 patients with various tumor types, including 5 studies in 782 NSCLC patients(4). HRs were lowest in those studies where early ToD corresponded to morning dosing.
We hypothesize that morning administration of pembrolizumab in combination with carboplatin-pemetrexed chemotherapy will significantly improve one year-survival rate as compared to afternoon dosing in NSCLC patients with metastatic non squamous cell tumor.

Methods: Immunotime will be a multicenter, randomized, controlled, open-label trial. This trial will compare the impact of the timing of administration of pembrolizumab in combination with chemotherapy in patients with metastatic NSCLC on 1-year survival (5). Patients will be randomized into two groups: (i) the morning group in which the start of administration will occur between 8:00 a.m. and 12:00 p.m. and (ii) the afternoon group in which the start of administration will occur between 1:00 p.m. and 5:00 p.m. The primary endpoint will be the one-year survival rate. Based on the Keynote-189 trial (6) and meta-analysis of retrospective available data (4), we hypothesize that the one-year survival rate for patients receiving morning and afternoon administration will be 80% and 64% respectively (HR = 0.50). A sample size of 242 randomized patients (65 events) is needed to detect an HR= 0.50 with 80% power and a two-sided log-rank test alpha of 0.05, including an estimated proportion of 5% drop-outs.

We also plan a companion study in order to identify the main circadian pharmacokinetics, immune-monitoring, and physiology mechanisms at work to be funded separately.

Discussion: This study is the first multicenter prospective randomized trial that investigates the impact of immunotherapy timing in combination with chemotherapy for efficacy and safety in lung cancer worldwide. If Immunotime confirms the significant positive impact of morning administration of ICIs, it will be a major change in the use of these therapies in the individual management of

these patients. These findings could also be extended to other types of cancer. It will also stimulate research into personalizing treatment schedules for cancer patients.

The economic impact of practice changes and the optimization of the cost-effectiveness of immunotherapies will be evaluated during a concomitant medico-economic study

To enhance the feasibility of our trial following the comments of the reviewers, we have increased the study population and have encompassed all metastatic patients with non-squamous lung carcinomas, regardless of tumor PD-L1 expression. Such choice was further supported by improved survival on morning administration of immuno-chemotherapy combination as first line treatment for 713 metastatic NSCLC (Karabaoué ASCO 23;Lévi ASCO 24 submitted). Immunotime involves 33 French centers included CLCC (Gustave Roussy, Institut Curie), CHU (including APHP, Lille, Marseille) and numerous centers nationwide from the GFPC cooperative group. Anticipated duration of recruitment will last 18 months with a 2-year follow-up. The patient association "moncancerdupoumon" has collaborated to the protocol through a nationwide survey that confirmed patient interest and acceptability of this trial through more than 400 responses.

3- INFORMATIONS SPÉCIFIQUES -*SPECIFIC INFORMATIONS*

Implication d'une structure de recherche clinique si approprié Si oui, cochez la case correspondante et précisez lesquels *Involvement of a clinical research structuer if appropriate.* *If yes, tick the appropriate box and specify which ones*	OUI/*YES* ☒	NON/*NO* ☐
CTD centre de traitement des données labellisé par l'INCa *Data management centre, designated by INCa*		
BCB Bases de données clinico-biologiques *Clinical-biological databases*		
CLIP² centre labellisé INCa de phase précoce *Early clinical trial center designated by INCa*		
SIRIC site de recherches intégrées sur le cancer French comprehensive cancer center		
Intergroupe Coopérateur *Cooperative Intergroup*	**GFPC Groupe Français de Pneumo-Cancérologie** **Secrétariat GFPC** 84 Avenue de la République _CS40701 63050 CLERMONT-FERRAND Cedex 2 France	
CIC-P *Clinical Investigation Centre - plurithematic*		
CIC-EC *Clinical Investigation Centre –* *Clinical epidemiology / clinical trial*		
CIC-BT *Clinical Investigation Centre - biotherapy*		
CIC-IT *Clinical Investigation Centre – technological innovation*		
CTRS/RTRS (préciser lesquels) *Thematic Research and Health Care Centres / Networks (specify which ones)*		
Unité de recherche clinique (préciser laquelle le cas échéant) *Clinical research unit (specify if relevant)*	**URC (Clinical Research Unit) GH APHP.Paris-Saclay** Hôpital Bicêtre Pavillon Lasjaunias (Secteur Bleu, Porte 89, 1er étage)	

	78 rue du Général Leclerc, 94275 Le Kremlin Bicêtre Cedex DRCI-URC project advisor: Pr Lamiae GRIMALDI
Autre *Other*	**Unité de recherche Clinique en économie de la santé d'ile de France (URC éco)** Pr Isabelle Durand-Zaleski

Ce projet comporte-t-il l'utilisation ou la préparation de produits de thérapie cellulaire, de thérapie génique ou de thérapie cellulaire xenogénique, ou la mise en œuvre ou l'évaluation de thérapeutique mettant en œuvre de telles thérapies, à des fins thérapeutiques, d'organes ou de tissus d'origine animale *Does the project involve the use or preparation of cell therapy, gene therapy or xenogeneic cell therapy products, or the implementation of such therapies or assessment of therapeutics, for therapeutic purposes, organs or animal tissues?*	OUI/*YES* ☐	NON/*NO* ☒
Recherches interventionnelles comportant une intervention sur la personne non justifiée par sa prise en charge habituelle au sens de l'article L.1121-1 du code de la santé publique (Jardé 1) *Interventional research with intervention not justified by the usual care of the person as mentioned in article. L.1121-1 of the French Public Health code*	OUI/*YES* ☒	NON/*NO* ☐
Si non *If no*		
Recherches interventionnelles ne portant pas sur des médicaments et ne comportant que des risques et des contraintes minimes au sens de l'article L.1121-1 du code de la santé publique (Jardé 2) *Interventional research not involving drugs and with only minimal risks or constraints as mentioned in articles L.1121-1 of the French Public Health code*	OUI/*YES* ☐	NON/*NO* ☐
Recherches non interventionnelles au sens de l'article L.1121-1 du code de la santé publique (Jardé 3) *Non interventional research as mentioned in art L.1121-1 of the French Public Health code*	OUI/*YES* ☐	NON/*NO* ☐

Dans le cas où il s'agirait d'un projet d'essai thérapeutique portant sur des produits mentionnés à l'article L.5311-1 du code de la santé publique, préciser les items suivants

In the case where the therapeutic trial project deals with products mentioned in article L.5311-1 of the French Public Health code, specify the following items

Essai réalisé sur des patients *Trial on patients*	OUI/*YES* ☒	NON/*NO* ☐
Essai Médicamenteux *Drug trial*	OUI/*YES* ☐	NON/*NO* ☒
Essai randomisé *Randomised trial*	OUI/*YES* ☒	NON/*NO* ☐
Phase : 1, 2 ,3 ,4	3	

Le risque encouru est justifié par : *The risk is justified by:*		
▪ **Le bénéfice escompté pour la personne qui se prête à la recherche** ▪ **The expected benefit for the person participating to the research**	OUI/*YES* ☒	NON/*NO* ☐
▪ **Le bénéfice escompté pour d'autres personnes** ▪ **The expected benefit for other persons**	OUI/*YES* ☒	NON/*NO* ☐
Indemnisation en compensation des contraintes subies prévue *Compensation for suffered damages*	OUI/*YES* ☐	NON/*NO* ☒
Nombre de personnes dont l'inclusion est prévue dans le protocole *Recruitment planned in the protocol*	242	

Projet multicentrique Multicentric *project*	OUI/*YES* ☒	NON/*NO* ☐
Si oui préciser la typologie des centres associée *If yes, specify the type of associated centres*		
CHU - *University hospital centre*	OUI/*YES* ☒	NON/*NO* ☐
CLCC - *cancer fight centres*	OUI/*YES* ☒	NON/*NO* ☐
CHG - *General hospital centre*	OUI/*YES* ☒	NON/*NO* ☐
ESPIC - *French private health structure of collective interest,*	OUI/*YES* ☒	NON/*NO* ☐
Structure privée - *Private structure*	OUI/*YES* ☒	NON/*NO* ☐
Médecins libéraux - *Professional physicians*	OUI/*YES* ☐	NON/*NO* ☒

Projet multidisciplinaire, si oui disciplines concernées *Multidisciplinary project : If yes, specify concerned areas*	OUI/*YES* ☒ Oncology Thoracic oncology pneumology	NON/*NO* ☐

4- CO-FINANCEMENT - *CO-FUNDING*

Le projet a-t-il été présenté à un autre financement *Has another funding been requested for the project?*	OUI/*YES* ☐	NON/*NO* ☒
Le projet a-t-il obtenu un autre financement *Has the project been awarded another funding?*	OUI/*YES* ☐	NON/*NO* ☒
Identité du ou des co-financeurs s'il y a lieu *Co-funders identities if relevant* Nom *Name* Adresse : *Address:*		
Montant du co-financement *Co-funding amount*		

Association avec les organismes de recherche (INSERM ou autre) *Cooperation with research organisms (INSERM or other)*	
Si oui préciser *If yes, please specify* Nom / *Name* Adresse *Address*	UPR "chronotherapy, cancer, transplantation" Paris Saclay University Will join INSERM U1193, Paul Brousse Hospital Villejuif in 2026
Montant du financement accordé par l'organisme de recherche *Total funding granted by the research organism*	0

Participation de l'industrie *Participation of the industry*	OUI/*YES* ☐	NON/*NO* ☒
Si oui, indiquer la nature de la participation (fourniture de médicament, circuit de distribution, autre…) ***If yes**, indicate the kind of participation (drug supply, distribution channels, other …)*		
Si non, justifier / *If no, justify*	The theme has been proposed to industry, who have not yet shown any interest. The protocol uses drugs in their current AMM in their indication. Result could lead to a modification of recommendation	
Un engagement écrit de l'industriel est-il disponible, *Is a written commitment from the industry available?*	OUI/*YES* ☐	NON/*NO* ☒
Si oui, il conviendra d'insérer ce document dans le dossier papier *If yes, please enclose a copy in the application*		

5- ÉQUIPES PARTICIPANTES - *PARTICIPATING TEAMS*

À adapter selon le nombre d'équipes participantes

Équipe *Team*	**Nom** *Name*	**Spécialité** *Specialty*	**Etablissement de santé** *Health institution*	**Téléphone / e-mail** *Phone / e-mail*	**Affiliation éventuelle à un organisme de cherche (INSERM CNRS …)** *Affiliation to research organism*
Équipe 1 (équipe de coordination) *Team 1 (coordination team)*	**Investigateur Coordonnateur** *Coordinator* *Dr Boris DUCHEMANN*	Thoracic oncologist	Avicenne Hospital, APHP, 125 rue de Stalingrad, 93000, Bobigny	+33 6 49 07 80 59 boris.ducheman@aphp.fr	INSERM
	Méthodologiste *Methodologist* Pr Lamiae GRIMALDI		URC (Clinical Research Unit) GH APHP Paris-Saclay Hôpital Bicêtre 94275 Le Kremlin Bicêtre Cedex	+33(0)171167728 Lamiae.grimaldi@aphp.fr	
	Autre, le cas échéant *Scientific coordinator* *Pr Francis LEVI* Dr Abdoulaye KARABOUE	Medical oncologist	INSERM unit	F.Levi@warwick.ac.uk abdoulaye.karaboue@ght-gpne.fr	
Équipe 2 *Team 2*	**Responsable** *Head* Pr Isabelle DURAND-ZALESKI		URC éco, APHP	Isabelle.durand-zaleski@aphp.fr	
	Autre, le cas échéant *Other*				

6- COMPÉTENCES DANS LE DOMAINE DE L'APPEL À PROJETS - *SKILLS AND EXPERTISES*

- **Coordonnateur(s) du projet *Coordinator(s)***

Principaux articles publiés et répertoriés dans des revues à comité de lecture international ou toutes autres publications significatives au cours des cinq dernières années (5 articles au maximum, titre et références) *Major scientific publications in indexed journals and peer-reviewed with international committees or any other significant publications during the past five years (titles and references)*
1. Effect of immunotherapy-infusion time of day on survival of patients with advanced cancers: a study-level meta-analysis. Landré T, Karaboué A, Buchwald ZS, Innominato PF, Qian DC, Assié JB, Chouaïd C, Lévi F, **Duchemann B**. ESMO Open. 2024 Jan 16;9(2):102220. doi: 10.1016/j.esmoop.2023.102220. Online ahead of print. PMID: 38232612
2. CD8+PD-1+ to CD4+PD-1+ ratio (PERLS) is associated with prognosis of patients with advanced NSCLC treated with PD-(L)1 blockers. **Duchemann B**, Naigeon M, Auclin E, Ferrara R, Cassard L, Jouniaux JM, Boselli L, Grivel J, Desnoyer A, Danlos FX, Mezquita L, Caramella C, Marabelle A, Besse B, Chaput N. J Immunother Cancer. 2022 Feb;10(2):e004012. doi: 10.1136/jitc-2021-004012. PMID: 35131864
3. Tyrosine Kinase Inhibitors for Acute Respiratory Failure Because of Non-small-Cell Lung Cancer Involvement in the ICU. Tandjaoui-Lambiotte Y, Akrour Y, Gibelin A, Gonzalez F, Stoclin A, Moreau AS, Jaubert P, Oppenheimer A, **Duchemann B**, Gaudry S. Chest. 2022 Jan;161(1):284-287. doi: 10.1016/j.chest.2021.06.079. Epub 2021 Jul 21.
4. Outcome following nivolumab treatment in patients with advanced non-small cell lung cancer and comorbid interstitial lung disease in a real-world setting. Assié JB, Chouaïd C, Nunes H, Reynaud D, Gaudin AF, Grumberg V, Jolivel R, Jouaneton B, Cotté FE, **Duchemann B**. Ther Adv Med Oncol. 2023 Jan 31;15:17588359231152847. doi: 10.1177/17588359231152847. eCollection 2023. PMID: 36743523
5. Anti-PD-(L)1 for KRAS-mutant advanced non-small-cell lung cancers: a meta-analysis of randomized-controlled trials. Landre T, Justeau G, Assié JB, Chouahnia K, Davoine C, Taleb C, Chouaïd C, **Duchemann B.** Cancer Immunol Immunother. 2022 Mar;71(3):719-726. doi: 10.1007/s00262-021-03031-1. Epub 2021 Aug 10. PMID: 34378081

- **Méthodologiste *Methodologist***

Principaux articles publiés et répertoriés dans des revues à comité de lecture international ou toutes autres publications significatives au cours des cinq dernières années (5 articles au maximum, titre et références) *Major scientific publications in indexed journals and peer-reviewed with international committees or any other significant publications during the past five years (titles and references)*
1. **Grimaldi L**, Papeix C, Hamon Y, Buchard A, Moride Y, Benichou J, Duchemin T, Abenhaim L. Vaccines and the Risk of Hospitalization for Multiple Sclerosis Flare-Ups. JAMA Neurol. 2023 Oct 1;80(10):1098-1104. doi: 10.1001/jamaneurol.2023.2968. PMID: 37669073; PMCID: PMC10481324.
2. Kolla E, Weill A, Zaidan M, De Martin E, Colin De Verdiere S, Semenzato L, Zureik M, **Grimaldi L**. COVID-19 Hospitalization in Solid Organ Transplant Recipients on Immunosuppressive Therapy. JAMA Netw Open. 2023 Nov 1;6(11):e2342006. doi: 10.1001/jamanetworkopen.2023.42006. PMID: 37934496; PMCID: PMC10630896.
3. Jaïs X, Brenot P, Bouvaist H, Jevnikar M, Canuet M, Chabanne C, Chaouat A, Cottin V, De Groote P, Favrolt N, Horeau-Langlard D, Magro P, Savale L, Prévot G, Renard S, Sitbon O, Parent F, Trésorier R, Tromeur C, Piedvache C, **Grimaldi L**, Fadel E, Montani D, Humbert M, Simonneau G. Balloon pulmonary angioplasty versus riociguat for the treatment of inoperable chronic thromboembolic pulmonary hypertension (RACE): a multicentre, phase 3, open-label, randomised controlled trial and ancillary follow-up study. Lancet Respir Med. 2022 Oct;10(10):961-971. doi: 10.1016/S2213-2600(22)00214-4. Epub 2022 Aug 1. PMID: 35926542

4. Sharshar T, Porcher R, Demeret S, Tranchant C, Gueguen A, Eymard B, Nadaj-Pakleza A, Spinazzi M, **Grimaldi L**, Birnbaum S, Friedman D, Clair B; MYACOR Study Group. Comparison of Corticosteroid Tapering Regimens in Myasthenia Gravis: A Randomized Clinical Trial. JAMA Neurol. 2021 Apr 1;78(4):426-433. doi: 10.1001/jamaneurol.2020.5407. PMID: 33555314; PMCID: PMC7871208.
5. **Grimaldi-Bensouda L**, Papeix C, Hamon Y, Benichou J, Abenhaim L. Association between vaccination and the risk of central demyelination: results from a case-referent study. J Neurol. 2023 Oct;270(10):4678-4686. doi: 10.1007/s00415-023-11822-y. Epub 2023 Jun 23. PMID: 37351662; PMCID: PMC10511379.

- **Scientific coordination**

Principaux articles publiés et répertoriés dans des revues à comité de lecture international ou toutes autres publications significatives au cours des cinq dernières années (5 articles au maximum, titre et références) *Major scientific publications in indexed journals and peer-reviewed with international committees or any other significant publications during the past five years (titles and references)*
1. Vlachou D, Veretennikova M, Usselmann L, Vasilyev V, Ott S, Bjarnason GA, Dallmann R, **Levi** F, Rand DA. TimeTeller: A tool to probe the circadian clock as a multigene dynamical system. PLoS Comput Biol. 2024 Feb 29;20(2):e1011779. doi: 10.1371/journal.pcbi.1011779. Epub ahead of print. PMID: 38422117.
2. **Lévi FA**, Okyar A, Hadadi E, Innominato PF, Ballesta A. Circadian Regulation of Drug Responses: Toward Sex-Specific and Personalized Chronotherapy. Annu Rev Pharmacol Toxicol. 2024 Jan 23;64:89-114. doi: 10.1146/annurev-pharmtox-051920-095416. Epub 2023 Sep 18. PMID: 37722720.
3. Bouchahda M, Ulusakarya A, Thirot-Bidault A, Attari A, Bossevot R, Tuligenga R, Hammel P, Adam R, **Lévi F**. Multicentre, interventional, single-arm study protocol of telemonitored circadian rhythms and patient-reported outcomes for improving mFOLFIRINOX safety in patients with pancreatic cancer (MultiDom, NCT04263948). BMJ Open. 2023 Jun 7;13(6):e069973. doi: 10.1136/bmjopen-2022-069973. PMID: 37286324; PMCID: PMC10254807.
4. Zhang Y, Cordina-Duverger E, Komarzynski S, Attari AM, Huang Q, Aristizabal G, Faraut B, Léger D, Adam R, Guénel P, Brettschneider JA, Finkenstädt BF, **Lévi F**. Digital circadian and sleep health in individual hospital shift workers: A cross sectional telemonitoring study. EBioMedicine. 2022 Jul;81:104121. doi: 10.1016/j.ebiom.2022.104121. Epub 2022 Jun 27. PMID: 35772217; PMCID: PMC9253495.
5. Karaboué A, Collon T, Pavese I, Bodiguel V, Cucherousset J, Zakine E, Innominato PF, Bouchahda M, Adam R, **Lévi F**. Time-Dependent Efficacy of Checkpoint Inhibitor Nivolumab: Results from a Pilot Study in Patients with Metastatic Non-Small-Cell Lung Cancer. Cancers (Basel). 2022 Feb 11;14(4):896. doi: 10.3390/cancers14040896. PMID: 35205644; PMCID: PMC8870559.

- **Équipe 2** ***Team 2***

1. Additional, Mechanized Upper Limb Self-Rehabilitation in Patients With Subacute Stroke: The REM-AVC Randomized Trial Rémy-Néris O, Le Jeannic A, Dion A, Médée B, Nowak E, Poiroux É, Durand-Zaleski I; REM Investigative Team*. Stroke. 2021 Jun;52(6):1938-1947. doi: 10.1161/STROKEAHA.120.032545. Epub 2021 Apr 29.
2. Cost-Utility Analysis of Transarterial Radioembolization With Yttrium-90 Resin Microspheres Compared With Sorafenib in Locally Advanced and Inoperable Hepatocellular Carcinoma Zarca K, Mimouni M, Pereira H, Chatellier G, Vilgrain V, Durand-Zaleski I; SARAH Trial Group. Clin Ther. 2021 May 28:S0149-2918(21)00213-7. doi: 10.1016/j.clinthera.2021.04.018. Online ahead of print.
3. The people left behind: refining priorities for health care during and after the pandemic Holmes DR Jr, Stefanini GG, Ntsekhe M, Wijns W, Mossialos EA, Durand-Zaleski I. EuroIntervention. 2020 Jul 17;16(4):e282-e284. doi: 10.4244/EIJY20M06-01.

4. Impact of Next Generation Sequencing on Clinical Practice in Oncology in France: Better Genetic Profiles for Patients Improve Access to Experimental Treatments Coquerelle S, Darlington M, Michel M, Durand M, Borget I, Baffert S, Marino P, Perrier L, Durand-Zaleski I; NGSEco Group. Value Health. 2020 Jul;23(7):898-906. doi: 10.1016/j.jval.2020.03.005. Epub 2020 Jul 18.
5. Cost and outcomes of the ultrasound screening program for birth defects over time: a population-based study in France Ferrier C, Khoshnood B, Dhombres F, Randrianaivo H, Perthus I, Jouannic JM, Durand-Zaleski I. BMJ Open. 2020 Jul 20;10(7):e036566. doi: 10.1136/bmjopen-2019-036566.

A compléter en fonction du nombre d'équipes

7- DESCRIPTION OF THE CLINICAL RESEARCH PROJECT

This part will include:

- Rationale, hypothesis and objective of the investigations
- Location of the work in the context of the current knowledge
- Detailed description of the methodology (number of necessary subjects :)
- Detailed description of the implemented techniques
- Plan of realization of the project:
  - the role of each team
  - a planned calendar adapted to the duration of the project
  - the identification of the key stages,
  - the modalities of coordination of the project
  - the capacity of patients' inclusion

It is expected to bring a solid argument on the feasibility of the project. This point will be a major axis of the decision of the committee of scientific evaluation

In case of re-submission, mention the additional aspects relevant to the previous recommendations of the Scientific Committee.

Detailed clinical research project, (max. 15 pages ; Simple line spacing, Tahoma 10 pts), including a synopsis at the beginning of text)

1- Principal project

1. Synopsis

Full title	Evaluation of the effect of time of administration on the activity on first line of pembrolizumab in association with carboplatin-pemetrexed in metastatic non-squamous lung cancer. A randomized multicenter phase III trial
Acronym/reference	IMMUNOTIME
Coordinating investigator	Dr Boris DUCHEMANN, - Thoracic and Medical Oncology Unit, Avicenne Hospital, Assistance Publique - Hôpitaux de Paris, 93000 - Bobigny, France - Research Unit "Chronotherapy, Cancer, Transplantation", Faculty of Medicine, Paris-Saclay University, Hospital Paul Brousse, 94800 - Villejuif, France
Scientific Director	Pr Francis LEVI - Research Unit "Chronotherapy, Cancer, Transplantation", Faculty of Medicine, Paris-Saclay University, Hospital Paul Brousse, 94800 - Villejuif, France
Sponsor	Assistance Publique – Hôpitaux de Paris

Scientific justification:

Pembrolizumab, alone or combined with chemotherapy, has become standard first-line treatment for non-small-cell-lung cancer (NSCLC). It blocks the PD-1 protein on T(CD8) lymphocytes (CD8), macrophages, and dendritic cells, resulting in cancer cells killing by the immune cells. Pronounced circadian rhythms in immune functions and cell trafficking are generated by cell-autonomous molecular clocks in T lymphocytes, macrophages and dendritic cells(1,2). Human T(CD8) cells-mediated responses to vaccinations are significantly improved following morning administration (1). In mice, the functionality and timing of the molecular clock in dendritic cells determine their tumor infiltration pattern over the 24 hours and drive circadian anti-tumour immune responses(1). In tumor-bearing mice, a PD-1 inhibitor was most effective following dosing in the early nocturnal-activity phase, due to the molecular clock dependency of both tumor infiltration by immune cells and PD-1 expression(3). Consistently with such temporal organization of immune functions, many studies have revealed the prolongation in progression-free survival (PFS) and overall survival (OS) through early vs late Time-of-Day (ToD) of administrations of Immune-

Checkpoint-Inhibitors (ICIs). Indeed, we have reported that early ToD of ICIs was associated with a Hazard-Ratio (HR) of an earlier death of 0.53 [95% CI, 0.34-0.80] in a meta-analysis of 13 studies from 9 countries in 4 continents comprising 1663 patients with various tumor types, including 5 studies in 782 NSCLC patients(4). HRs were lowest in those studies where early ToD corresponded to morning dosing.

Main objective and primary endpoint: To assess the efficacy of pembrolizumab combined with chemotherapy administered in the morning vs afternoon on 1-year survival rate. The primary endpoint will be 1-year milestone survival rate, defined as the survival probability at one year.

Secondary objectives and secondary endpoints: To compare the morning and the afternoon groups with regards to (i) progression-free survival (PFS), (ii) overall survival (OS), (iii) best tumor response over follow-up, (iv) early tumor response rate at 6 weeks, (v) time to treatment stop (and its cause), (vi) percentages of worst toxicity grades per toxicity category and per patient, with time to reach them, both over 1 year, and up to 2 years, (vii) quality of life every 3 months (i.e 4 course of treatment) up to 1 year, (viii) quality of sleep every 3 months up to 1 year, (ix) chronotype measured at baseline, and at 3 and 6 months and (x) to investigate optimal time of administration, (xi) evaluate cost-utility warranted by the assumption that the intervention will impact both survival and quality of life, each of which affects the total cost.
Secondary endpoints will be: (i) PFS defined as delay from randomization until all-cause death or tumor progression whichever comes first, (ii) OS defined as delay from randomization until all-cause death, (iii) best tumor response rate according to RECIST criteria (assessed with TAP/brain scan and/or PET scan and/or MRI every 2 cycles over 1 year), (iv) early response rate according to RECIST criteria (assessed with TAP/brain scan and/or PET scan and/or MRI) at the first evaluation (i.e. usually at 6 weeks), (v) TTD as the delay from randomization until treatment stop irrespective of cause (progression, toxicities, loss of follow up, refusal, end of protocol or death), (vi) adverse events categorized by severity and type (grade 1, 2, 3, 4, or 5 according to CTCAE v5.0 criteria), (vii) quality of life measured using EORTC-QLQ C30 and assessed at baseline and every 4 cycles up to 1 year, (viii) quality of sleep measured using Pittsburgh questionnaire and assessed at baseline and every 4 courses of treatment up to 1 year, (ix) chronotype measured using Horne-Ostberg questionnaire and assessed at baseline, and after 4th and 8th course and (x) analysis of PFS/OS as a function of the real time of pembrolizumab infusion start, taken as a continuous variable (xi) The incremental cost-utility ratio is calculated as Δ costs (between arms) / Δ QALYs (between arms).

Design of the study: Immunotime will be a French randomized controlled open label multicentric trial involving 33 centers.

Study population: Patients with advanced non squamous cell lung cancer (NSCLC).
Main inclusion criteria:
- stage IV NSCLC,
- histologic or cytologic proof of non-squamous cell carcinoma,
- tumor PDL-1 status available from histochemical evaluation,
- WHO performance status of 0 or 1,

Main non-inclusion criteria:
- small cell lung cancer or mixed type histology including small cell lung cancer EGFR activating mutation,
- ALK or ROS1 gene rearrangement,
- previous immunotherapy (including adjuvant or neoadjuvant),
- no measurable metastasis,
- autoimmunity at risk of severe exacerbation including (but not limiting to myasthenia gravis, and vasculitis, or inflammatory bowel disease).

Interventional group: prembrolizumab infusion starting between 08:00 and 12:00 (Morning group)
Control group: pembrolizumab infusion starting between 13:00 and 17:00 (Afternoon group)

Expected benefit for patients: For Participating patients, impact of treatment and timing treatment will be scrutinized, thus allowing an optimization of their medical and care management. For society, the demonstration of a time window that would significantly improve pembrolizumab efficacy and avoid loss of chance due to wrong timing will bear massive beneficial consequences both for NSCLC patients on ICIs therapies as a whole, and for the cost-effectiveness of such immunotherapies.

Risk added by research: The increase in efficacy could be accompanied by an increase in toxicity.
There is also a possible impact on the patient's quality of life when the administration schedule is set by randomization.

Sample size calculation: Based on the Keynote-189 trial, we hypothesize that the one-year survival rate of the patients in the morning and in the afternoon groups will be 80% and 64% respectively (HR = 0.50). A sample size of 242 randomized patients (65 events) is needed to detect an HR= 0.50 with 80% power and a two-sided log-rank test alpha of 0.05, considering 5% drop-outs.

Statistical analysis of the primary endpoint: The primary statistical analysis will compare the 1-year survival rate estimated in each group (morning versus afternoon administration groups). One-year survival rate will be estimated with the non-parametric Kaplan-Meier method with Rothman's 95% Confidence Interval. The two estimates will be considered significantly different if their confidence intervals do not overlap. The main analysis will be performed on the intention-to-treat population and will also be performed in per protocol way, as a sensitivity analysis

Number of participant centers: 33
Number of inclusions expected per center and per month: 0.4/month

Schedule of study: Inclusion period: 18 months; Participation period: 24 months (including 12 months of treatment and 12 month of follow-up); Total duration : 42 months

2. Rationale

Pembrolizumab, alone or combined with chemotherapy, has become standard first-line treatment for NSCLC. It blocks the PD-1 protein on T(CD8) lymphocytes (CD8), macrophages, and dendritic cells, resulting in cancer cells killing. Pronounced circadian rhythms in immune functions and cell trafficking are generated by cell-autonomous molecular clocks in T lymphocytes, macrophages and dendritic cells (1,2). Human T(CD8) cells-mediated responses to vaccinations are significantly improved following morning administration (1). In mice, the functionality and timing of the molecular clock in dendritic cells determine their tumor infiltration pattern over the 24 hours and drive circadian anti-tumour immune responses(1). PD-1 inhibitors were most effective following dosing in the early nocturnal-activity phase, due to clock-dependent tumor-infiltrating immune cells density and PD-1 expression (3). Consistently with such temporal organization of immune functions, thirteen observational clinical studies in 1663 metastatic cancer patients have shown a similar improvement in progression-free survival (PFS) and overall survival (OS) through early vs late Time-of-Day (ToD) of administrations of Immune-Checkpoint-Inhibitors (ICIs). In a meta-analysis of 5 published studies in 782 NSCLC patients, early ToD of ICIs was associated with a Hazard-Ratio (HR) of an earlier death by 0.53 (95% CI, 0.34-0.80)(4). HRs were lowest in those studies where early ToD corresponded to morning dosing. Immunotime aims at establishing the clinical relevance of anti-PD-1 timing for efficacy and tolerability in NSCLC patients through a randomized comparison between morning and afternoon administration. These results were confirmed In a recently published retrospective study involving 361 cancer patients including 80% with NSCLC (6). Results showed that morning infusions were associated with increased OS as compared to afternoon (median 30.3 vs 15.9 months, p=0.0024; HR 1.56 [1.17-2.1], p=0.003). Moreover, we now have retrospective evidence that morning pembrolizumab combined with chemotherapy also nearly doubles overall survival as compared to afternoon dosing in a total of 713 NSCLC patients (ASCO 2024 and manuscript submitted).

3. Hypothesis for the study

We hypothesize that morning administration of pembrolizumab in combination with carboplatin-pemetrexed chemotherapy, will significantly improve one year-survival rate in metastatic non squamous lung cancer patients, as compared to afternoon dosing.

4. Location of the work in the context of the current knowledge

This study is the first prospective multicenter randomized trial that investigates the impact of immunotherapy timing for efficacy and safety in lung cancer worldwide. The Atlanta team is developing a similar randomized trial in malignant melanoma. A monocentric randomized trial of ICI timing is ongoing in China, thus further supporting the need for the multicenter Immunotime trial in non-Asian populations, due to distinct genomic, habitus, and tumoral specificities between European and Chinese populations. If Immunotime leads to positive results, it will provide a definitely innovative major shift in immunotherapy use, impacting safety, tolerability and efficacy and providing novel chronobiomarkers for personalizing immunotherapy for lung cancer, but also for many other cancer types. Gaining insights into the main mechanisms of ICIs timing effects will help link efficacy and tolerability to measurable telemonitored circadian biomarkers in individual patients, a path toward the personalization of immunotherapy and chemotherapy timing. Sparing the administration of ICIs at times of poor efficacy would further optimize their cost-effectiveness. Such secondary endpoint will be evaluated through a medico-economic study to be conducted during the trial.

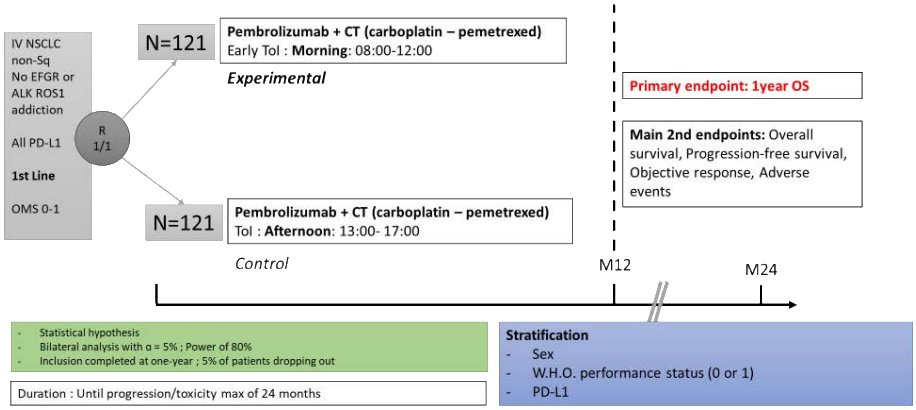
5. Methodological aspects

Design of the study
Immunotime is a multicenter, open-label, randomized, superiority study comparing the efficacy of starting administration in the early part of the day (08.00 -12.00) versus late part of the day (13.00-17.00) of pembrolizumab with chemotherapy administered in patients with stage IV non-squamous cell lung cancer.
Patients with stage IV non-squamous cell lung cancer meeting the eligibility criteria will be included in the study.

Patients will be randomized into 2 groups (1:1):
- A morning group: pembrolizumab infusion will start between 08:00 and 12:00
- An afternoon group: pembrolizumab infusion will start between 13:00 and 17:00

Randomization will be done centrally and by minimization with a 80:20 random element and stratified according to: sex (male or female), W.H.O. performance status (0 or 1) and tumor PD-L1 expression category (<1% or 1%-49% or ≥ 50%).

The first administration will start within a maximum of 3 weeks following randomization. After randomization between both timing groups, each patient will be treated at the same time for all the treatment courses given over the first treatment year.



Main objective and primary endpoint
To assess the efficacy of starting pembrolizumab infusion followed by chemotherapy in the early part of the day (morning group: 08:00 to 12:00) versus the later part of the day (afternoon group: 13:00 to 17:00) of pembrolizumab based upon 1-year survival rate.
The main endpoint will be 1-year milestone survival rate, defined as the survival probability at one year. One-year milestone survival rate has recently been shown as a robust surrogate of overall survival for cancer immunotherapy in a metanalysis of 77 Phase III trials (7).

Secondary objectives and endpoints
Secondary objectives
To compare the Morning group with the Afternoon dosing group on:
- Progression-free survival (PFS)
- Overall survival (OS)
- Best tumor response rate over follow-up
- Early tumor response rate at 6 weeks
- Time to treatment discontinuation (TTD) (and cause of treatment withdrawal)
- Percentages of worst toxicity grades per toxicity category and per patient, with time to reach them, both over 1 year, and up to 2 years.
- Quality of life every three months up to 1 year
- Quality of sleep every three months up to 1 year
- Chronotype measured at baseline, and at 3 and 6 months

- Exploratory analysis of optimal time of administration
- Evaluate cost-utility

Secondary endpoints

- PFS defined as delay from randomization until all-cause death or tumor progression whichever comes first.
- OS defined as delay from randomization until all-cause death
- Best tumor response rate according to RECIST criteria (assessed with TAP/brain scan and/or PET scan and/or MRI every 2 cycles over 1 year).
- Early response rate according to RECIST criteria (assessed with TAP/brain scan and/or PET scan and/or MRI) at the first evaluation (i.e. usually at 6 weeks).
- TTD as the delay from randomization until treatment stop irrespective of cause (progression, toxicities, loss of follow up, refusal, end of protocol or death).
- Adverse events categorized by severity and type (grade 1, 2, 3, 4, or 5 according to CTCAE v5.0 criteria).
- Quality of life measured using EORTC-QLQ C30 and assessed at baseline and every 4 cycles up to 1 year.
- Quality of sleep measured using Pittsburgh questionnaire and assessed at baseline and every 4 courses of treatment up to 1 year.
- Chronotype measured using Horne-Ostberg questionnaire and assessed at baseline, and after 3 and 6month.
- Analysis of PFS/OS as a function of the real time of pembrolizumab infusion start, taken as a continuous variable.
- Incremental cost-utility ratio is calculated as Δ costs (between arms) / Δ QALYs (between arms)

Population of the study

We propose to study a homogeneous population of patients with advanced non squamous cell lung cancer. The benefit of immunotherapy in bronchial cancer is certain for the population as a whole, but it varies largely between individual patients. We have chosen non-squamous cell carcinomas, in order to ensure a homogeneous treatment in terms of molecules and duration of administration regarding both the immune checkpoint inhibitor, i.e. pembrolizumab, and the chemotherapy protocol that combines pemetrexed and carboplatin. The daily timing data of immune checkpoint inhibitors are most extensive and consistent for non small cell lung cancer patients, both as single agents as shown in our metanalysis of single agent immune checkpoint inhibitor (4), and in a recent article (6) as well as for our preliminary report of immunochemotherapy(8), now confirmed by us in an international multicentre retrospective cohort of 713 NSCLC patients (manuscript submitted).

Inclusion criteria

- Stage IV NSCLC.
- Have a histologically-confirmed or cytologically confirmed diagnosis of stage IV (M1a or M1b- AJCC 8th edition) non-squamous NSCLC.
- Have confirmation that EGFR or ALK-directed or ROS 1 therapy is not indicated (documentation of absence of tumor activating EGFR mutations AND absence of ALK gene rearrangements OR presence of a K-Ras mutation).
- Tumor PD-L1 status available from histochemical evaluation.
- Age greater than 18 years.
- WHO performance status of 0 or 1.
- Life expectancy of at least 3 months.
- At least one measurable lesion according to iRECIST criteria.
- Adequate hematology workup: neutrophil count ≥ 1,500/mm3, platelet count ≥ 100,000/mm3, Hemoglobin concentration ≥ 10.0 g/dl.
- Adequate liver function tests: bilirubin ≤ 1.5xLSN and AST/ALAT ≤ 2.5 Upper Normal Limit (UNL) (≤ 5xUNL if liver metastasis(es)).
- Adequate renal function: blood creatinine ≤ 1.5xUNL.
- Affiliated or beneficiary of a social security plan.
- Good understanding of the protocol and expected psychological compliance.
- Signature of informed consent.

Non-inclusion criteria

- Small cell lung cancer or mixed type histology including small cell lung cancer

- EGFR activating mutation (del 19, L858R mutation or L861X in exon 21), ALK or ROS1 gene rearrangement by immunochemistry, FISH or NGS sequencing in the tumor.
- Previous immunotherapy, including adjuvant or neoadjuvant.
- No measurable metastasis.
- Autoimmunity at risk of severe exacerbation including but not limiting to myasthenia gravis, and vasculitis, or inflammatory bowel disease.
- Other active cancer disease or cancer cured within the last 3 years (except for skin cancers).
- Hematological alterations: neutrophil count < 1,000/mm3, platelet count < 100,000/mm3, hemoglobin concentration < 10 g/dl.
- Hepatic tests alterations: bilirubin > 1.5x UNL, AST/ALT > 2.5 UNL (> 5xUNL if liver metastasis).
- Renal alterations: creatinine >1.5xUNL.
- Treatment with immunosuppressive drugs, except for glucocorticosteroids if: Intranasal, inhaled, topical or local injections, Systemic morning administrations of physiologic doses (≤10 mg/day prednisone or equivalent), Premedication for prevention of a hypersensitivity reaction.
- Participation in another interventional clinical study.
- Patient under guardianship, curatorship or other legal protection, deprived of liberty by legal or administrative decision.
- Pregnancy, nursing.

Number of participants included
Based on the Keynote-189 trial (5) we hypothesize that the one-year survival rate for patients receiving morning and afternoon administration will be 80% and 64% respectively (HR = 0.50). A sample size of 242 randomized patients (65 events) is needed to detect an HR= 0.50 with 80% power and a two-sided log-rank test alpha of 0.05, and considering 5% drop-outs.

6. Statistical analysis

Statistical analyses will be performed by the biostatistician at the URC APHP Paris-Saclay with SAS® software. SAS® version will be the one currently used at the time of the analyses.

Analysis of the primary endpoint
The primary statistical analysis will compare the 1-year survival rate estimated in each group (morning versus afternoon administration groups). One-year survival rate will be estimated with the non-parametric Kaplan-Meier method with Rothman's 95% Confidence Interval(9).
The estimates in each group will be considered significantly different if their confidence intervals do not overlap. The primary statistical analysis will be performed when the primary endpoint will be collected for all randomised patients (i.e, when all the randomised patients will complete one year of follow-up or die before reaching one year of follow-up) or when the expected number of events to show a significant difference between groups, will be achieved even if not all randomised patients reach the one-year follow-up. Subjects without documented death will be censored at the last follow-up date.

Analysis of the secondary endpoints
1- The non-parametric Kaplan-Meier method will be used to estimate the PFS curve in each group (morning versus afternoon administration groups). The difference in PFS will be assessed by the log-rank test. A Cox proportional hazards model will be used to assess the magnitude of the effect of the period of administration (ie, hazard ratio) between the two arms. The Cox model will be adjusted on the variables used in the randomisation stratification (i.e sex, W.H.O performance status, expression of tumor PD-L1). For the subject who have disease progression, the true date of disease progression will be approximated by the date of the first assessment at which disease progression is objectively documented. Death will be considered as a confirmed disease progression event. Subjects without documented disease progression or death will be censored at the last disease assessment date.
2- The non-parametric Kaplan-Meier method will be used to estimate the OS curve in each group (morning versus afternoon administration groups). The difference in OS will be assessed by the log-rank test. A Cox proportional hazards model will be used to assess the magnitude of the effect of the period of administration (ie, hazard ratio) between the two arms. The Cox model will be adjusted on the variables used in the randomisation stratification (i.e sex, W.H.O performance status, expression of PD-L1 tumor). Subjects without documented death will be censored at the last disease assessment date.
3- Best tumor response rate will be analysed using the stratified Miettinen and Nurminen method with strata weighting by sample size(10). The stratification factors used for randomisation (i.e sex, W.H.O performance status, tumor PD-L1 expression category) will be applied to the analysis.
4- Early response rate will be analysed using the stratified Miettinen and Nurminen method with strata weighting by sample size(10). The stratification factors used for randomisation (i.e sex, W.H.O performance status, tumor PD-L1 expression category) will be applied to the analysis.

5- The non-parametric Kaplan-Meier method will be used to estimate the TTD curve in each group (morning versus afternoon administration groups). The difference in TTD will be assessed by the log-rank test.
6- Descriptive analysis of the severe adverse events reported.
7- A joint model for longitudinal and discrete survival data will be used to test the effect of the administration group (morning versus afternoon groups) on the quality of life. The model will be adjusted on the score at baseline, the timing of questionnaire assessment and the randomisation stratification factors (i.e sex, W.H.O performance status, tumor PD-L1 expression category).
8- Same statistical methods as the ones planned for the secondary endpoints n°7 (see above)
9- The distribution of chronotypes in both groups will be compared at each timepoint using a chi-square test (or Fisher's exact test if the application conditions are not met).
10- An advanced mathematical model will be developed as already published(6)

Anticipated level of statistical significance
The global level of statistical significance will be 0.05. All tests are two-sided.

Choice of individuals to be included in the analyses
According to the intention to treat principle, all randomized patients will be taken into account.
Depending on the number and type of protocol deviations, the analysis of the primary endpoint might be duplicated in the Per Protocol set. These sensitivity analyses will be detailed in the SAP.
Safety analyses will be performed on the safety set, i.e all randomised patients who will receive at least one immunotherapy administration. Subjects will be included in the group corresponding to the time period during which they actually received their immunotherapy.
A more detailed statistical analysis plan (SAP) will be written during the study and before the database lock.

7. Detailled description of the implemented techniques

Interventions added for the study
The only intervention added by the study will be the randomization of the time of infusion. Infusion and drug themselves are not changed from the standard of care.

Investigational medicinal product(s)
Treatment medications will be administered using the products and doses recommended after the results of the KEYNOTE-189 trial, which established the combination of pembrolizumab and pemetrexed-carboplatin chemotherapy, as the recommended treatment for first line chemotherapy for metastatic non-squamous cell lung cancer (11). This treatment has been approved in France by the National Agency for the Safety and Health Products (ANSM).

Drug	Dose/Potency Dose	Frequency	Route of Administration	Regimen/ Treatment Period
Pembrolizumab[1]	200 mg (over 30min)	Q3W	IV	infusion Day 1 of each 21 day cycle
Carboplatin	AUC 5 (over 30 to 60 min)	Q3W	IV infusion	Day 1 of each 21 day cycle for 4 cycles
Pemetrexed	500 mg/m 2 (over 10 min)	Q3W	IV infusion	Day 1 of each 21 day cycle
[1]Pembrolizumab to be administered prior to chemotherapy.				

Flowcharts
The recruitment will be performed in pneumology or oncology departments and in outpatient clinic of the participating French center. The screening visit will be realize within 1 month prior to randomization, potential subjects will be evaluated to determine whether they fulfil the inclusion criteria. Written consent will be obtained prior to performing any specific protocol procedure. Laboratory tests are to be performed within 10 days prior to the first dose of trial treatment. Tumor imaging must be performed within 30 days prior to the first dose of trial treatment. During the baseline visit medical history will include all active conditions, and any condition diagnosed within the prior 10 years that are considered to be clinically significant by the Investigator and concomitant medication. Details regarding the subject's lung cancer will be recorded separately and not listed as medical history. All prior anti-cancer treatments including systemic treatments, radiation, and surgeries, regardless of the time prior to first dose of trial treatment will be recorded. The Investigator or qualified designee will perform a complete physical exam during the screening period including Eastern Cooperative Oncology Group (ECOG) Performance Scale. During follow up visit, for cycles that do not require a full physical exam per the Trial Flow Chart, the

	Screening phase	Treament Cycles (3 week Cycles)								End of Treatment	Post Treatment		
Treatment Cycle	Screening (visit1)	**1**	**2**	**3**	**4**	**5**	**6 to 17**	**18 to 35**	**36+**	Discon	Safety Follow-up	Follow-up visits	Survival follow-up
Scheduling Window (Days)	-28 to -1	+3	±3	±3	±3	±3	±3	±3	±3	At Time of Discon	30 Days Post Discon	Every 6 Weeks Post Safety Follow-up	Every 12Weeks
Administrative Procedures													
Informed Consent	X												
Informed Consent for Future Biomedical Research (optional)	X												
Inclusion /Exclusion Criteria	X												
Subject Identification Card	X												
Demographics and Medical Histolory	X												
Prior and Concomitant Medications	X	X	X	X	X	X	X	X	X	X	X	X	X
NSCLC Disease Details and Prior Treatment	X												
Randomization	X												
Subsequent antineoplastic therapy status										X	X	X	X
Survival Status													X
Clinical Procedures/ Assessments													
Review Adverse Events		X	X	X	X	X	X	X	X	X	X	X	
Full Physical Examination	X												
Directed Physical Examination		X	X	X	X	X	X	X	X	X			
Vital Signs and Weight	X	X	X	X	X	X	X	X	X	X			
12-Lead ECG	X												
ECOG Performance Status	X	X	X	X	X	X	X	X	X	X	X	X	
Laboratory Procedures/Assessments : Analysis Perdormed by local Laboratory													
Pregnancy Test- Urine or Serum β-HCG	X												
PT/INR and aPTT /PTT	X												
CBC with Differential	X		X	X	X	X	X	X	X	X	X		
Comprehensive Chemistry Panel	X		X	X	X	X	X	X	X	X	X		
Creatinine Clearance Calculation	X		X	X	X	X	X	X	X	X	X		
T3 or FT3, FT4and TSH	X		X		X		X[8]	X[8]	X[8]		X		
Analysis Performed by Central Laboratory													
Pembrolizumab Pharmacokinetics [1]		X	X	X	X								
Immunomonitoring, cytokines, Soluble and exosomal PDL-1 serum concentrations [1]		X			X								
cortisol circadian rhythm, computed from 4 self-collected daily saliva sampels over 2 days[1]		X			X								
Efficacy Measurements													
Tumor Imaging	X			X		X	X	X	X	X		X	
Study Drug Administration													
Pembrolizumab		X	X	X	X	X	X	X					
Patient Reported Outcome (PRO)		X			X								
EuroQol(EQ)-5D,		X			X		X		X				
Questionnary: quality of sleep, chronotype		X			X		X						
[1]for patient in ancillary study monito-immunotime													

Table1

Investigator or qualified designee will perform a directed physical exam as clinically indicated prior to trial treatment administration. The flowchart is describe in the table 1.

Expected benefits for the participants and for society
Participating patients will benefit from trial inclusion since their response to immunotherapy and its impact on their daily life will be scrutinized, thus optimizing their medical and care management. In addition, the evaluation of treatment effects through e-PROs and circadian telemonitoring, thus creating new data-based dialogue between the engaged patients and their oncology teams. This process usually leading to improved confidence and patient satisfaction, as well as treatment optimization with the aim to personalize the timing of administration. This ancillary study will be proposed and conducted in a subset of 50 patients.
The demonstration of a time window that would significantly improve pembrolizumab efficacy and avoid loss of chance due to wrong timing will bear massive beneficial consequences both for NSCLC patients on ICIs therapies as a whole, and for the cost-effectiveness of such immunotherapies. Critical questions will regard optimal timing issues of immune-chemotherapy combinations, that will complement those already systematically reviewed for radiotherapy (12) and chronomodulated chemotherapy (13).
Expectedly, timing issues could become mandatory to be explored for immunotherapeutic drugs in development, and for their use in many cancer types, and different cancer patient characteristics. Currently, preliminary retrospective studies show that ICIs timing matters not only for melanoma and NSCLC, but also for kidney, breast, urologic, oesophageal, stomach,and liver cancers among others. It will become needed to investigate timing issues prospectively (Landré Esmo Open 2024). It will be necessary to further define optimal timing at individual level by using "chronobiomarkers", based on mechanistic studies such as those implemented in Immunotime.

Risks and burdens added by the study
The increase in efficacy could be accompanied by an increase in toxicity.
There is also a possible impact on the patient's quality of life when the administration schedule is set by randomisation and continued during the course of treatment. Need to complete questionnaires over time may engender fatigue and anxiety although this has not been the case in our prior multicentric experience in patients with metastatic colorectal or pancreatic cancer.

8. Plan of realization of the project

The role of each team
Team 1: Team 1 includes the medical coordinator, the scientific coordinator, and the sponsor (including the methodology, statistics, data management, and clinical research assistants teams). The medical coordinator, scientific coordinator, and sponsor will be in charge of the organization of the study and will meet regularly (every two weeks) over the initial 6 months and monthly thereafter.
The team will be responsible for answering questions from investigators from the 33 centers involved. The steering committee will meet every 6 months to monitor accrual, compliance and data quality from the centers and possibly propose amendments. The team will also respond to the IDMC questions on safety every 6 months.

Team 2: The medical economic clinical research unit team will be in charge of the medico-economic analysis which will be carried out during the study and analyzed at the end of the study.
We will carry out a cost-utility analysis. This is warranted by the assumption that the intervention will impact both survival and quality of life, each of which affects the total cost. This health economic evaluation will be prospective, conducted in parallel with the clinical study, and will compare resources, as well as clinical and quality of life results in each arm, for the duration of the follow-up. We follow the recommendations of the national health authority 40. All patients included in the trial are eligible for the health economic evaluation. Analysis of the costs and outcomes will follow the intent to treat principle. The analysis will be conducted from the perspective of the healthcare system (production). Resources and outcomes will be collected prospectively at the patient level using both the study eCRF and hospital discharge data.
The table below summarizes the types and resources collected and the costing methodology.

Type of resource	Resource collected	Source	Valuation	Source
Hospital (acute for treatments, rehabilitation, hospital at home)	Diagnosis related group, diagnosis related days (rehabilitation), days (hospital-at-home), length of stay	Local (PMSI/eCRF) and national (SNDS) reimbursement database	Actual cost adjusted for length of stay	National cost database (ENC)

Out of hospital/ ambulatory care	Imaging, biology, clinic visits, physical therapy, transportation	eCRF and / or reimbursement database SNDS	Fee and list price, reimbursement information	Social health insurance schedule eg. NABM, AMELI, journal officiel or SNDS
Sick leave and days off work	Number of days	eCRF and/or reimbursement database SNDS	Social health insurance daily compensation and average per capita GDP	Social health insurance schedule and INSEE

For hospital admissions, the total length of stay and discharge information (diagnosis-related group) will be extracted from the hospital information system for all event-related subsequent admissions during the follow-up period. The cost of each admission will be estimated from the national cost study, using the actual diagnosis-related group tariff, modified for the length of stay and for the length of stay within an ICU unit.
Out-of-hospital resources will be collected at each visit using patients' interviews. These resources will be valued using the official tariffs for consultations and other interventions.

In a best-case scenario, it would be possible to obtain deterministic linkage of the study database with the claims database of the social health insurance. This would allow exhaustive compilation of all healthcare resources used and accurate calculation of the amounts of healthcare services paid by the social health insurance. Authorization for linkage and data extraction has in the past proven long and difficult to obtain which is why, while we will initiate the process, we prefer to rely on other data sources. Health-related quality of life will be estimated using the EQ-5D-5L questionnaire which will be submitted at inclusion and during a planned follow-up visit at 1 month, 3 months, 6 months and 12 months after inclusion. We will use the published French value set (14) to convert EQ5D-5L items to utility values. The utilities will accumulated into QALYs for each arm using the area under the curve (AUC) method. This method assumes a linear relationship between values at different time points(15).
The primary outcome measure for the economic evaluation will be 12-year cumulated QALYs. The gain of QALYs will be the difference between the utilities accumulated in each time period for each arm.

The incremental cost-utility ratio is calculated as Δ costs (between arms) / Δ QALYs (between arms). Deterministic sensitivity analyses and uncertainty analysis will be conducted on costs and cost-effectiveness data. The uncertainty surrounding the incremental cost-utility ratio will be assessed by nonparametric bootstrapping with 1,000 resamples and the result presented on the cost-effectiveness plane. In addition to the cost-effectiveness plane, we will plot acceptability curves, which relates the probability of having the highest net monetary benefit (strategy being the most cost-effective) to the decision threshold (in QALY/euro).

Planned calendar of the study
Duration of the study
- Inclusion period: 18 months
- Participation period: 24 months (including 12 months of treatment and 12 month of follow-up)
- Total duration: 42 months

The identification of the key stages

Objectives	Milestones	Start Date	Completion Date	Describe how you are going to monitor progress (Deliverables)	Constraints	Contingency plan to address delays
Regulatory steps for trial launch	Ethic approval by regulatory and ethics committee.	09/30/24	01/15/25	Trial contractualisation with the sponsor (APHP); Dates of ethics approval ; date of ANSM approval ; clinical trial number of clinicaltrial.gov	Date of ethic committee and possible delay in ANSM response	Protocol and intent letter are ready for submission by 9/2024. Ethics committee report due within 2 months

						following submission.
Contract and opening visit at participating center	Opening centers	01/15/25	03/01/25	Opening of centers, centers ready for inclusion	Availability of administrative and clinical team for regulatory aspect	Adequate support clinical research support plan for each center
Trial inclusions	Succes in inclusion rate	03/01/25	01/15/26	Half of patient (121) recruited	Motivation of patient and oncology team	Duration of inclusions has been extended to 18 months to secure full accrual in the trial. Patient population include the majority of NSCLC patient. Centers involved are leading center highly experienced in clinical trial.
Trial accrual	Completed accrual	01/15/26	10/31/26	242 patients included	Motivation of patient and oncology team	Duration of inclusions has been extended to 18 months to secure full accrual in the trial. Patient population include the majority of NSCLC patient. Centers involved are leading center highly experienced in clinical trial.
Primary endpoint data	One year survival for all randomized patient	03/01/26	10/31/27	Data monitoring, queries ; database freezing	High number of missing data requiring iterative queries and site visits in some centers	Dedicated research assistants to timely support eCRF completion in near real time.
Data analysis	Statistical report	10/31/27	01/31/28	Statistical report on main endpoint	Insuffisant number of events	Assess secondary endpoints (OS, PFS)
Results dissemination	Result communication and publication	01/31/28	03/01/29	Main publication and congres communication	Manuscript and abstract rejection	Re submission with secondary endpoint and translationnal data

The modalities of coordination of the project

The **coordinating investigator** with the collaboration of the methodologist, the project coordinator, and the Clinical Research Assistants of the clinical research unit and the referent Study site coordinator has several tasks:
a) To organize the Set-up of the trial at each of the participating centers
b) To check the enrollment in each center.
c) To prepare and send a monthly newsletter to the investigators centers
d) To organize steering committee in order to check if the study is running correctly.
e) To organize with Study coordinator referent the coordination of the team in its own center.
The study will be conducted with the clinical research unit of Paris Saclay (URC APHP.Paris-Saclay).

9. The capacity of patients' inclusion

Number of enrolments expected per site and per month
242 patients during 18 months in 33 centers.
A safe average number of 0.4 patient per center per month is expected.
These centres altogether could potentially include a total of 549 patients over 18 months.

Participating centers

N°	First name	Last name	Town	Country	Hospital	Specialty
1	Boris	Duchemann	Bobigny	France	Avicenne	Thoracic Oncology
2	Thierry	Collon	Montfermeil	France	GHI Le Raincy Montfermeil	Oncology
3	Etienne	Giroux-Leprieur	Boulogne	France	Ambroise-Paré	Thoracic oncology
4	Pascal	Hammel	Villejuif	France	Paul Brousse	Oncology
5	Monnet	Isabelle	Créteil	France	CHI Créteil	Oncology
6	Morel	Hugues	Orléans	France	CH Orléans	Oncology
7	Robinet	Gilles	Brest	France	CHU Morvan	Oncology
8	Audigier Valette	Clarisse	Toulon	France	CH Toulon	Oncology
9	Urban	Thierry	Angers	France	CHU Angers	Oncology
10	Léna	Hervé	Rennes	France	CHU Rennes	Oncology
11	Vergnenègre	Alain	Limoges	France	CHU Limoges	Oncology
12	Dujon	Cécile	Le Chesnay	France	CH Versailles	Oncology
13	Abdiche	Samir	Libourne	France	CH Libourne	Oncology
14	Cloarec	Nicolas	Avignon	France	CH Avignon	Oncology
15	Arpin	Dominique	Villefranche sur Saone	France	Hôpital Nord	Oncology
16	Stancu	Alma	Avignon	France	Institut Saint Catherine	Oncology
17	Stoffaes	Louis	Marseille	France	Institut Paoli Calmette	Oncology
18	Le Treut	Jacques	Marseille	France	Hôpital Européen	Oncology
19	Paysse	Magalie	Périgueux	France	CH Périgueux	Oncology
20	Curcio	Hubert	Caen	France	Centre François Baclesse	Oncology
21	Bakebe	Alain	Longjumeau	France	CH Longjumeau	Oncology
22	Kaluzinski	Laure	Cherbourg	France	CH Cotentin	Oncology
23	Guisier	Florian	Rouen	France	CHU Rouen	Oncology
24	Saboundji	Karima	Mantes La jolie	France	CH François Quesnay	Oncology
25	Vieillot	Sabine	Perpignan	France	Centre Catalan d'oncologie	Oncology
26	Greillier	Laurent	Marseille	France	APHM	Oncology

27	Doubre	Hélène	Suresnes	France	Hôpital Foch	Oncology
28	François	Géraldine	Amiens	France	CHU Amiens	Oncology
29	Labourey	Jean Luc	Carcassone	France	CH Carcassone	Oncology
30	Hauss	Pierre Alexandre	Elbeuf	France	CHI Elbeuf	Oncology
31	Daniel	Catherine	Paris	France	Curie Institute	Thoracic oncology
32	Scherpereel	Arnaud	Lille	France	CHU Lille	Pulmonary and Thoracic Oncology
33	Besse	Benjamin	Villejuif	France	Paris-Saclay university	Medical oncology
*	Karaboué	Abdoulaye	Villejuif	France	Paris-Saclay university	Scientific committee
*	Lévi	Francis	Villejuif	France	Paris-Saclay university	Scientific committee

Feasability and representativeness of the target population
To enhance the feasibility of our trial and the representativeness of the target population, we have increased the study population and have encompassed all metastatic patients with adenocarcinomas, regardless of tumor PD-L1 expression. Such choice was supported by improved survival on morning administration of immune-chemotherapy combination as first line treatment for metastatic NSCLC (Karabaoué ASCO23, Lévi ASCO 2024 submitted), consistently with reported ToD dependent efficacy of single agent nivolumab or pembrolizumab.

We had an inclusion period of 18 months with 33 centers including highly experimented center including CLCC (Gustave Roussy, Institut Curie), CHU (APHP and others) and the GFPC a large nationwide and active cooperative group which has already included similar populations within comparable timeframes.

Patient association involvement
We construct the trial with the "Patients en réseau" association is a nationwide organization which was created in February 2014 on the basis of real-life cancer patients experiences. The group "monreseaucancerdupoumon" is part of this association. It has 3000 actives members and the management team is made up of 7 peoples.
Patients were involved at an early stage of the research during protocol writing and in three subsequent stages:
1) The research team contacted the expert patients in my lung cancer network. They assessed the issue of chronotherapy and the expected impact. They suggested to follow up with the association board .
2) The board assessed both the overall interest for the patient community and the possible advisory role of the association during the project design and submission of the project and its dissemination.
3) Then we prospectively questioned patients who were members of the association.
We received 417 responses within 2 weeks to the following questions:
1) Do you think that a study aimed at personalizing the time of administration is justified? (84% yes);
2) Would you be interested in taking part in a protocol evaluating the impact of immunotherapy administration times? (67% yes);
3) Would it be acceptable for you to be randomly assigned to start administration in the morning between 08:00 and 12:00 or in the afternoon between 13:00 and 17:00? (76% yes);
4) Other comments in the form of a verbatim report.
Among these comments, we noted that patients are treated at different times of the day, rarely have a choice of timetable and that the question of access accommodation needs to be addressed. During the trial we will submit a reactions to research participation questionnaires (RRPQ) to assess the views of clinical research studies(16).
The objectives have been chosen to be meaningful for patients, including survival but also quality of life. We are also going to study an aspect that is often neglected, which is customization to the patient's rhythm of life (impact of chronotype and circadian parameters).

10. Improvement since last submission

Response to the reviewers of the last submission (2022-2023)
We have made important modifications to the design of the Immunotime trial. Firstly, we have now chosen one year survival rate as the main endpoint, which is now currently considered as a dependable surrogate for overall survival (Zhang Z, EClinicalMedicine 2023). In a recent meta-analysis of 13 studies (Landre ESMO open 2024), early administration of ICI vs late was associated with a hazard ratio (HR) of was 0.50 [95% CI: 0.42–0.58]; $p < 0.00001$) for overall survival. We computed our sample size based on an expected HR of 0.50.

Regarding the justification for a phase III trial, the number of reported study raised and allows us to perform a meta-analysis, which has shown a consistent survival benefit from early Time of Day administration of ICIs in 13 studies comprising 1663 patients with various tumor types, including 782 patients with NSCLC(4). Our preliminary report of immunochemotherapy (8), now confirmed by us in an international multicentre retrospective cohort of 713 NSCLC patients (manuscript submitted) confirm us in the design. We will conduct an analysis using the real time of administration like a continuous variable. This will allow us to perform sensitivity analyses and mathematical modeling to improve precision in optimal timing definition, and relate it to (chrono)biological biomarkers.

Response to the reviewers of the intention letter (2023-2024)
R1 : ICI infusions are currently administered in the morning or in the afternoon, pending upon each center's logistic and patient's presentation to outpatient clinics. A randomized trial such Immunotime is mandatory to avoid any loss of chance that might result from a non-adapted timing of treatment based on logistic rather than pharmacologic consideration.

R2 : We agreed with the comments so the +/- 1 hour of ToI must remain in the time frame from randomization i.e. between 08:00 and 12:00 for the morning and between 13:00 and 17:00 for the afternoon group.

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2- Ancillary study if relevant

Full title	Biological and clinical telemonitoring of patient treated with immunotherapy and chemotherapy combination for advanced non-small cell lung cancer
Acronym/reference	Monito-immunotime
Coordinating investigator	Dr Boris DUCHEMANN
Scientific Director	Pr Francis LEVI
Sponsor	Assistance Publique – Hôpitaux de Paris

Monito-immunotime

An ancillary study (Monito-immunotime) will be proposed to consenting patients in order to identify those circadian biomarkers that will help determine the individual optimal timing of immunochemotherapy delivery in each patient. The 'Monito-immunotime' will both reveal the main circadian mechanisms at work, and the methods and technologies needed to design personalized cancer chronoimmunochemotherapy protocols. For instance, we will telemonitor circadian rhythms in rest-activity and body temperature (and possibly also heart rate and respiratory rates) and determine the circadian rhythms in salivary cortisol from 50 remote patients before and after 4 treatment courses (25 in each group). This will tell us about the between- and within-patients variabilities in circadian rhythms, as well as possible dosing-time dependent treatment effects on the circadian timing system. The circadian timing system rhythmically coordinates organism and cellular physiology, as well as molecular expressions and drug responses.
This ancillary study aims at the identification of biomarkers towards the personalization of treatment timing.
The following parameters will be compared between both arms in a subset of 50 consecutive consenting patients (from up to 8 centres): 1) Immune blood cell dynamics, 2) CRP and LDH dynamics 3) Serum cytokines concentrations dynamics 4) Soluble and exosomal PDL-1 serum dynamics 5) Circadian synchronization 6) Symptoms and impact on daily life 7) Pharmacokinetics of pembrolizumab 8) Patient-reported outcomes 9) Telemonitored circadian rhythms in rest-activity and body temperature

As indicated in the letter of intent, since PHRC-K funding cannot cover translational aspects, we will apply for dedicated funding if the trial is accepted.

8- DÉTAILS DU FINANCEMENT SOLLICITÉ - *BUDGET REQUESTED*

Compléter la « Grille Budgétaire » à télécharger sur : https://www.e-cancer.fr/Institut-national-du-cancer/Appels-a-projets/Appels-a-projets-en-cours/PHRCK2023

Information relative au traitement des données personnelles qui seront renseignées dans le dossier de candidature

Dans le cadre de ses missions d'intérêt public, l'Institut national du cancer conduit des appels à projets dans le domaine de la cancérologie.
Afin d'effectuer l'évaluation des projets reçus et d'assurer le recensement et le suivi des appels à projets financés par l'Institut, ce dernier doit recueillir des données relatives à l'identité et la vie professionnelle du coordonnateur, du représentant légal ou de la personne dûment habilitée de l'organisme bénéficiaire, de la personne chargée du suivi administratif du dossier, du responsable d'équipe et, le cas échéant, du personnel de l'équipe et des personnes désignées par le coordonnateur ne devant pas avoir connaissance du projet, ces dernières pouvant de par leurs liens en tirer un avantage direct ou indirect.
Les personnes dont les données personnelles figurent dans le dossier de candidature doivent être informées par celui qui les a désignées que l'Institut les utilisera selon les modalités ici décrites.
L'Institut est le responsable de l'utilisation de ces données. Il les conservera 10 ans à compter de la dernière intervention sur un ou plusieurs projets de la personne qui a déposé la lettre d'intention (par exemple signature d'un engagement, dépôt d'un document sur le Portail Projets).
Sauf opposition de votre part, vos données (nom, prénom, mail) alimenteront l'outil de gestion de contacts de l'Institut qui permet également de vous adresser des informations plus ponctuelles concernant les activités de l'Institut.
Conformément au Règlement général sur la protection des données 2016/679 et à la loi informatique et libertés n°78-17 modifiée, vous disposez durant la durée du traitement d'un droit d'opposition, d'un droit d'accès, de rectification, d'effacement et d'un droit à la limitation du traitement de vos données. Pour les exercer, veuillez adresser votre demande par mail à l'adresse suivante : dpo@institutcancer.fr. Vous trouverez les coordonnées de l'Institut, de son représentant et de sa déléguée à la protection des données sur e-cancer.fr.

Vous disposez, par ailleurs, du droit d'introduire une réclamation auprès de la Commission nationale de l'informatique et des libertés (CNIL).

DATE LIMITE DE SOUMISSION : 12 MARS 2024 – 18H

Document à transmettre en format électronique par téléchargement via portail PROJETS https://projets.e-cancer.fr/

- **Le dossier de candidature : descriptif projets - format Word ou bien PDF(8Mo)**
- **La grille budgétaire dûment complétée - Format Excel**
- **Document engagement spécifique pour le PHRC**

8.1.2 Lettre de couverture du projet « *IMMUNOTIME* »

université
PARIS-SACLAY

FACULTÉ DE
MÉDECINE

UPR 'Chronothérapie,
Cancers et Transplantation'
(Directeur : Prof. René Adam)
Campus CNRS,
7 rue Guy Moquet
94800 – Villejuif

Villejuif, March 1st , 2024

To whom it may concern,

Please find attached the detailed Immunotime protocol information as requested on the PHRC-K platform. We have also added a full protocol version to be amended if needed prior to submission to the regulatory authorities, once trial funding is secured. We outline below the main features of Immunotime.

Why did we develop Immunotime?
The need for the Immunotime randomized trial has stemmed for the pioneering retrospective study report by Dr Abdoulaye Karaboué, showing a prominent difference in non-small cell lung cancer (NSCLC) patients' survival as a function of the daily time of nivolumab infusion timing (Proc ASCO 2021, Cancers 2022). Dr Karaboué is a medical oncologist currently completing his PhD project on "survival prolongation mechanisms through cancer chronotherapy" under the direction of Professors Adam and Lévi at Paris-Saclay University (Paris South).

Boris Duchemann is a thoracic oncologist, Assistant Professor, from Avicenne Hospital, APHP, HUPSSD. He has joined our team since mid 2023, and has led a meta-analysis of 13 published studies that has highlighted a significant survival improvement through morning rather than afternoon/evening single agent immunotherapy (ESMO Open 2024).

Over the past three years, the combination of immune checkpoint inhibitors with chemotherapy has become the main reference treatment for patients with non-small cell lung cancer (NSCLC). For this reason, we have now focused the Immunotime trial on the timing of pembrolizumab-chemotherapy as first line treatment for non-squamous NSCLC. To ensure that the daily timing of immune checkpoint inhibitors remained influential on patient survival, Dr Karaboué conducted a preliminary study that identified a major role for the time of day of administration of the 4 initial courses of immunochemotherapy (Proc ASCO 2023). We have just confirmed that morning administration of immunochemotherapy significantly improved overall survival, progression-free survival and tumor response rates as compared to afternoon dosing in an international retrospective study involving 713 NSCLC patients (abstract to ASCO 2024 Annual Meeting and manuscript submitted).

In order to improve the feasibility of this trial compared to last year's version, (i) all the patients with non-squamous NSCLC will be included, regardless of PD-L1 expression; (ii) we chose an inclusion period to 18 months corresponding to 0.4 patient per center, way below their inclusion possibilities ; (iii) the selection of carboplatin-pemetrexed as the single chemotherapy regimen to be associated to pembrolizumab should enhance the homogeneity of treatment effects.

Our research unit has pioneered research on cancer chronotherapy worldwide since the 1990's (Lancet 1997). Our research is also linked with the University of Chicago (USA), with which we have founded the first International Network for Personalized Cancer Chronotherapy, now gathering members from 56 centers in 16 countries (2022-2026), and the University of Warwick (UK) for artificial intelligence in cancer chronotherapy modelling and rhythms telemonitoring.

What are the main features of the Immunotime trial?
We do feel that a randomized trial has become mandatory for the prospective assessment of the relevance of daily time of administration for patients' survival. This is needed for supporting recommendations in daily practice, for promoting basic and clinical cancer chronotherapy research, and ultimately for tailoring treatment delivery to the individual patient's circadian rhythms.

Immunotime is a multicentre, open-label, randomised, superiority trial designed to assess the role of the time of administration of first line immunotherapy-chemotherapy with pembrolizumab and pemetrexed-carboplatin in patients with metastatic non squamous cell NSCLC. This tumor type is encountered in over 65% of NSCLC.

The consenting patients will be allocated to a Morning or an Afternoon group. Pembrolizumab infusions will start between 08:00 and 12:00 for the patients allocated to the Morning group, and between 13:00 and 17:00 for those allocated to the Afternoon group. No per-protocol pembrolizumab infusion will start between 12:00 and 13:00. Pembrolizumab timing will remain consistent (+/- 1 h) from one course to the next for each patient.

The main endpoint is one-year survival rate, a reliable surrogate of overall survival according to a meta-analysis of 77 Phase III trials of immunotherapy (Zhang et al. eClinMed 2023). Overall survival, progression-free survival, best response rate, and adverse events represent secondary endpoints.
We hypothesize that one-year survival rate will be 80% in the Morning group and 64% in the Afternoon group, with a Hazard Ratio of 0.50. The number of patients needed is 242 to demonstrate a statistically significant difference ($p<0.05$) with 80% power.

We aim to complement this trial with an ancillary study to be funded separately from the current PHRC-K call and proposed to consenting patients. The purpose is to identify those circadian biomarkers that will help determine the individual optimal timing of immunochemotherapy delivery in each patient. The 'Monito-immunotime' will both reveal the main circadian mechanisms at work, and the methods and technologies needed to design personalized cancer chronoimmunochemotherapy protocols. For instance, we will telemonitor circadian rhythms in rest-activity and body temperature (and possibly also heart rate and respiratory rates) and determine the circadian rhythms in salivary cortisol from 50 remote patients before and after 4 treatment courses (25 in each group). This will tell us about the between- and within-patients variabilities in circadian

rhythms, as well as possible dosing-time dependent treatment effects on the circadian timing system, and on the immune system variables that will also be measured.

We feel confident that Immunotime will be successful, and that such randomized trial is needed because of the retrospective nature of all currently available evidence. In addition to the accumulating clinical data on immunotherapy timing for lung, kidney, bladder, oesophagus, liver, stomach cancers and melanoma, experimental studies have revealed that prominent circadian rhythms characterize several cellular and molecular mechanisms that account for immune checkpoint inhibitors (Tsuruta et al. Mol Cancer Res 2022; Wang et al. Nature 2023).

If our project is selected for funding, we expect to complete all protocol-related documents and regulatory aspects and reach trial approval by January 2025, and start inclusions by March 2025. Accrual would be completed by October 2026, and main endpoint reached by October 2027. As a result, we foresee communication and first publication of main endpoint results by March 2028, whilst other scientific communications and publications are foreseen upon trial initiation, and at 2, 3, and 4.5 years after project start.

Which are the main entities and persons and involved in the Immunotime project ?
The Research Unit "Chronotherapy, Cancer and Transplantation" in the Faculty of Medicine, Paris Saclay University is leading the project, that mainly involves

- Dr Boris Duchemann, MD, PhD. He is the Principal Investigator of the Immunotime trial. He is an active thoracic oncologist at Avicenne University Hospital (Assistance Publique-Hôpitaux de Paris). He will interact with all parties involved to ensure that the main goals of the trial are reached with excellent quality. He will report and communicate the main results of the trial.
- Dr Abdoulaye Karaboué, MD, PhD in training, will both contribute to the translational study and to patient accrual.
- Professor Francis Lévi, MD PhD. He is the scientific director of the project. He is involved in all aspects of Immunotime design, analyses and interpretation of patient-centered and translational circadian research questions. He will contribute to scientific interactions with the trial participants and entities, and to the dissemination of the results.
- Professor René Adam, MD, PhD is the Head of the Research Unit and a world leader in liver surgery and transplantation, with a long standing commitment in cancer chronotherapy research. He will ensure that the project develops smoothly within the Paris Saclay University and Assistance Publique Hôpitaux de Paris settings. He will play role in the dissemination of the results towards the surgery, gastro-intestinal and hepatobiliary medical and scientific communities.

The Clinical Research Unit from Paris Saclay University Hospitals has worked very closely with
The Research Unit "Chronotherapy, Cancer and Transplantation" to develop the full Immunotime protocol. To finetune the trial hypotheses, they have also evaluated the first retrospective evidence of immunochemotherapy timing for survival outcomes in lung cancer patients, jointly with us (abstract submitted to ASCO 2024 Annual Meeting, and manuscript submitted).

- Prof Lamiae Grimaldi's expertise involves clinical trials methodology and regulatory aspects, as well as conduct, analyses and interpretation. She and her team have critically contributed to the hypotheses, design and logistics of the trial.
- Mrs Celine Piedvache and Dr Adrien Lecoeuvre have more specifically developed the statistical plan.

- Mrs Sonia Makhlouf, has described and costed the logistics of the trial conduct, implementation and reporting.

Other collaborators in Immunotime
We also collaborate closely with a large panel of clinical teams and colleagues with internationally-acknowledged expertise in oncology, including Gustave Roussy (Professor B. Besse and F. Barlesi), Assistance Publique-Hôpitaux de Paris, Curie Institute, and the Groupe Français de Pneumo-Cancérologie (GFPC), a main French cooperative group in thoracic oncology, which has gathered 30 affiliated thoracic oncology units, since the initial conception of Immunotime.

How we foresee the role of the patients and their association
There is actually no recommendation on treatment timing, and the pharmaceutical industry has been reluctant to enter the chronotherapy domain thus far. Establishing a difference in efficacy through Immunotime would lead to a major change in practice with existing therapies, as well as for drug development and registration, and oncology care organizations.

We are convinced that a main strength of our project is that it answers to a question which most patients ask their doctor : 'is there a best time to treat me?' Because the issue of treatment timing is a patient-centered issue, we have set up close collaboration between a national cancer patient association, on the one hand, and our research team and clinical network of investigators on the other hand. The "monreseaucancer du poumon" association has assessed the patients' perception of the project, through questioning 400 expert patients and the association's board prior to submission. We have been strongly encouraged by the vast majority of positive patient responses to the concept of such randomized trial aiming at testing the relevance of treatment timing for efficacy.

Quality of life, sleep and chronotype questionnaires will be self-assessed by the patients at different stages of their participation to Immunotime. They will also fill a questionnaire evaluating their perception of the participation to the research (RRPQ), that has been used successfully by our team in two prior cancer chronotherapy studies. These questionnaires results will provide a patients'views on the ImmunoTime trial, and will be shared with the Association for interpretation and possible actions.

We look forward to the evaluation of the Immunotime trial project.

Best wishes

Boris Duchemann, MD, PhD and Francis Lévi, MD, PhD

8.1.3 Lettre d’acceptation de financement

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, @> 6 BF6DE@?D 23@FE E96 D2> A=6 DK6 42=4F=2E@? 2?5 E96 DE2EDE42= 2?2=JDD @7 E96 > 2:? 6?5A@?E #4@> AFE65 E96 D2> A=6 DK6 H:E9 " + AC@3 23=J @G6C @AE> :DE4 4@?D56C?8 :E :D 32D65 @? 2 > 6E2 2?2=JDD H:E9 2 ?@? ?F=DE2EDE42=96E6C@36?6:EJ E2F1 " :88:?D#I 2?5 7@F?5 2 D> :=2C ?F> 36C @7 6G6?ED H:E9 @C A2E6?ED (BF6CJ =@3 C2?< E6DE @C 6I A@?6?E2= G6DA64EG6=J 2DDF> :?8 ?@5C@A @FED =D@ #5@?@E F?56CDE2?5 E96 ?F> 36C@7 A2E6?ED7@C2 " + #E96? 4@?D56G65 2 D2> A=6 DK6 @7 2 3:?2CJ G6DA@?D6 GD 3FE# @3E2:? ? 7@C2 :D96CD6I 24E 2DDF> :?8 ?@5C@A @FED , @ E9:D4@?46C? 23@FEE96 D2> A=6 DK6 :D 2=D@G6=2E65 E@> J BF6DE@? 23@FE E96 DE2EDE42=2?2=JDD @7 E96 > 2:? 6?5A@?E #D:E 2 3:?2CJ G6DA@?D6 @C2 46?D@G65 6?5A@?E - 96 D64E@? 2?2=JDD @7 E96 AC> 2CJ 6?5A@?E :D2 =EE=6 3:E 4@?7FD65 #? 42D6 @7 2 E> 6 E@6G6?E6?5A@?E 2=C2?5@> :D65 A2E6?ED92G6 E@36 :?4=F565 6G6? :7D@> 6 2G6 46?D@G65 :? E96 7:CDEJ62C - 9:D:DE96 AC?4:A=6 @7 E> 6 E@6G6?E2?2=JDD

@?4=FD@?D

* C@64E92DE@36 7F?5:?8 E@4@?5F4EE9:DAC@A64EG6 DEF5J :? @56CE@G2=52E6 @C?@E E96 36?67:E @7 62G=J 25> :?:DEC2E@? @7 A6> 3C@=KF> 23 3FE E96 2FE9@D92G6 E@36 4=62G6C23@FE E96 EJA6 @7 E96 > 2:? 6?5A@?E2?5 :ED2?2=JDD3642FD6 :E:D4FCG6?E=J ?@E4=62C

+2AA@DE6FCD

* @?ED7@DED

- 9:D :D 2 H6=56D8?65 D> A=6 DEF5J E@56E6C> :?6 :? 2 AC@DA64EG6 72D9:@? H96E96CE> :?8 2?5 D4965F=?8 @7#) 25> :?:DEC2E@? 4@F=5 :> A24E@? 677:424J

* @?ED72:3=6D

&24< @7 * 92C> 24@<:?6E4DDFAA@DEE@86EC5 @7 A@DD3=6 4@?7@F?5:?8 724E@DD

@?4=FD@?D

- 9:D :D 2 G6CJ :?E6G6DE?8 DEF5J =<6=J E@ 92G6 2 DEC@?8 :> A24E H:E9 62DJ :> A=6> 6?E2E@? 2E 365D56 - 9:DAC@64ED9@F=5 36 DFAA@DE65 :? AC@CEJ

* @?ED7@DED

#E :D 2 DEC2E68:4 BF6DE@? 7:EE?8 H6=:? 2? 24256> :4 DEF5J - 96 C2E@?2=6 :D > @?6 C@?FDE H96? 4@> A2C65 E@=2DE J62CDF3> :DD@? 2?5 E96 E62> H6=255C6DD65 E96 =2E6DE C6G6H6CD 4@> > 6?ED ? :> A24E @? A2E6?E > 2?286> 6?E :D 6I A64E65 44CF2=D9@F=5 36 762D3=6 H:E9 A2E6?ED :? 46?E6CD A2E6?E 46?E6C> @?E9 - 96C6 :DC6=6G2?E2DD@4:2E65 EC2?D=2E@?2=C6D62C49

* @?ED72:3=6D

' 6E9@5 @=@8J :D D4@C65 3J E96 > 6E9@5 @=@8:DE C6G6H6C H:E9 2 4@?46C? @? E96 D2> A=6 DK6 42=4F=2E@? 2?5 @? E96 2?2=JDD@7E96 AC> 2CJ 6?5A@?E

@?4=FD@?D

- 9:D A92D6 ## C2?5@> :K65 @A6?=236=EC2=255C6DD?8 2 49C@?@A92C> 24@=@8:42=BF6DE@? :D 2?2> 3:E@FD AC@64E H:E9 A@E6?E2= 4=?:42= :> A24E - 96C6 C6> 2:? > 6E9@5 @=@8:42= :DDF6D E@ 36 4=2C7:65

@> :EO5 OG2=F2E@?

2E6 5F 4@> :EO

GD5F 4@> :EO

* C@6E7:?2?4O

7.2 ANNEXE II : RESUMES ET POSTERS SOUMIS A CONGRES INTERNATIONAUX PENDANT LA THESE

1. **Abdoulaye Karaboué,** Gabrielle Danino, Marie-Sara Malin, Adrien Lecoeuvre, Marthe Rigal, Lamiae Grimaldi, René Adam, Francis Lévi and Boris Duchemann : Critical Relevance of Immunochemotherapy Daily Timing for Survival Prolongation in Lung Cancer Patients, 2024 SRBR Biennial Conference, San Juan, Puerto Rico (USA), May 18-22, 2024 (Oral presentation).

2. Thierry Landre, **Abdoulaye Karaboué**, Zachary S Buchwald, Pasquale Innominato, David C Qian, Jean-Baptiste Assié, Christos Chouaid, Francis Lévi, and Boris Duchemann. Time-dependent efficacy of immune checkpoint inhibitors in the treatment of metastatic cancers: A meta-analysis. Annual meeting, ASCO 2023. Journal of Clinical Oncology 41, 2562-2562(2023)

3. **Karaboué A**, Collon T, Pavese I, Bodiguel V, Cucherousset J, Zakine E, Innominato PF, Bouchahda M, Adam R, Lévi F. Nivolumab timing as a major survival predictor in patients with stage IV non–small cell lung cancer. Annula meeting, ASCO 2022. Journal of Clinical Oncology 40, no. 16_suppl (June 01, 2022) 9058-9058.

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